PMA-STIMULATED PROTEIN KINASES AND THE REGULATION OF TRANSFECTED GLUCAGON RECEPTORS

Edward Spencer Tobias

B.Sc.(Hons.) M.B.,Ch.B.(Commend.) M.R.C.P.(U.K.)

Division of Biochemistry and Molecular Biology

Institute of Biomedical and Life Sciences

University of Glasgow

This thesis is submitted in partial fulfilment of the requirements of the University of Glasgow for the degree of Doctor of Philosophy

July, 1997

In memory of my father.

Abstract

Previous observations had suggested that the reduction in glucagon-stimulated adenylyl cyclase activity that was elicited by challenge of hepatocytes with phorbol 12-myristate 13-acetate (PMA) may have been mediated by the action of protein kinase C. In order to gain further insight into the nature of the protein kinase species which conferred such an inhibitory effect in these cells, I used a model system consisting of COS-7 cells transiently transfected so as to overexpress glucagon receptors. In these cells glucagon elicited a profound, dose-dependent, increase in the intracellular cAMP concentration with an EC_{50} of 1.8±0.4 nM glucagon. This value was comparable to those reported previously for the response observed in intact hepatocytes. In the transfected cells, levels of cAMP accumulation were maximal after approximately 10 minutes of stimulation with glucagon and thereafter remained stably elevated. These studies were undertaken in the presence of the non-selective phosphodiesterase inhibitor, IBMX, which elicited an inhibition of >96% of total cAMP phosphodiesterase (PDE) activity in these cells.

Glucagon challenge of the transfected COS-7 cells failed to elicit a significant stimulation of IP_3 production. However, in the presence of 0.3% (v/v) butan-1-ol, the hormone elicited an increase in the level of [^{3}H]-PtdOH, although not of [^{3}H]-PtdBut, suggesting that the hydrolysis of PtdCho by PLC, but not by PLD, was stimulated by glucagon in these cells.

Intriguingly, in contrast to previous observations made using hepatocytes, treatment with PMA did not inhibit the ability of glucagon to increase intracellular cAMP levels in these transfected cells. Furthermore, PMA-induced inhibition of the response was not conferred by varying the quantity of transfected DNA or by treating with the potent protein phosphatase inhibitor, okadaic acid. Nor was it observed following the co-transfection of the cells with cDNAs encoding various protein kinase C isoforms (PKC-α, PKC-βII and PKC-ε) or the PMA-activated G-protein receptor kinases, GRK2 and GRK3.

A striking PMA-induced inhibition (51%) of the glucagon-stimulated cAMP accumulation was, however, observed in COS-7 cells which had been co-transfected with a cDNA encoding the novel diacyl glycerol/phorbol ester-stimulated protein kinase, protein kinase D (PKD). This PMA-induced inhibitory effect in these co-transfected COS-7 cells was dependent upon the catalytic activity of the kinase since PMA failed to elicit a reduction in glucagon-stimulated cAMP accumulation in COS-7 cells which had been co-transfected with the glucagon receptor and a kinase-inactive form of PKD. Moreover, the effect appeared to be directed at the level of cAMP synthesis rather than its

degradation as studies of homogenate cAMP PDE activity showed no change in total PDE activity and IBMX was able to inhibit a similar fraction of the total activity. In the transfected cells, treatment with PMA did not inhibit either [^{125}I]-glucagon binding or GTP-induced glucagon dissociation. Furthermore, the intracellular cAMP accumulation elicited by either cholera toxin or forskolin was not reduced by treatment with the phorbol ester.

No statistically significant PMA-induced reduction in the isoprenaline-stimulated cAMP accumulation was detectable in COS-7 cells transfected with PKD and either the β_2AR or the β_3AR. This is consistent with the possibility that, in the co-transfected COS cells in which the inhibitory effect is observed, PKD phosphorylates the glucagon receptor itself.

PKD transcripts were detected in RNA isolated from hepatocytes but not from COS-7 cells. Transcripts for GRK2 were present in hepatocytes but not in COS-7 cells, whilst transcripts for GRK3 were not found in either cell type. Immunoblotting studies indicated that PKC-α, PKC-βII and PKC-ε were expressed both in hepatocytes and in COS-7 cells, although the level of PKC-βII appeared to be lower in the latter cell type.

Such studies also indicated that the levels of PKC-α, PKC-βII, PKC-ε and PKC-ζ in hepatocytes isolated from streptozotocin diabetic rats were 1.4-2.0 fold higher than in hepatocytes obtained from healthy control animals. In contrast, the level of PKD-specific transcript in hepatocytes and adipose tissue appeared to be markedly reduced in diabetic rats, and was restored by insulin treatment. It is suggested that PKD may play a role in the regulation of glucagon-stimulated adenylate cyclase activity *in vivo* and that the loss, in the diabetic state, of such a regulatory mechanism might constitute an important factor in the pathogenesis of hyperglycaemia.

Acknowledgements

I would like to express my gratitude to Professor Miles Houslay and Professor John Connell for their supervision and critical analysis of my work throughout the period of this research. I would also like to thank Professor Houslay for providing me with the facilities with which to carry out this project and for his valuable comments on the manuscript. In addition, I am grateful to the Medical Research Council for providing me with the Training Fellowship.

I wish to thank Dr. Susan Pyne for allowing me to carry out a number of experiments in her laboratory at the University of Strathclyde.

I would also like to thank all of the members of Professor Houslay's laboratory for their encouragement and discussions throughout the project and Grant Scotland for his assistance with the automated DNA sequencer.

Finally, I am indebted to my family and my fiancée, Ruth, for their continuous encouragement and support during the project and the period of my writing-up.

Table of Contents

Abstract iii

Acknowledgements v

Table of contents vi

List of tables xvii

List of figures xviii

Abbreviations xxii

CHAPTER 1

Introduction 1

1.1. G-protein-coupled receptors 2

1.1.1. Structure 2

1.1.1.1. General features 2

1.1.1.2. Ligand binding site structure 3

1.1.1.3. Residues which are involved in mediating the agonist-induced conformational change in GPCRs 4

1.1.1.4. Identification of the G-protein coupling site 5

1.1.2. Receptor-G-protein coupling 6

1.1.2.1. Conformational equilibrium of GPCRs 8

1.1.2.2. Receptor-G-protein selectivity 8

1.2. Guanine nucleotide-binding proteins 10

1.2.1. The G protein activation cycle 10

1.2.2. Covalent modifications of G-proteins 11

1.2.3. G protein subunit structure and lipid modification 12

1.2.4. Structural characteristics of the heterotrimer, and its interaction with receptors 14

1.2.5. Stimulation of effectors by G protein α subunits 14

1.2.6. Effector stimulation by $\beta\gamma$ heterodimers 15

1.3. Adenylyl cyclases 17

1.3.1. Structural features 17

1.3.2. Regulation of adenylyl cyclases 19

1.4. Receptor desensitization 21

1.4.1. Protein kinase C 22

1.4.1.1. Introduction 22

1.4.1.2. Structural features of PKC isoforms 23

1.4.1.3. Expression of PKC isoforms 25

1.4.1.3.1. Tissue distribution of PKC isoforms 25

1.4.1.3.2. Regulation of PKC gene expression 25

1.4.1.4. Activators and substrates of the PKC isoenzymes 26

1.4.1.4.1. Activation of PKC isoforms 26

1.4.1.4.2. Substrate-specificity of PKC 28

1.4.2. cAMP-dependent protein kinase (PKA) 30

1.4.2.1. The structure, regulation and distribution of PKAs 30

1.4.2.2. A-kinase anchoring proteins (AKAPs) 31

1.4.2.3. The substrate specificity of PKA 32

1.4.3. G-protein coupled receptor desensitization by PKC and PKA **33**

1.4.4. Protein kinase D **34**

1.4.4.1. Cloning of PKD and its expression in mouse tissues 35

1.4.4.2. Structural features of PKD 35

1.4.4.3. Functional differences between PKD and PKCs 36

1.4.4.4. Regulatory domain of PKD 37

1.4.4.5. The similarity between PKD and PKC-μ 38

1.4.4.6. Implications of the properties of PKD for cellular signalling 39

1.4.5. G-protein coupled receptor kinases **39**

1.4.5.1. The family of GRKs 40

1.4.5.2. Membrane attachment 41

1.4.5.3. Regulation of GRK activity 42

1.4.5.4. Receptor specificity 42

1.5. The glucagon receptor 45

1.5.1. The glucagon receptor gene 45

1.5.2. Structural aspects 47

1.5.3. Effector systems of the glucagon receptor 50

1.5.4. Glucagon receptor desensitization 51

1.5.4.1. The timecourse for glucagon-stimulated intracellular cAMP accumulation and its implications 51

1.5.4.2. Glucagon receptor desensitization elicited by pre-exposure to the hormone 52

1.5.4.3. Evidence for phosphorylation of the receptor 53

1.5.4.4. The potential role of PKC 54

CHAPTER 2

Materials and Methods 62

2.1. Materials 63

2.1.1. Radiochemicals 63

2.1.2. General reagents 63

2.1.3. Biochemical reagents 64

2.1.4. Molecular biology reagents 67

2.1.5. Plasmid DNA 68

2.1.6. Tissue culture 69

2.1.6.1. Media 69

2.1.6.2. Plastic ware 69

2.1.7. Animal resources 70

2.1.8. Cell lines 70

2.2. Methods for genetic manipulation 71

2.2.1. Water purification 71

2.2.2. Plasmid purification 71
2.2.2.1. Phenol/chloroform extraction 72
2.2.2.2. Ethanol precipitation 73
2.2.3. Determination of DNA concentration and purity 73
2.2.4. Restriction digests 73
2.2.5. Agarose gel electrophoresis 74
2.2.6. Purification of DNA from an agarose gel 75
2.2.7. Phosphatase treatment of DNA 75
2.2.8. DNA ligation 75
2.2.9. Preparation of competent E. coli 76
2.2.10. Transformation 77
2.2.11. Transient transfection 78
2.2.12. RNA extraction 78
2.2.13. Determination of RNA concentration 79
2.2.14. First strand cDNA synthesis 80
2.2.15. Polymerase chain reaction (PCR) 80
2.2.15.1. Design of oligonucleotide primers for PCR 80
2.2.15.2. Preparation of PCR oligonucleotide primers 81
2.2.15.3. PCR conditions 81
2.2.16. Site-directed mutagenesis 81
2.2.17. Automated DNA sequencing 82
2.2.17.1. PCR reactions 82
2.2.17.2. Sequencing gel preparation 83
2.2.17.3. Sequence sample purification and loading 83
2.2.17.4. Data collection and analysis 84
2.3. Methods for antiserum production 85
2.3.1. Generation of peptide antisera 85
2.3.2. Coupling peptides to KLH 85
2.3.2.1. Solutions for coupling 85

2.3.2.2. Coupling method 85

2.3.3. Expression and preparation of GST fusion protein 86

2.3.3.1. Induction of E. coli 86

2.3.3.2. GST fusion protein extraction 87

2.3.3.3. Verification of induction 87

2.3.3.4. Gel staining, destaining and drying 87

2.3.3.5. Fusion protein purification 87

2.3.4. Immunisation 88

2.3.5. Preparation of anti-serum 88

2.4. Methods for biochemical analysis 89

2.4.1. SDS polyacrylamide electrophoresis 89

2.4.2. Protein molecular weight markers 91

2.4.3. Western blotting 91

2.4.4. De-hybridisation of antibodies from nitrocellulose membranes 92

2.4.5. Immunoprecipitation 92

2.4.6. Induction and treatment of diabetes in rats 93

2.4.7. [^{125}I]-glucagon binding 93

2.4.8. Protein determination 94

2.4.9. Preparation of hepatocytes 95

2.4.10. Isolation of adipocytes by collagenase digestion 95

2.4.11. Preparation of membrane and cytosol fractions 96

2.4.12. Lactate dehydrogenase (LDH) assay 96

2.4.13. Membrane cyclase assay 97

2.4.14. Determination of intracellular cAMP production 97

2.4.14.1. COS cell stimulation 98

2.4.14.2. Extraction of cyclic AMP 98

2.4.14.3. Neutralisation of the sample 98

2.4.14.4. Preparation of cAMP binding protein 99

2.4.14.5. Reagent preparation for the cAMP binding assay 99

2.4.14.6. Assay procedure 100
2.4.14.7. Calculation of cyclic AMP levels 101
2.4.14.8. Determination of cell number and viability 101
2.4.15. Cyclic AMP phosphodiesterase assay 102
2.4.15.1. Stimulation and lysis of cells for the PDE assay 102
2.4.15.2. PDE assay reagent preparation 103
2.4.15.3. Assay procedure 104
2.4.15.4. Calculations 104
2.4.16. COS and 293 cell culture 105
2.4.17. PtdCho-PLC and PtdCho-PLD measurement 105
2.4.18. Measurement of inositol phospholipid hydrolysis 106
2.4.18.1. Ins(1,4,5)P_3 measurement by binding assay 106

CHAPTER 3

Approaches towards the construction of a full-length cDNA encoding the glucagon receptor, and the incorporation of an epitope tag at its amino-terminus 111

3.1. Introduction 112

3.2. Results 115

3.2.1. RT-PCR of the 5' region of the cDNA encoding the GR 115
3.2.2. Preparation and analysis of cDNA fragments of GR prior to ligation 116
3.2.3. Design of PCR oligonucleotides for the amplification of the entire GR coding sequence 117
3.2.4. Epitope tagging the glucagon receptor 119

3.3. Discussion 122

3.3.1. The attempted construction of the entire coding sequence of the rat hepatic glucagon receptor 122
3.3.2. Incorporation by PCR of an N-terminal epitope tag into the coding sequence of the glucagon receptor 123

CHAPTER 4

Glucagon stimulation of COS-7 cells transfected with cDNA encoding the glucagon receptor and PKC isoforms..... 135

4.1. Introduction..... 136

4.1.1. Evidence for rapid glucagon receptor desensitization..... 136

4.1.2. Glucagon receptor desensitization observed upon repeated glucagon administration..... 137

4.1.3. The potential involvement of PKC in the mechanism of desensitization..... 138

4.1.4. Aims and objectives..... 140

4.2. Results..... 141

4.2.1. The transient transfection of GR cDNA..... 141

4.2.1.1. The cAMP phosphodiesterase activity in GR-transfected COS cells and the effect of IBMX..... 141

4.2.1.2. Dose-response experiments in GR-transfected COS cells..... 142

4.2.1.3. Timecourse of glucagon-stimulated cAMP accumulation in GR-transfected COS cells..... 142

4.2.1.4. Glucagon-stimulated PtdCho and PIP_2 hydrolysis..... 143

4.2.1.4.1. Determination of PtdCho hydrolysis..... 143

4.2.1.4.2. Determination of PIP_2 hydrolysis..... 144

4.2.1.5. Determination of adenylate cyclase activity in plasma membranes..... 144

4.2.1.5.1. Membrane adenylate cyclase activity in different homogenisation buffers in glucagon receptor-transfected COS-7 and HEK-293 cells..... 145

4.2.1.6. The effect of PMA on glucagon-stimulated cAMP accumulation in glucagon receptor-transfected COS cells..... 146

4.2.1.6.1. The effect of PMA on glucagon-stimulated cAMP accumulation in glucagon receptor-transfected 293 cells..... 147

4.2.1.6.2. Phosphatase inhibition by okadaic acid..... 147

4.2.1.6.3. Varying the quantity of DNA transfected..... 148

4.2.2. Western blotting for PKC isoforms in COS cells..... 148

4.2.3. Co-transfections of COS cells with GR and PKC isoform cDNAs 149

4.2.3.1. Co-transfections of COS cells with GR and PKC-α cDNAs 149

4.2.3.1.1. Confirmation of expression of PKC-α cDNA 150

4.2.3.1.2. Glucagon-stimulated cAMP responses in GR/PKC-α co-transfected COS cells 150

4.2.3.2. Co-transfections of COS cells with GR and PKC-βII and PKC-ε cDNAs 151

4.3. Discussion 152

4.3.1. Glucagon-stimulated cAMP accumulation in transfected COS cells 152

4.3.2. Investigation of glucagon-induced lipid signalling 153

4.3.3. Measurement of adenylate cyclase activity in plasma membranes of transfected cells 154

4.3.4. The effect of pre-incubation of transfected COS cells with PMA 155

CHAPTER 5

Co-expression of the GR with GRK2, GRK3 and PKD in COS-7 cells, and the effects of PMA on the individual steps leading from glucagon binding to cAMP accumulation 173

5.1. Introduction 174

5.1.1. Aims and objectives 177

5.2. Results 179

5.2.1. Detection of PMA-sensitive kinases other than PKCs in hepatocytes 179

5.2.1.1. Western blotting 179

5.2.1.2. Design of oligonucleotide PCR primers 179

5.2.1.3. RT-PCR analysis of GRK2, GRK3, PKD and β-actin expression in hepatocytes and native COS cells 181

5.2.2. Co-transfection of COS-7 cells with plasmids encoding both the glucagon receptor and GRK2 181

5.2.3. Co-transfection of COS-7 cells with plasmids encoding both the glucagon receptor and GRK3 182

5.2.4. Co-transfection of COS-7 cells with plasmids encoding both the glucagon receptor and PKD..... 182

5.2.5. Co-transfection of COS-7 cells with plasmids encoding PKD and either the β_2-AR or the β_3-AR..... 183

5.2.6. Investigation of the effects of PMA on the individual steps leading from glucagon binding to cAMP accumulation..... 184

5.2.6.1. The effect of PMA on glucagon binding..... 184

5.2.6.2. The effect of PMA on G_s-cyclase coupling and on adenylate cyclase function..... 184

5.2.6.3. The effect of PMA on the GTP-induced reduction in receptor affinity for glucagon..... 185

5.2.7. RT-PCR analysis of PKD expression in CHO cells..... 185

5.3. Discussion..... 187

5.3.1. The possible role of PKD in hepatocytes..... 191

CHAPTER 6

Relationship between the induction of diabetes and the expression levels of PMA-activated kinases in rats..... 205

6.1. Introduction..... 206

6.1.1. The use of streptozotocin to induce diabetes..... 206

6.1.2. Changes in expression and activity of PKC isoforms in diabetes..... 207

6.1.3. The use of semi-quantitative RT-PCR to determine levels..... 208

6.1.4. Aims and objectives..... 208

6.2. Results..... 210

6.2.1. Determination of changes in PKC isoform levels in diabetes..... 210

6.2.1.1. Immunoblotting of hepatocytes..... 210

6.2.1.2. Immunoblotting of adipocytes..... 211

6.2.2. PKD expression studies..... 213

6.2.2.1. Optimisation of PKD-specific PCR..... 213

6.2.2.2. PKD tissue distribution..... 214

6.2.3. Detection of expression of PMA-activated kinases and the glucagon receptor in control and diabetic animals 215

6.2.3.1. Transcript detection in hepatocytes 215

6.2.3.2. PKD-specific transcript detection in epididymal fat 216

6.3. Discussion 219

6.3.1. Immunoblotting for PKC isoforms 219

6.3.2. PKD expression studies 221

6.3.3. Implications of results for the pathogenesis of diabetes 223

6.3.3.1. Postulated physiological role of PKD in glycaemic homeostasis 223

6.3.3.2. Implications of diminished expression of PKD 224

6.3.3.3. Homologous glucagon desensitization in diabetic rats 225

CHAPTER 7

Conclusions 239

7.1. Analysis and manipulation of the glucagon receptor cDNA 240

7.2. Transient transfection studies 241

7.3. Investigations into the mechanism of the PKD-mediated inhibition 244

7.4. Inferences regarding the possible roles of PKD in hepatocytes 245

7.5. Postulated physiological and pathological importance of PKD 246

7.6. Future studies 247

APPENDIX

Attempts to obtain a glucagon receptor-specific antiserum..... 252

A.1. Testing antisera raised against GR-based peptides 253

A.2. Testing antisera raised against a GST-GR fusion protein..... 255

A.3. Testing the ability of fusion protein antisera to immunoprecipitate the GR 256

A.4. Phosphatase treatment of membrane proteins prior to immunoblotting 256

A.5. Re-coupling of peptides to the carrier molecule KLH 256

Discussion 257

REFERENCES..... 271

APPENDIX B (rear pocket)

Amino acid sequence of the rat hepatic glucagon receptor

List of Tables

Table 1.1. The functions of the G protein α subunits.....56

Table 1.2. The regulation of mammalian adenylyl cyclases.....57

Table 4.1. Glucagon and GTP-stimulated adenylate cyclase activities in membranes prepared from control and PMA-treated untransfected and GR-transfected COS cells.....159

Table 4.2. Glucagon and GTP-stimulated adenylate cyclase activities in membranes prepared from GR-transfected COS and HEK-293 cells.....160

Table 4.3. The effect of PMA on the glucagon-stimulated cAMP response in COS cells co-transfected with cDNA encoding the GR and PKC-α, PKC-βII or PKC-ε.....161

Table 5.1. PCR conditions for amplification of GRK2, GRK3, PKD and β-actin.....193

Table 5.2. The effect of PMA on the hormone-stimulated cAMP response in COS cells co-transfected with cDNA encoding either the GR or a βAR in addition to GRK2, GRK3 or PKD.....194

Table 5.3. The effect of PMA treatment on the forskolin and cholera toxin-induced cAMP response in control and PKD-transfected COS cells.....195

Table 5.4. The effect of PMA treatment on the GTP-mediated inhibition of specific ^{125}I glucagon binding (fmol/mg) to membranes isolated from GR/PKD co-transfected COS cells and from hepatocytes.....196

Table 5.5. RxxS consensus searches in primary sequences of adenylate cyclase isoforms.....197

Table 6.1. RT-PCR of PKD-specific cDNA from rat epididymal fat.....226

Table A.1. Amino acid sequences and corresponding locations of the synthetic peptides based on the primary sequence of the glucagon receptor.....260

List of Figures

Figure 1.1. Structural features of G-protein-coupled receptors 58

Figure 1.2. Schematic illustration of the Gβγ heterodimer 59

Figure 1.3. Structural comparison of the members of the PKC family 60

Figure 1.4. Sequence comparison of the first intracellular loop of the glucagon receptor with that of the two calcitonin receptor isoforms 61

Figure 2.1. Analysis of DNA sequencing error frequency 108

Figure 2.2. Standard curve for Bradford protein assay 109

Figure 2.3. Standard curve for the Ins (1,4,5) P_3 assay 110

Figure 3.1. Strategy for the construction of the full length glucagon receptor coding sequence 126

Figure 3.2. The use of RT-PCR to amplify the 5' region of the sequence encoding the rat hepatic glucagon receptor 127

Figure 3.3. Pst I digestion of the 941 bp RT-PCR product. 128

Figure 3.4. Restriction digestion of the cDNA clone encoding the C-terminal region of the glucagon receptor 129

Figure 3.5. Hypothesis for the rationalisation of the digestion products of the C-terminal GR cDNA clone 130

Figure 3.6. Confirmation of the presence of intronic DNA by individual BamH I and HinD III digests of the pUC 18 – GR construct 131

Figure 3.7. Attempted RT-PCR of the full-length cDNA encoding the glucagon receptor 132

Figure 3.8. Strategy for the incorporation of a VSV epitope tag into the coding sequence of the glucagon receptor by PCR 133

Figure 3.9. Analysis of epitope tag incorporation by PCR amplification and restriction digestion 134

Figure 4.1. The pCDM8-GR construct 162

Figure 4.2. Dose response curve for glucagon-stimulated cAMP production in untransfected and GR-transfected COS cells. 163

Figure 4.3. Timecourses for glucagon-stimulated cAMP production in GR-transfected COS cells. 164

Figure 4.4. PtdOH and PtdBut production in mock and glucagon receptor-transfected COS cells upon stimulation with varying concentrations of glucagon 165

Figure 4.5. Timecourse of PtdOH and PtdBut production in glucagon receptor-transfected COS cells in response to glucagon..... 166

Figure 4.6. Dose response of Ins(1,4,5)P_3 production upon glucagon stimulation of mock and GR-transfected COS cells..... 167

Figure 4.7. Glucagon-stimulated cAMP production in control and PMA-treated untransfected and GR-transfected HEK-293 cells..... 168

Figure 4.8. The effect of okadaic acid, PMA and varying the quantity of transfected GR DNA on the glucagon-stimulated cAMP response in COS cells..... 169

Figure 4.9. Immunoblots for PKC α, PKC βII, PKC ε and PKC ζ isoforms in untransfected COS-7 and hepatocyte extracts..... 170

Figure 4.10. The effect of PMA on the glucagon-stimulated cAMP response in PKC alpha and glucagon receptor co-transfected COS cells..... 171

Figure 4.11. Confirmation of expression of transfected cDNA encoding PKC α, PKC βII and PKC ε by Western blotting..... 172

Figure 5.1. RT-PCR detection of GRK2, GRK3, PKD and β-actin in hepatocyte and untransfected COS cell RNA..... 198

Figure 5.2. RT-PCR detection of GRK2, GRK3 and β-actin in transfected and untransfected COS cell RNA..... 199

Figure 5.3. The effect of PMA on the glucagon-stimulated cAMP response in GRK2 and glucagon receptor co-transfected COS cells..... 200

Figure 5.4. The effect of PMA on the glucagon-stimulated cAMP response in GRK3 and glucagon receptor co-transfected COS cells..... 201

Figure 5.5. Immunoblot confirming the expression of transfected cDNA encoding PKD in transfected COS cells..... 202

Figure 5.6. Timecourses for glucagon-stimulated cAMP production in the presence and absence of PMA in COS cells transfected with cDNAs encoding both the GR and PKD..... 203

Figure 5.7. RT-PCR detection of PKD and β-actin transcripts in hepatocyte and CHO cell RNA..... 204

Figure 6.1. Immunoblots for PKC alpha, beta II, epsilon and zeta in extracts of hepatocytes prepared from control and streptozotocin diabetic rats..... 227

Figure 6.2. Immunoblots for PKC beta II and epsilon in extracts of epididymal fat prepared from control and streptozotocin diabetic rats..... 228

Figure 6.3. Amplification by PCR of the cDNA encoding protein kinase D..... 229

Figure 6.4. Amplification by PCR of the cDNA encoding protein kinase D..... 230

Figure 6.5. Effect of varying the $MgCl_2$ concentration upon PKD-specific RT-PCR..... 231

Figure 6.6. Amplification by PCR of the cDNA encoding protein kinase D from RNA isolated from various tissues 232

Figure 6.7. Immunoblots for PKD in homogenates of rat hepatocytes and control and transfected COS cells 233

Figure 6.8. Amplification by RT-PCR of the cDNA encoding protein kinase D from RNA isolated from control and diabetic rat hepatocytes 234

Figure 6.9. Amplification by RT-PCR of the cDNAs encoding GRK2 and GRK3 from RNA isolated from control and diabetic rat hepatocytes 235

Figure 6.10. Amplification by RT-PCR of glucagon receptor cDNA from RNA isolated from control and diabetic rat hepatocytes 236

Figure 6.11. RT-PCR of the cDNA encoding protein kinase D from a range of quantities of RNA isolated from rat epididymal fat 237

Figure 6.12. Amplification by RT-PCR of the cDNAs encoding protein kinase D and β-actin from RNA isolated from control, diabetic and insulin-treated diabetic rat epididymal fat 238

Figure 7.1. The proposed role of PKD in a homeostatic mechanism regulating hepatocyte gluconeogenesis 250

Figure 7.2. Strategy for the site-directed mutagenesis of the coding sequence of the rat hepatic glucagon receptor 251

Figure A.1. Immunoblot of liver membranes using as primary antibody either pre-immune serum or an antiserum raised against peptide 4 of the glucagon receptor 261

Figure A.2. Immunoblot of liver membranes using as primary antibody either pre-immune serum or an antiserum raised against peptide 5 of the glucagon receptor 262

Figure A.3. The effect of peptide competition on immunodetection by YE627 antiserum 263

Figure A.4. Immunoblot of COS cell extracts using as primary antibody either pre-immune serum or an antiserum raised against peptide 5 of the glucagon receptor 264

Figure A.5. Immunoblots of COS cell extracts using as primary antibodies, antisera YE621, 622, 625, 628 265

Figure A.6. Protein-staining of hepatocyte membranes and COS cell extracts, confirming the expression of transfected glucagon receptor cDNA 266

Figure A.7. Immunoblots of hepatocyte and COS cell extracts using as primary antibody an antiserum raised against a GST-GR fusion protein 267

Figure A.8. [^{125}I]-glucagon specific binding following immunoprecipitation from hepatocyte membranes using the antisera raised against a GST-GR fusion protein 268

Figure A.9. Immunoblots of COS cell membrane fractions following phosphatase treatment using an antiserum raised against a GST-GR fusion protein..... 269

Figure A.10. Immunoblots of COS cell extracts using antisera raised against re-conjugated peptide 4 and peptide 5 of the glucagon receptor 270

Abbreviations

AC	adenylyl cyclase
ATP	adenosine 5'-triphosphate
β_2AR	beta$_2$-adrenergic receptor
βARK	beta-adrenergic receptor kinase
bp	base pairs
BSA	bovine serum albumin
cAMP	adenosine 3', 5'-cyclic monophosphate
cDNA	complementary DNA
CHAPS	(3-[(cholamidopropyl)dimethylammonio]-1-propane-sulfonate)
CHO	Chinese hamster ovary cell line
CIAP	calf intestinal alkaline phosphatase
cm^2	square centimetres
cpm	counts per minute
DAG	*sn*-1,2-diacylglycerol
DEAE	diethylaminoethyl
DEPC	di-ethyl pyrocarbonate
dH_2O	distilled water
dl	decilitre
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside trisphosphate
dpm	disintegrations per minute
DTT	dithiothreitol
EC_{50}	concentration eliciting 50% activation
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetra-acetic acid
EGTA	ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
FCS	foetal calf serum
GDP	guanosine 5'-diphosphate
G_i	inhibitory G protein to adenylyl cyclase
GPCR	G-protein coupled receptor
GppNHp	5'-guanylylimidodiphosphate
G-protein	guanine nucleotide-binding regulatory protein
GR	glucagon receptor

GRK	G-protein coupled receptor kinase
G_s	stimulatory G protein to adenylyl cyclase
GST	glutathione S-transferase
GTP	guanosine 5'-triphosphate
h	hour(s)
HCl	hydrochloric acid
HEK	human embryonal kidney
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane-sulphonic acid
HRP	horseradish peroxidase
IBMX	3-isobutyl-1-methylxanthine
IDDM	insulin-dependent diabetes mellitus
$Ins(1,4,5)P_3$	inositol 1, 4, 5-trisphosphate
kb	kilobase(s) or kilobase pair(s)
kD	kiloDalton(s)
KLH	keyhole limpet haemocyanin
l	litre(s)
LDH	lactate dehydrogenase
M-MuLV	Moloney Murine Leukaemia Virus
mg	milligram(s)
min	minute(s)
ml	millilitre(s)
mM	millimolar
mRNA	messenger RNA
Mwt	molecular weight
NIDDM	non-insulin-dependent diabetes mellitus
nM	nanomolar
nm	nanometre(s)
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PC	phosphatidylcholine
PCA	perchloric acid
PCR	polymerase chain reaction
PDE	phosphodiesterase
pH	negative logarithm of hydrogen ion concentration (in moles per litre)
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKA	cAMP-dependent protein kinase A
PKC	protein kinase C

PKD	protein kinase D
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate (also termed TPA)
PMSF	phenylmethylsulphonyl fluoride
PS	phosphatidylserine
PtdBut	phosphatidylbutanol
PtdCho	phosphatidylcholine
PtdOH	phosphatidate
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulphate
SE	standard error
TBS	Tris-buffered saline
TE	Tris/EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
TPA	12-O-tetradecanoylphorbol 13-acetate (also termed PMA)
Tris	Tris(hydroxymethyl)aminomethane
UV	ultraviolet
v/v	volume/volume
w/v	weight/volume
µg	microgram(s)
µl	microlitre(s)
µM	micromolar
°C	degrees centigrade
PI	phosphatidylinositol
PLD	phospholipase D
VSV	vesicular stomatitis virus

CHAPTER 1
Introduction

1.1. G-protein-coupled receptors

1.1.1. Structure

1.1.1.1. General features

Through electron microscopy imaging studies, the three-dimensional structure of the first membrane protein, namely the light-activated H^+ translocator, bacteriorhodopsin, was deduced in 1975 (Henderson and Unwin, 1975). Intriguingly, the primary sequence of the first G-protein coupled receptor (GPCR) to be cloned, namely the β-adrenoceptor (βAR) showed seven similar hydrophobic stretches of amino acids (Dixon *et al.*, 1986). Each of these stretches was of a length sufficient to form a single transmembrane α-helix, as deduced for the structure of bacteriorhodopsin. Thus it was by analogy with the transmembrane protein, bacteriorhodopsin, that the subsequent proposal of the likely structure and orientation of the purified and sequenced β-adrenoceptor, was formulated (Dixon *et al.*, 1986).

By virtue of the widespread application of molecular cloning techniques, the nucleotide sequences of over 300 GPCRs have been elucidated. The subsequent comparison of their deduced amino acid sequences has revealed that, despite the diversity of the ligands which activate these receptors, the receptors themselves share a surprising degree of structural homology, both at the primary and tertiary level. Thus, all of these receptors contain seven domains of 22-28 predominantly hydrophobic amino acids, which are each postulated to form α-helices that span the plasma membrane and which are connected by hydrophilic extracellular and intracellular loops (Houslay, 1992). It is in the membrane-spanning regions that the majority of the amino acid sequence homology resides, with greater diversity in the hydrophilic regions. These regions extend out into the extracellular space at the amino-terminus and between helices 2-3, 4-5 and 6-7, whereas those hydrophilic loops which are located between helices 1-2, 3-4 and 5-6 extend intracellularly and the C-terminal tail is found at the cytosolic surface of the plasma membrane (figure 1.1). Such a tertiary structure was also indicated by a more recent low

resolution projection map, obtained by two-dimensional electron crystallography, of bovine rhodopsin (Schertler *et al.*, 1993). This also implied that while four of the helices are almost perpendicular to the membrane, the other three are tilted (Schertler *et al.*, 1993).

Primary sequence data for various GPCRs has led to their further classification into three sub-families: the rhodopsin/β-adrenergic receptor sub-family, the secretin/vasointestinal peptide receptor (VIP) sub-family and the metabotropic glutamate (mGlu) receptor sub-family.

1.1.1.2. Ligand binding site structure

The rhodopsin/β-adrenergic receptor sub-family, which contains the majority of the GPCRs identified to date, has been the subject of the most extensive studies done concerning structure-function relationships. Owing to the difficulty in obtaining high-quality crystals for the structural determination of membrane proteins, however, there is a paucity of structural data relating to the tertiary structure of GPCRs. Therefore, computer molecular modelling, based upon the three-dimensional structure of bacteriorhodopsin and the primary sequences of GPCRs, has been combined with biochemical, pharmacological and genetic analyses in order to identify the key structural elements of these receptors. An important observation was that the periodic distribution of hydrophobicity within the seven relatively hydrophobic regions was consistent with an α-helical conformation in all cases (Rees *et al.*, 1989). Thus, the structure of bacteriorhodopsin has been used as a template around which to orient the hydrophobic regions in the GPCRs (Strader *et al.*, 1994). Such modelling has directed subsequent mutagenesis studies, leading to the identification of key residues in the βAR that form the ligand binding site. Thus, by the systematic mutagenesis of negatively charged residues, Asp^{113}, which is located near to the extracellular end of the third transmembrane helix of the βAR, has been shown to constitute an important contact point for the basic amines in the agonist molecules (Strader *et al.*, 1988). Although this ionic interaction is a major source of binding energy between the ligand and the receptor, however, it does not

appear to be critical for agonist activation of the βAR (Strader *et al.*, 1988). Subsequently, from analyses of the interactions of differing analogues of isoproterenol with mutant forms of the receptor, it was inferred that the catechol ring of the agonist interacts with Phe^{290} in transmembrane helix 6 (Strader *et al.*, 1989). In addition, these studies indicated that the catechol hydroxyl groups of the agonist appear to form hydrogen bonds with the side chains of two Ser residues at positions 204 and 207 in transmembrane helix 5 (Strader *et al.*, 1989). Thus it appears that the ligand binding site of the βAR is formed by a juxtaposition of amino acids residing in at least three helices, defining its specificity and affinity for agonists.

Many members of the rhodopsin/β-adrenergic sub-class of GPCRs interact with peptide ligands, which range from 3 to 40 amino acid residues in length. As discussed above, catecholamines and related biogenic amines bind primarily within the transmembrane domains of their receptors. However, as most peptide ligands are significantly larger than catecholamines, it might be envisaged that the former are less able to fully enter the helical core of the receptor. It is not surprising, therefore, that the binding of one such ligand, substance P, has been shown by site-directed mutagenesis of its receptor, the NK1 neurokinin receptor, to be dependent upon residues located in both the extracellular and transmembrane regions of the receptor (Fong *et al.*, 1992).

1.1.1.3. Residues which are involved in mediating the agonist-induced conformational change in GPCRs

The binding of agonist to a GPCR causes a conformational change allowing the formation of an agonist-receptor-G protein complex with a low affinity for ligand (Gilman, 1987). It has been difficult, using mutagenesis techniques, to distinguish between residues which participate solely in the initial binding step and those which are involved in the subsequent conformational change. However, two amino acids in the NK1 neurokinin receptor have been identified whose substitution leads to a complete loss of the ability of the receptor to mediate a signal transduction response but only a modest reduction in the binding affinity for agonists (Strader *et al.*, 1995). These two amino

acids, Glu^{78} in transmembrane helix 2 and Tyr^{205} in helix 5, therefore, appear to be crucial for the receptor activation process rather than for ligand binding per se.

1.1.1.4. Identification of the G-protein coupling site

Site-directed mutagenesis studies of the βAR have indicated that regions of approximately 8 and 12 residues, located at the N- and C-terminal ends of the third intracellular loop, respectively, determine the specificity of interaction with Gα subunits (O'Dowd *et al.*, 1988; Kosugi *et al.*, 1992; Baldwin, 1994). Thus, the reduction in length of this loop in the m3-muscarinic acetylcholine receptor, from 239 to 22 amino acids, did not appear to affect the function of the receptor, provided that the charged residues near either end were preserved (Baldwin, 1994). Additional studies of the contribution made by the third intracellular loop have included the transfection of Chinese hamster ovary cells with the cDNA which encodes the rat thyrotropin (TSH) receptor resulting in TSH-stimulated cAMP and $Ins(1,4,5)P_3$ responses (Kosugi *et al.*, 1992). In this system it was found that the substitution of Ala^{623}, which is located at the C-terminal end of the third intracellular loop of the receptor, results in the loss of TSH-stimulated formation of inositol phosphate but not that of cAMP (Kosugi *et al.*, 1992).

It appears, in addition, that the C-terminal tail of the GPCRs may also influence coupling. For example, in the βAR, this region contains a number of residues, including Thr^{384}, Ser^{396}, Ser^{401} and Ser^{407}, whose phosphorylation by β-adrenergic receptor kinase (βARK) leads to an attenuation of the signalling by the receptor (Roth *et al.*, 1991; Inglese *et al.*, 1993; Fredericks *et al.*, 1996). In addition, a highly conserved cysteine residue, that can be post-translationally modified by palmitoylation, is located in the C-terminal tail of a large number of receptors (O'Dowd *et al.*, 1989; Baldwin, 1994). This is found approximately 12 amino acids from the end of helix 7 (O'Dowd *et al.*, 1989; Baldwin, 1994). Mutation of this residue reduced coupling, implying that it is functionally important (O'Dowd *et al.*, 1989). It is conceivable that the lipid moiety associated with this residue inserts into the bilayer allowing the formation of a small

fourth intracellular loop. This may be expected to engender a structural change which may enhance the interaction of the GPCR with its appropriate G-protein.

Significant differences exist in the structure of the G protein binding site between members of different GPCR sub-families. This is perhaps not too surprising, considering the range of G-protein α-subunits to which different GPCRs are able to couple. For instance, activation of G proteins by members of the metabotropic glutamate receptor sub-family appears to be determined by regions in the second intracellular loop (Pin *et al.*, 1994) rather than in the third loop, which, as discussed above, appears to be an important determinant of G-protein coupling for the receptors of the rhodopsin/β-adrenergic sub-family. There is still, however, no clearly defined primary sequence motif that appears to determine the specificity of a particular GPCR for an individual G-protein. Indeed, the implied degeneracy in the structure of the G-protein coupling site might be envisaged to account for the ability of an individual receptor, when over-expressed, to activate non-cognate additional G-proteins (Milligan *et al.*, 1991; Chabre *et al.*, 1992; Gudermann *et al.*, 1992; Zhu *et al.*, 1994).

1.1.2. Receptor-G-protein coupling

Activation of GPCRs by agonists such as light, odorants, hormones or neurotransmitters causes a conformational change that leads to their ability to couple to their cognate G-protein. The association of the receptor with a G protein was originally demonstrated using a number of different approaches. These included the reconstitution of G_s in plasma membranes isolated from cells deficient in adenylyl cyclase activity (Ross *et al.*, 1978) and the agonist-induced apparent increase in receptor size as assessed by both radiation inactivation (Houslay *et al.*, 1977) and gel filtration (Limbird *et al.*, 1980). The activation process, which is now known to involve the replacement of GDP by GTP on the Gα subunit (Gilman, 1987), was shown in early studies to require the presence of divalent cations such as Mg^{2+} or Mn^{2+} (Pohl *et al.*, 1971). This activation of G_s, by agonist-bound receptor, is considered to be a catalytic event, as the formation of the receptor-G_s complex is transient (Levitzki, 1988). It was suggested that during this

encounter, the guanine nucleotide exchange is brought about by an "opening" of the GTP binding site i.e. a conformational change in the protein that allows the replacement of GDP with GTP (Cassel and Selinger, 1978). Support for this hypothesis was later gained from the demonstration, by a kinetic analysis of the βAR system in turkey erythrocytes, that the G_s protein can, indeed, attain an open and a closed state (Braun *et al.*, 1982). The GTP/GDP exchange and the activation cycle of the Gα subunit are described in section 1.2.1.

Although binding of GTP, or one of its non-hydrolysable analogues, by G_s, causes this protein to dissociate rapidly from the βAR (Limbird *et al.*, 1980), a temporal amplification of around 100-fold is achieved owing to the relatively low GTPase activity of the G protein (Levitzki, 1988). Moreover, since the receptor-G_s interaction is transient, a single receptor can activate several G_s molecules within the lifetime of the ligand-bound (occupied) receptor, thus leading to a further tenfold amplification in the system (Levitzki, 1988). In the rhodopsin-transducin system the potential for such an amplification is even greater, with up to 300 molecules of cGMP phosphodiesterase becoming activated by activated rhodopsin within the first 500 milliseconds following a flash (Yee and Liebman, 1978; Fung and Stryer, 1980).

It is the case, generally, that the addition of GTP and related analogues decreases the affinity of binding of a hormone or neurotransmitter to specific receptors in isolated membrane preparations (Rodbell, 1980). One of the first receptors for which this was demonstrated was the glucagon receptor, where the binding of [^{125}I]-glucagon was found to be markedly reduced by GTP (Rodbell *et al.*, 1971c). Thus, it was suggested that, in the presence of GTP, the receptor-G protein complex has a lower affinity for ligand than the free receptor (Limbird *et al.*, 1980). Following the occupation of the G protein by GTP, the ligand is released from the receptor, permitting the cycle to be re-initiated (Limbird *et al.*, 1980).

1.1.2.1. Conformational equilibrium of GPCRs

There is now evidence that GPCRs, even in their unoccupied state, elicit a significant basal level of signal transduction. For example, the activation of membrane adenylate cyclase by unoccupied transfected receptors has been demonstrated in NG108-15 cells expressing either the human β_2 adrenoceptor (Adie and Milligan, 1994) or the δ-opioid receptor (Mullaney *et al.*, 1996) and also in Chinese hamster ovary cells transfected so as to over-express the histamine H_2 receptor (Smit *et al.*, 1996). This phenomenon appears to relate to the abnormally high expression levels of GPCR in these transfected cells. Thus it has been postulated that GPCRs exist in an equilibrium between R_i, an inactive conformation that is unable to couple to G proteins, and R_a, an active conformation which can couple (Mullaney *et al.*, 1996). Ligand binding stabilises the R_a state. However, even in the absence of ligand, an increase in the concentration of GPCRs will lead to an increase in the number of unoccupied receptors which adopt the active conformation and lead to signal transduction. Studies using cell lines stably transfected with the β_2AR have demonstrated this to be the case (Samama *et al.*, 1993; Adie and Milligan, 1994).

According to the model, an agonist stabilises the active conformation, while an inverse agonist reduces the basal activity of the receptor by stabilising the inactive conformation of the receptor (Milligan *et al.*, 1995a). It could be envisaged that an antagonist would not stabilise either conformation. Like agonists, inverse agonists may cover a range of efficacies, and may thus be termed "partial inverse agonists". For the β_2AR, a rank order of inverse efficacy has been described of timolol > propranolol > alprenolol > pindolol (Chidiac *et al.*, 1994).

1.1.2.2. Receptor-G-protein selectivity

Activation of multiple cellular signalling cascades is a frequent outcome of the exposure of cells to a single agonist. The mechanisms by which a single GPCR species may initiate more than one signalling cascade include (i) the ability of one receptor to couple to a number of G protein types and (ii) the capacity, described in sections 1.2.5 and 1.2.6, of the α and βγ functional subunits of a G-protein to regulate distinct effector

enzymes. A large number of studies have been reported to demonstrate the first of these mechanisms. Many of these studies, however, have involved the analysis of receptors that have been over-expressed following transfection of their cDNA, thus limiting the physiological implications of the data. It is clear from these and other studies, however, that certain GPCRs do have the intrinsic capacity for coupling to alternative G-protein types. For instance, the agonist occupation of the β-adrenoceptor leads, in turkey erythrocytes, to the activation of a phospholipase C, in addition to that of adenylyl cyclase (Rooney *et al.*, 1991; Vaziri and Downes, 1992). While the activation of phospholipase C could, as discussed in section 1.2.6, result from the action of Gβγ, it has since been demonstrated that antibodies directed against the C-terminus of $G\alpha_{11}$, which is the $G\alpha_q$-like G protein expressed by these cells (Maurice *et al.*, 1993), abrogated the phospholipase C response without affecting the activation of adenylyl cyclase (James *et al.*, 1994).

The ligand occupation of another GPCR, the glucagon receptor, has been shown, in hepatocytes, to lead not solely to a stimulation of adenylate cyclase, but also to the production of diacylglycerol (Bocckino *et al.*, 1985; Pittner and Fain, 1991), an increase in inositol phosphates (Wakelam *et al.*, 1986; Whipps *et al.*, 1987) and a marked elevation of intracellular calcium (Charest *et al.*, 1983; Mauger *et al.*, 1985; Sistare *et al.*, 1985; Blackmore and Exton, 1986; Mine *et al.*, 1988). It remains to be demonstrated, however, whether this apparent phospholipase C activation is mediated by $G_s\beta\gamma$ or by the coupling of the receptor to one of the members of the Gq family.

1.2. Guanine nucleotide-binding proteins

Since the early observation by Rodbell, Birnbaumer and colleagues that GTP is required for the hormonal activation of adenylyl cyclase (Rodbell *et al.*, 1971a), it has become clear that guanine nucleotide-binding proteins (G proteins) mediate the regulation of a variety of other enzymes, ion channels (Birnbaumer *et al.*, 1990b; Krapivinsky *et al.*, 1995) and vesicular transport processes (Helms, 1995) by seven-transmembrane domain receptors which have been activated by hormones, neurotransmitters or sensory signals (Gilman, 1987; Birnbaumer *et al.*, 1990a; Houslay, 1990). G proteins consist of three polypeptides, arranged in two functional units: an α subunit which binds and hydrolyses GTP, and by the nature of which the G proteins are defined, and a $\beta\gamma$ heterodimer which functions as a single unit (Sternweis, 1994). The heterotrimeric G proteins constitute part of a larger superfamily of proteins possessing GTPase activity that includes the small GTP-binding proteins such as Ras, Rho, Rab, Rap and Arf (Bokoch, 1993; Takai *et al.*, 1995). Numerous heterotrimeric G protein monomers have been cloned in recent years, resulting in the identification of at least 20 different α, 5 different β and 12 different γ subunits (Hamm and Gilchrist, 1996). Although, in theory, the existence of such numbers of isoforms would permit the generation, by random association, of over a thousand different heterotrimeric G proteins there is evidence that the number of distinct G protein complexes is actually more limited, owing to preferential association of individual monomers (Pronin and Gautam, 1992). For example, while the $G\beta_1$ subunit can form a dimer with either $G\gamma_1$ or $G\gamma_2$, the $G\beta_2$ subunit can interact only with $G\gamma_1$ (Pronin and Gautam, 1992; Schmidt *et al.*, 1992). This is likely to be at least partly due to the three-dimensional structural characteristics of the isoforms (Lee *et al.*, 1995).

1.2.1. The G protein activation cycle

The activation state of the complex is determined by a GTPase cycle (Gilman, 1987). Briefly, in the inactive state, GDP is bound to the $G\alpha$ subunit, and the

heterotrimeric complex is stabilised. Agonist occupancy of the receptor, however, and the consequent adoption of an "open" conformation by the Gα subunit, cause a reduced affinity of the G protein subunit for GDP (Brandt and Ross, 1986). This nucleotide then dissociates and is replaced by GTP, since the concentration of GTP in cells is much higher than that of GDP (Gilman, 1987). The binding of GTP causes the Gα subunit to adopt its activated conformation, with two consequences: (1) the G-protein coupled receptor adopts a conformation with a lower affinity for its agonist, thus encouraging agonist dissociation and permitting the re-instigation of the cycle (Rodbell *et al.*, 1971c; Rodbell *et al.*, 1974; Limbird *et al.*, 1980) and (2) each of the G protein functional units (α and βγ) becomes free to activate target effectors (Gilman, 1987). This active state is maintained until the GTP is hydrolysed to GDP by the intrinsic GTPase activity of the Gα subunit, with the consequent re-association of the Gα subunit with the Gβγ dimer. In fact, while all Gα subunits possess the ability to hydrolyse GTP, the rate of this intrinsic activity varies between the Gα subunit types (Carty *et al.*, 1990).

1.2.2. Covalent modifications of G-proteins

The activation of G proteins can also be effected experimentally by non-hydrolysable GTP analogues such as GTPγS and Gpp[NH]p. The activation can, in addition, be effected by fluoroaluminate (AlF_4^-) (Gilman, 1987), which, together with GDP, forms a non-covalent complex that mimics GTP in the binding site of Gα (Antonny and Chabre, 1992). In addition, bacterial toxins, which covalently modify Gα subunits by catalysing the transfer of the ADP-ribose moiety from NAD to Gα, can be used to alter the functioning of these G-proteins. Thus, cholera toxin, produced by *Vibrio cholerae*, ADP-ribosylates $G\alpha_s$ on Arg^{187} which is located in the GTPase domain, causing the loss of its GTPase activity and the consequent constitutive activation of the G protein (Moss and Vaughan, 1977; Cassel and Pfeuffer, 1978; Gill and Meren, 1978; Gilman, 1987; Hamm and Gilchrist, 1996). In contrast, pertussis toxin, from *Bordetella pertussis,* by ADP-ribosylating $G\alpha_i$-1 and $G\alpha_i$-2 on Cys^{351} and Cys^{352}, respectively, at their extreme C-termini, causes the uncoupling of these proteins from their receptors,

thereby preventing their activation (Katada and Ui, 1982b; Katada and Ui, 1982a; Gilman, 1987).

The role of covalent modification of G proteins in disease is not, however, limited to the ADP-ribosylation reactions effected by these bacterial toxins. The observations that $G\alpha_i$ can be phosphorylated *in vitro* by protein kinase C (Katada *et al.*, 1985) and that $G\alpha_i$-2 is phosphorylated in intact hepatocytes in response to a number of agonists (Bushfield *et al.*, 1990b), have been shown to have pathophysiological relevance. Thus the induction of diabetes in rats by streptozotocin is associated with the phosphorylation of $G\alpha_i$-2 and the consequent inactivation of this G protein subunit (Bushfield *et al.*, 1990a). A similar phosphorylation was observed in obese Zucker rats (Bushfield *et al.*, 1990c). This modification has been shown to occur at the protein kinase C phosphorylation site (Morris *et al.*, 1996) and is considered to be the principal change underlying the observed aberrant functioning of G_i in these animals (Houslay, 1994). Interestingly, in transgenic animals where the expression of $G\alpha_i$-2 was oblated solely in the liver, hyperglycaemia was evident (Moxham and Malbon, 1996). This indicates that $G\alpha_i$-2 is important in glucose homeostasis.

1.2.3. G protein subunit structure and lipid modification

The G protein α subunits are a family of proteins of 39-52 kDa, which can be divided according to their amino acid sequences into four classes, named $G\alpha_s$, $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12}$.

Differential co- and post-translational processing occurs on these monomers, including myristoylation and palmitoylation (Wedegaertner *et al.*, 1995). Thus those G protein α subunits belonging to the G_i subfamily are co-translationally myristoylated at an amino-terminal glycine residue (Buss *et al.*, 1987; Jones *et al.*, 1990; Mumby *et al.*, 1990) and most are also palmitoylated at the cysteine residue nearest the amino terminus (McCallum *et al.*, 1995; Wedegaertner *et al.*, 1995). Such lipid modifications, like the association with Gβγ, are believed to contribute to the membrane attachment of Gα (Milligan *et al.*, 1995b). It has been suggested that the palmitoylation of Gα, which is

known to be reversible, may, by changing the affinity of the subunit for the plasma membrane, modulate its signalling (Bigay *et al.*, 1994; Grassie *et al.*, 1994). It is interesting to note in this connection that agonist-induced activation of the β-adrenoceptor can alter the palmitoylation status of the stimulatory G-protein G_s (Degtyarev *et al.*, 1993).

Crystallography, primarily of Gαt and Gαi, has revealed that the Gα subunit consists of two domains. The first is a GTPase domain composed of five α-helices surrounding a six-stranded β-sheet (Noel *et al.*, 1993; Coleman *et al.*, 1994; Lambright *et al.*, 1994; Sondek *et al.*, 1994; Mixen *et al.*, 1995). This domain contains not only the binding site for the guanine nucleotide, but also the sites for the interaction with the receptors, effectors and the βγ heterodimer (Sondek *et al.*, 1994). The second domain is entirely α-helical and is considered, on the basis of its structure, to function as a molecular "lid" that encloses the guanine nucleotide (Noel *et al.*, 1993).

The approximately 36 kDa Gβ subunit consists of an amino-terminal amphipathic α-helical region followed by seven repeating units of approximately 43 amino acids each, which are examples of the sequences known as WD motifs (Neer *et al.*, 1994). Crystal structure analysis has shown that these seven WD repeats form a "β propeller" comprising 7 β sheets (figure 1.2.) (Sondek *et al.*, 1996).

The Gγ subunits share less homology at the level of primary structure than their Gβ counterparts, which suggests that it may be these subunits which determine the functional specificity of the Gβγ complexes. The γ subunits are only 6-9 kDa in size and have been shown to undergo isoprenylation at a carboxy-terminal cysteine residue that is part of the CAAX-COOH sequence (Kalman *et al.*, 1995). This isoprenylation can take the form of either farnesylation or geranylgeranylation depending on the identity of the X residue. Thus it has been shown that a C_{15} farnesyl group is added to proteins in which a Ser or Met residue is found in this position, while proteins which end with a Leu residue undergo, instead, a modification with a C_{20} geranylgeranyl moiety (Kohl *et al.*, 1991; Seabra *et al.*, 1991). Such isoprenylation appears to be necessary for the interaction of

the Gγ subunit with the lipid membrane, the Gα subunit and also with effector molecules (Higgins and Casey, 1994).

1.2.4. Structural characteristics of the heterotrimer, and its interaction with receptors

The crystal structures of $G\alpha_{i1}\beta_1\gamma_2$ (Wall *et al.*, 1995) and $G\alpha_t\beta_1\gamma_1$ (Lambright *et al.*, 1996) suggest that the Gγ subunit lies extended along one side of the β propeller with the Gα subunit located on the opposing side. Extensive non-covalent interactions between the β and γ subunits, including the formation by their α-helical amino termini of a coiled-coil, results in a tight association between these two units (figure 1.2.) (Garritsen *et al.*, 1993; Sondek *et al.*, 1996). The sites of lipid modification of the α and γ subunits are located on their amino- and carboxy-termini, respectively. Since these sites are both located on the same face of the complex, it has been postulated that the lipid moieties may serve to orientate the heterotrimer with respect to the plasma membrane surface (Lambright *et al.*, 1996).

Synthetic peptides have been used previously to map the sites of interaction between the receptor, rhodopsin, and the Gα subunit, transducin (Hamm *et al.*, 1988). More recent studies, using the transducin-βγ subunit complex, have however, indicated that the receptor-G protein interaction is not mediated solely by the Gα subunit, but also by Gβγ interactions (Phillips and Cerione, 1992). Furthermore, there is now evidence, from peptide studies, that the carboxy-terminal region of the Gγ subunit is a significant determinant of the ability of the heterotrimer to couple to receptors (Kisselev *et al.*, 1994).

1.2.5. Stimulation of effectors by G protein α subunits

The initial demonstration that G_s is required for the hormonal stimulation of adenylyl cyclase under physiological conditions (Ross *et al.*, 1978), was followed by the findings that the hormonal inhibition of adenylyl cyclase could be abolished by treating platelet membranes with pertussis toxin and that a subsequent restoration of the hormonal

inhibition could be effected by reconstituting the membranes with purified pertussis toxin substrate (Katada *et al.*, 1984a; Katada *et al.*, 1984b). This protein is now termed G_i.

Much has been learned regarding the functions of individual G proteins since these discoveries. The four classes of Gα subunits mentioned above are now known to stimulate a variety of effector molecules, including a number of second messenger enzymes and ionic channels (table 1.1). It is believed that the effector-binding region of $G\alpha_s$ comprises the α helices 2, 3 and 4 (Neer, 1995) and there is evidence from peptide studies which suggests that the specificity of the interaction may be conferred by the α4 helix (Rarick *et al.*, 1992).

1.2.6. Effector stimulation by βγ heterodimers

For many years it was considered that the activation of effector molecules by G protein heterotrimers was attributable solely to the Gα subunit, and that the βγ dimer functioned merely as a negative regulator. Although the βγ released from an activated heterotrimer can, indeed, deactivate the Gα subunits of other G proteins by forming heterotrimers (Gilman, 1987), this paradigm was fundamentally changed in 1987 by the finding that the βγ dimer could activate the muscarinic K^+ channel (Logothetis *et al.*, 1987). Subsequently, the βγ heterodimer has been shown to be a positive regulator of a large number of other effectors, including adenylyl cyclase II (Taussig *et al.*, 1993; Chen *et al.*, 1995b), phospholipase C-β_2 and β_3 (Camps *et al.*, 1992; Smrcka and Sternweis, 1993; Murthy and Makhlouf, 1995; Stehnobittel *et al.*, 1995), phospholipase A_2 (PLA_2) (Jelsema and Axelrod, 1987; Kim *et al.*, 1989), phosphoinositide 3-kinase (PI3-kinase) (Morris *et al.*, 1995a; Zhang *et al.*, 1995) and β-adrenergic receptor kinase (βARK) (Pitcher *et al.*, 1992b).

In addition, evidence is accruing which supports the ability of the βγ dimer to mediate the activation of the mitogen-activated protein kinase (MAPK) pathways by G_i (and possibly G_q) -coupled receptors (Faure *et al.*, 1994; Inglese *et al.*, 1995; Coso *et al.*, 1996; Sadoshima and Izumo, 1996). The mechanism of this activation is poorly defined, but may involve the Gβγ-induced tyrosine phosphorylation of Shc, leading to an

increased functional association between Shc, Grb2 and Sos (Touhara *et al.*, 1995; Van Biesen *et al.*, 1995; Sadoshima and Izumo, 1996). It has recently been proposed, on the basis of studies involving the expression of a dominant negative mutant form of PI 3-kinase gamma, that this enzyme mediates the activation of the tyrosine kinase by Gβγ (Lopez-Ilasaca *et al.*, 1997). The identity of the tyrosine kinase remains to be ascertained but it has been suggested that it may be the recently discovered protein tyrosine kinase, PYK2 (Lev *et al.*, 1995). Such a mechanism is speculative and is unlikely to represent the only route by which this Gβγ-induced Shc phosphorylation takes place, especially in view of the fact that PYK2 is expressed almost exclusively in the brain (Bourne, 1995). In fact, other tyrosine kinases such as Btk and Tsk have also been shown recently to be βγ-stimulated (Tsukada *et al.*, 1994; Langhans-Rajasekaran *et al.*, 1995). Finally, the MAPK pathway activation by Gβγ may occur via a more direct mechanism, as it has been demonstrated by yeast two-hybrid analysis, and by co-immunoprecipitation, that βγ can interact with Raf, localising this Ser/Thr kinase to the plasma membrane (Pumiglia *et al.*, 1995).

1.3. Adenylyl cyclases

1.3.1. Structural features

The diterpene forskolin has been shown to activate all forms of adenylyl cyclase through an interaction with the catalytic unit of this enzyme. Such a property was exploited to purify adenylyl cyclase to homogeneity through the use of a forskolin affinity resin, and led to the isolation of the full-length cDNA encoding the type I isoform (Krupinski *et al.*, 1989). Subsequently, the cDNAs encoding a number of other isoforms, types II-IX have been isolated by homology cloning under low-stringency conditions (Bakalyar and Reed, 1990; Feinstein *et al.*, 1991; Gao and Gilman, 1991; Katsushika *et al.*, 1992; Premont *et al.*, 1992; Yoshimura and Cooper, 1992; Cali *et al.*, 1994; Watson *et al.*, 1994; Völkel *et al.*, 1996). Based on sequence homology, the isoforms have been classified into three principal subfamilies: the type I-like group (types I, III and VIII), the type II-like group (types II, IV and VII) and the type V-like group (types V and VI) (Mons and Cooper, 1995). The more recently characterised type IX isoform has been ascribed to an independent sub-class on the basis of the degree of divergence of its sequence from the other members of the family (Premont *et al.*, 1996).

While all adenylyl cyclases isolated to date appear to be expressed in neural tissue, many of the isoforms appear to exhibit region-specific expression within the nervous system. Thus, while the type III enzyme is expressed principally in the olfactory neuroepithelium (Bakalyar and Reed, 1990), the recently isolated type VII adenylyl cyclase is expressed in the retinal pigment epithelium (Völkel *et al.*, 1996) and type IX is predominantly expressed in hippocampus, cerebellum and neocortex (Premont *et al.*, 1996).

Adenylyl cyclases are large transmembrane proteins of 1080-1248 amino acids (Cooper *et al.*, 1995). Interestingly, all the isoforms characterised to date exhibit the same inferred topology. This is proposed to consist of two regions, each comprising a transmembrane (M1 and M2) domain that crosses the plasma membrane six times and a

large cytoplasmic (C1 and C2) domain (Cooper *et al.*, 1995; Whisnant *et al.*, 1996; Zhang *et al.*, 1997). The regions containing the highest degree of homology between the isoforms are the cytosolic domains, in contrast to the GPCRs where it is the transmembrane domains that are the most highly conserved regions (Zhang *et al.*, 1997). The complex deduced double motif structure of adenylyl cyclases is reminiscent of that proposed for membrane transporters such as the P-glycoprotein and the cystic-fibrosis transmembrane conductance-regulator (CFTR) chloride channel (Riordan *et al.*, 1989). There is, however, no evidence to date which suggests that the mammalian cyclases might also serve as either channels or transporters.

It is believed on the basis of sequence conservation and expression studies, that the C1 and C2 domains represent the catalytic regions (Tang and Gilman, 1995). Consistent with this is the observation that point mutations in these regions leads to a severe impairment of enzymatic activity and an increase in the K_m for substrate (Tang *et al.*, 1995). Furthermore, an interaction between these two cytosolic domains appears to be a pre-requisite for catalysis, since the independent expression of either of these domains, even in the additional presence of one of the transmembrane regions, is insufficient for the detection of significant enzymatic activity (Tang *et al.*, 1991). However, the co-expression of both a C1 and a C2 domain, tethered by a linker, permits regulated high-level catalytic activity (Tang and Gilman, 1995). It has been observed that within the C1 intracellular domain is a less conserved region named C1b (Taussig and Gilman, 1995). Although deletion of this region did not prevent the assembly of C1 with C2 to form a catalytic enzyme that responded to Gαs and forskolin (Whisnant *et al.*, 1996), point mutations in C1b did interfere with the activation of type I adenylyl cyclase by calmodulin (Wu *et al.*, 1993).

The complex transmembrane nature of adenylyl cyclases has hitherto prevented the elucidation of the three-dimensional structure of these molecules. However, very recently, the C_2 -catalytic region of the rat adenylyl cyclase type II has been crystallised in complex with forskolin (Zhang *et al.*, 1997). Structural analysis of the complex, to a resolution of 2.7 Å, indicates that the catalytic region forms a dimer in which alpha

helices and β-sheets are arranged in a wreath containing a central cleft (Zhang *et al.*, 1997). It is in the hydrophobic pockets situated at either end of this cleft that forskolin appears to bind (Zhang *et al.*, 1997).

1.3.2. Regulation of adenylyl cyclases

All adenylyl cyclase sub-types that have been characterised are positively regulated by both forskolin and GTP-bound Gαs and are inhibited by the adenosine analogues termed P-site inhibitors. Individual isoforms reveal differing regulatory effects by other means, including G-protein βγ complexes, calcium/calmodulin, PKC-mediated phosphorylation and intracellular calcium levels. These are shown in table 1.2.

The marked inhibition of type I adenylyl cyclase by G protein βγ subunits that was observed in SF9 cell membranes (Tang *et al.*, 1991) was later shown, using highly purified proteins, to be due to a direct interaction of adenylyl cyclase with the βγ subunit complex (Taussig *et al.*, 1993). Intriguingly, this inhibitory effect appeared to be specific for the type I isoform, with the demonstration of stimulatory effects elicited by βγ, particularly in the presence of $G\alpha_s$, upon the type II (Tang and Gilman, 1995) and type IV (Gao and Gilman, 1991) isoforms.

It has also been shown that calcium has a stimulatory effect upon types I and VIII (Tang *et al.*, 1991; Cali *et al.*, 1994) but inhibits the activity of types V and VI (Ishikawa *et al.*, 1992; Katsushika *et al.*, 1992; Yoshimura and Cooper, 1992). Recently, it has been demonstrated that elevated calcium also inhibits type III adenylyl cyclase *in vivo* (Wayman *et al.*, 1995), and that this is mediated by activation of the calmodulin (CaM)-dependent protein kinase II (Wei *et al.*, 1996). This mechanism was deduced from the demonstration that not only is this isoform phosphorylated *in vivo* when the intracellular Ca^{2+} concentration is increased, but that both the calcium-stimulated phosphorylation and the inhibition of adenylyl cyclase type III were abrogated by the site-directed mutagenesis of the CaM kinase II consensus site of this isoform (Wei *et al.*, 1996).

The regulation of adenylyl cyclase isoforms by protein kinase C is discussed in a great deal of literature, much of which is contradictory. One prevailing conclusion is,

however, that in intact cells, type II adenylyl cyclase can be stimulated by PKC (Jacobowitz *et al.*, 1993; Lustig *et al.*, 1993). However it is not yet clear whether this activation is a direct or indirect effect of PKC.

In addition, earlier evidence from *in vitro* studies that PKC activation leads to a stimulation of the activity of type V adenylyl cyclase (Kawabe *et al.*, 1994) has recently been confirmed in stably transfected intact cells (Kawabe *et al.*, 1996). The evidence with regard to type I adenylyl cyclase is less clear. Thus while some workers have reported enhanced forskolin- (Choi *et al.*, 1993) or calmodulin- (Jacobowitz *et al.*, 1993) stimulated type I adenylyl cyclase activity in response to activation of protein kinase C, others have been unable to demonstrate such an effect (Yoshimura and Cooper, 1993).

1.4. Receptor desensitization

During the continuous exposure to stimuli, a number of biological processes involving cell surface receptors undergo a regulatory process which results in the attenuation of their function despite the continuous presence of the stimulatory agent. This phenomenon is termed desensitization and has been observed in such diverse biological systems as visual light adaptation (Hargrave and Hamm, 1994), bacterial chemotaxis (Stock, 1994) and yeast mating behaviour (Liggett and Lefkowitz, 1994). It is also widespread amongst GPCRs, having been demonstrated in both intact tissue and cell culture systems. In the case of GPCRs, desensitization can occur by one or more of three principal mechanisms. These are: (1) phosphorylation of the receptor, leading to its functional uncoupling from the G-protein, (2) receptor internalisation (sequestration) to a pool where it cannot generate a signal and (3) a reduction in total cellular receptor number (down-regulation) (Liggett and Lefkowitz, 1994).

Receptor internalisation involves the removal of receptors from the cell surface into endosomes, where the receptors may be dephosphorylated prior to their return to the cell surface or, in the case of prolonged receptor stimulation, where they may eventually be degraded (Von Zastrow and Kobilka, 1992; Pippig *et al.*, 1995). In the human A431 carcinoma cell line internalisation of the β_2-adrenoceptor has been shown to occur at a slower rate, with a half-life of approximately 10 minutes, than was seen for the phosphorylation-induced uncoupling of the response, which has a half-life of between 15 seconds and 2 minutes (Lohse, 1990; Lohse *et al.*, 1990; Roth *et al.*, 1991).

In those systems where it occurs, the even slower process of down-regulation is evident only after 30-60 minutes of receptor stimulation (Hadcock and Malbon, 1991). This reduction in receptor number is achieved, it appears, by agonist-induced lysosomal degradation of the receptors, in combination with reduced receptor mRNA levels, resulting from both reduced transcription (Hosoda *et al.*, 1995) and a shorter half-life of the mRNA (Hadcock *et al.*, 1989).

Phosphorylation of G-protein coupled receptors is now known to be mediated by serine/threonine kinases of two categories: (a) the non-receptor-specific effector kinases (such as protein kinase C and protein kinase A), for which such receptors are likely to form only one of many classes of substrate, and (b) the G-protein coupled receptor kinases (GRKs) whose only known cellular substrates are GPCRs (Lohse, 1993). The characteristics of both of these groups of kinases will be discussed in this section.

1.4.1. Protein kinase C

1.4.1.1. Introduction

Protein Kinase C (PKC), when first discovered in 1977, was considered to be a proteolytically activated serine/threonine kinase whose proteolytic cleavage yielded the active molecule termed Protein Kinase M (PKM) (Inoue *et al.*, 1977). Subsequently, however, a "phorbol ester receptor" was identified and demonstrated to have properties similar to those of PKC (Leach *et al.*, 1983; Sando and Young, 1983). It was later shown that intact PKC possessed kinase activity but its activation was found to require calcium and the presence of the cofactors 1,2-diacylglycerol (DAG) and phosphatidylserine (PS) (Nishizuka, 1984). The requirement for DAG, a product of the phospholipase C-mediated hydrolysis of phosphatidyl inositol 4,5-bisphosphate (PIP_2) (Berridge and Irvine, 1984), suggested that PKC might play an important role in lipid-mediated signalling pathways.

PKC activity is now known to be provided by a family of structurally related serine/threonine kinases. All known PKC isoforms contain 3 or 4 highly conserved and thus presumably functional amino acid stretches (C1-C4), separated by regions of greater variability (V1-V5) (Nishizuka, 1988) (figure 1.3.). These kinases are involved in a variety of signal transduction processes which include proliferation, differentiation and secretion. A total of 12 members of the PKC gene family have been identified to date. The members show differences in their structure, mode of activation, substrate specificity and patterns of expression. They are classified (Asaoka *et al.*, 1992) on the basis of their

requirement for calcium and DAG into three groups: (1) the "classical" PKCs (cPKC-α, βI, βII and γ) which are dependent upon calcium, PS and DAG for their activation; (2) the calcium-independent "novel" PKCs (nPKC-δ, ε, η/L and θ); and (3) the more recently identified calcium- and DAG-independent "atypical" PKCs (aPKC-ι, ζ and λ). PKC-μ will be discussed separately in connection with protein kinase D (see section 1.4.4).

1.4.1.2. Structural features of PKC isoforms

The C1 region, which is common to all known PKC isoenzymes, is the putative binding site for the physiological activators (diacylglycerol and phospholipid) and also for the activating phorbol esters, which are structural analogues of DAG. In both the conventional and novel PKC isoforms this region contains two cysteine-rich zinc finger-like sequence motifs, whereas in the atypical PKCs, such as PKC-ζ, only one such sequence is evident (Ono *et al.*, 1989). Motifs of this kind are known to mediate the DNA-binding of transcription factors such as GAL4, although DNA-binding to PKC remains to be demonstrated. It does appear, however, that the presence of two of these cysteine-rich sequences forms a so-called "zinc butterfly" between His^{102} and Cys^{151} which is necessary for the binding of both DAG and phorbol ester to PKC (Bell and Burns, 1991; Quest *et al.*, 1994).

Classical and novel PKC isoforms show selectivity for binding of phorbol esters with an order of potency for PKC-βII, PKC-γ and PKC-ε of sapintoxin A (Sap A) > 12-deoxyphorbol-13-O-phenylacetate (DOPP) > 12-tetradecanoylphorbol-13-O-acetate (TPA) > phorbol-12,13-dibutyrate (PDBu) (Dimitrijevic *et al.*, 1995). While both DAG and phorbol esters do bind within the cysteine-rich region of the C1 domain, it has been suggested that these two classes of activator may bind rather differently. This conclusion was reached as a consequence of the observation that the activation of PKC elicited by both DAG and phorbol ester together is in excess of that which can be achieved by either of these activators alone and also by the finding that butanol inhibits DAG-induced PKC activity but not that induced by PMA (Slater *et al.*, 1994).

The C1 region also contains, near to its NH_2-terminus, a "pseudosubstrate" sequence. This, owing to its similarity to the postulated PKC phosphorylation site motif, occupies the substrate-binding site in the catalytic domain of the enzyme in its non-activated state (House and Kemp, 1987). Since the serine or threonine in the substrate motif is substituted by an alanine, the pseudosubstrate sequence cannot itself be phosphorylated and thus the enzyme is retained in an inactive state (Orr and Newton, 1994). Consistent with such a function is the observation that synthetic peptides based upon pseudosubstrate sequences, can, indeed, act as inhibitors of activated PKC (House and Kemp, 1987). This activation of PKC by DAG is envisaged as occurring through a conformational change which removes the pseudosubstrate from the active site of the enzyme.

The C2 region contains the putative calcium-binding site of the cPKC isoenzymes. Although it does not contain any classical E-F hand (Van Eldik *et al.*, 1982) calcium-binding motif, it does consist of many acidic amino acids which are likely to participate in calcium binding (Ohno *et al.*, 1987). Analysis of divalent cation binding using GST-PKC fusion proteins suggests, however, that the C1 region alone can bind Mn^{2+}, Mg^{2+} or Ca^{2+} and that it is only Ca^{2+} specificity that is conferred by the C2 region (Luo and Weinstein, 1993). The C2 domain must, nevertheless, contribute to the Ca^{2+} dependency of the cPKCs, since the nPKCs, which possess no C2 region, can be activated in the absence of this cation (Koide *et al.*, 1992; Ogita *et al.*, 1992).

The conserved region C3 contains the ATP-binding site while the phosphate transfer and substrate-binding regions are contained within the C4 region (Hug and Sarre, 1993). Contained within the centre of the phosphate transfer region is the central sequence DFG, which is highly conserved amongst protein kinases (Kemp and Pearson, 1990). Within this amino acid motif, it is the Asp residue which is believed to be directly responsible for phosphate transfer (Kemp and Pearson, 1990).

The C-terminal catalytic domain is separated from the N-terminal regulatory domain by the variable V3 region. The V3 region is a stretch of amino acids which has been demonstrated to be susceptible to proteolysis by trypsin and by the calcium-dependent

neutral proteases calpain I and II, both *in vivo* and *in vitro* (Nishizuka, 1988). In fact, proteolytic cleavage of the molecule at this site produces the constitutively activated C-terminal catalytic fragment mentioned above, termed PKM (Inoue *et al.*, 1977).

It has been shown that the deletion of the regulatory domain from PKC-α leads to not only the constitutive activation of this kinase, but also its nuclear targeting (James and Olson, 1992). Furthermore, it is inferred from the properties of other deletion mutants that such targeting is mediated by the V3 region, which only becomes exposed upon activation of the kinase by DAG or phorbol ester (James and Olson, 1992). Thus, it is conceivable that, *in vivo*, proteolytic cleavage of activated PKCs may lead to the nuclear targeting of the catalytic domain, allowing the latter to phosphorylate nuclear transcription factors.

1.4.1.3. Expression of PKC isoforms

1.4.1.3.1. Tissue distribution of PKC isoforms

The tissue-distribution patterns of individual PKC isoenzymes have been elucidated by the use of Northern and Western blot analyses. It appears that PKC-α, δ and ζ are the most ubiquitous iso-enzymes (Dekker and Parker, 1994) and it has been suggested that these PKC iso-enzymes may serve "house-keeping" functions while other PKC isoforms, which are expressed in only particular differentiated tissues, serve more specialised functions. For example, PKC-γ is found predominantly in the central nervous system (Wetsel *et al.*, 1992), PKC-η is preferentially expressed in skin and lung (Osada *et al.*, 1990) and PKC-θ is expressed predominantly in skeletal muscle (Osada *et al.*, 1992). In addition to tissue-specific expression patterns, it is apparent that within a given tissue the PKC isoform expression patterns vary according to the developmental stage (Nishizuka, 1988).

1.4.1.3.2. Regulation of PKC gene expression

Only limited information is currently available regarding the mechanisms which regulate PKC isoenzyme expression. The promoter regions of the rat PKC-γ (Chen *et al.*, 1990) and the human PKC-β (Niino *et al.*, 1992; Obeid *et al.*, 1992) genes have,

however, been cloned. Both promoters lack a TATA box but possess binding sites for the AP1, AP2 and SP1 transcription factors. Interestingly, the DNA-binding of AP1 can be induced by the phosphorylation of this transcription factor complex by MAP kinase (Meek and Street, 1992), which, itself, is involved in a cascade of phosphorylation that can be initiated by the PKC-α-mediated activation of Raf-1 kinase (Kolch *et al.*, 1993). This implies that PKC-β and PKC-γ transcription may be positively regulated by PKC-α and, possibly, by other PKC isoforms. The PKC-γ promoter contains, in addition, a cAMP response element while the PKC-β promoter contains a CAAT box.

PKC gene expression is regulated not only at the transcriptional level but also by differential splicing. Thus in any given tissue or cell line one of the two PKC-β splice forms, which differ at their 3' ends, is preferentially expressed (Kubo *et al.*, 1987), and differential splicing also plays a role in PKC-ε expression in brain and lung tissue (Schaap *et al.*, 1990).

1.4.1.4. Activators and substrates of the PKC isoenzymes

A great deal of information regarding the biochemical features of the PKC isoenzymes has been obtained by detailed analyses of individual recombinant isoforms purified from transfected COS cells or baculovirus-infected insect cells.

1.4.1.4.1. Activation of PKC isoforms

A pre-requisite for the effector-dependent activation of PKC isoforms is the post-translational phosphorylation of key residues within the catalytic domain by a non-PKC kinase (Pears *et al.*, 1992). Thus PKC-α, when purified from rat brain, is a functional phosphoprotein (Kikkawa *et al.*, 1982) that can be completely inactivated upon treatment with the serine/threonine-specific protein phosphatase 1 (Pears *et al.*, 1992). More recently, site-directed mutagenesis has been used to map the phosphorylated residues in PKC-α to a region in the catalytic domain which contains Thr^{494}, Thr^{495} and Thr^{497} (Cazaubon and Parker, 1993). Phosphorylation of Thr^{497}, in particular, appears to be critical for the generation of functional PKC-α although Thr^{495} may also be involved (Cazaubon *et al.*, 1994).

In addition to the trans-phosphorylation of residues within the catalytic domain PKC-βII has been shown to undergo auto-phosphorylation on the C-terminal residues Thr^{634} and Thr^{641} (Dutil *et al.*, 1994). In contrast to trans-phosphorylation, this auto-phosphorylation is not a pre-requisite for the generation of the functional kinase but rather is dependent upon the presence of the activators of the kinase (Newton and Koshland, 1989).

The current model for the mechanism of activation for the cPKCs envisages that activation is initiated by the binding of calcium to the C2 region of the PKC with the consequent translocation of the protein to the plasma membrane. In this model, DAG and phosphatidylserine (PS), being constitutively present in the membrane, complete the activation process by binding to the C1 region (Bell and Burns, 1991; Gschwendt *et al.*, 1991). This results in the stabilisation of the conformation in which the pseudosubstrate site is not bound by the catalytic domain. In addition, it is possible that the phospholipids which activate PKC may bind, by virtue of their acidic nature, to the released pseudosubstrate site. The latter contains basic sequences which have been shown to bind these phospholipids *in vitro* (Mosior *et al.*, 1990).

The presence of calcium is not necessary in achieving the activation of cPKCs by phorbol esters. These agents mimic the effect of DAG in stimulating the activation of both the cPKC and nPKC isoforms. However, they persist for a greater duration in the cellular membrane than DAG (Gschwendt *et al.*, 1991) and lead to a more prolonged activation of these isoenzymes (Nishizuka, 1995).

The nPKC isoforms, as discussed above, lack the calcium-binding C2 region that is a feature of the cPKCs, and can be activated by DAG and the co-factor PS without the requirement for calcium (Osada *et al.*, 1990; Koide *et al.*, 1992; Ogita *et al.*, 1992).

It is now accepted that DAG is not produced solely by the action of PLC on PIP_2. It appears that the PLC-mediated hydrolysis of PIP_2 gives rise only to an "early", transient, phase of DAG production and that a more sustained, "late", phase of DAG production can occur largely as a consequence of the action of phospholipases C and D on phosphatidylcholine (PC) (Billah and Anthes, 1990). This PC-derived DAG is likely

to be important physiologically as current evidence supports the hypothesis that the sustained activation of PKC is an essential requirement of signal transduction pathways which lead to cell division or differentiation (Aihara *et al.*, 1991; Asaoka *et al.*, 1991).

In contrast to the cPKC and nPKC isoforms, PKC-ζ, which is a member of the aPKC class, is not stimulated by calcium, DAG or even phorbol ester (Nakanishi and Exton, 1992). Instead, this isoform can be activated by phosphatidic acid (Liscovitch and Cantley, 1994), ceramide (Müller *et al.*, 1995), PS, unsaturated fatty acids such as arachidonic acid and by phosphatidyl 3,4,5-trisphosphate (PIP_3) (Nakanishi *et al.*, 1993). Thus the signal transduction pathways in which PKC isoforms might be involved *in vivo* are not limited to those leading to the generation of DAG. They include, for example, the insulin signalling pathway, which leads to the activation of phosphatidylinositol 3-kinase (PI-3-kinase) and the generation of PIP_3 (White and Kahn, 1994).

1.4.1.4.2. Substrate-specificity of PKC

The cPKC isoforms are relatively non-specific Ser/Thr kinases *in vitro*. They can seemingly phosphorylate a wide range of proteins and peptides provided that these contain a motif of the type K/RxxS/TxK/R or K/RxS/TxK/R (Kemp and Pearson, 1990). In contrast, members of the nPKC group display a relatively poor kinase activity towards several efficient substrates of cPKC isoforms, including histone IIIS, myelin basic protein, protamine and protamine sulphate (Hug and Sarre, 1993). There is evidence which suggests that the limited kinase activity of nPKCs towards these substrates may be due to a restrictive effect exerted by the regulatory domain of these kinases rather than an intrinsic property of their catalytic domains. For example, upon the proteolytic removal of the regulatory domain of PKC-ε (Schaap *et al.*, 1990) or of PKC-η (Dekker *et al.*, 1993) by trypsin treatment, the catalytic fragment displays a much enhanced capacity for histone phosphorylation. Furthermore, a chimaeric enzyme, formed by the fusion of the regulatory domain of PKC-ε to the catalytic domain of PKC-γ, exhibited a substrate specificity similar to that of PKC-ε (Pears *et al.*, 1991).

A large number of putative physiological substrates have been revealed by ^{32}P labelling of cells treated with PKC stimulators. The substrates can be divided arbitrarily into the following classes: (1) ion channels and pumps (Winkel *et al.*, 1993; Hell *et al.*, 1994; Torchia *et al.*, 1994) (2) the family of myristoylated alanine-rich C-kinase substrates (MARCKS) which mediate the attachment of cytoskeletal actin to the plasma membrane (Aderem, 1992; Blackshear, 1993) and whose ability to bind calmodulin is abrogated by PKC-mediated phosphorylation (Blackshear, 1993) (3) proteins which regulate gene expression and mitogenesis, such as Raf-1 kinase (Kolch *et al.*, 1993) (4) cell surface receptors such as the epidermal growth factor, insulin, serotonin, nicotinic acetyl choline and β_2-adrenoceptors (Ido *et al.*, 1987; Houslay, 1991; Hsieh *et al.*, 1991) and (5) GTP binding proteins such as G_i-2α (Morris *et al.*, 1996).

It has been proposed that PKC-α can phosphorylate and activate Raf-1 kinase (Kolch *et al.*, 1993), which, in turn, initiates a protein kinase cascade that culminates in the activation of mitogen-activated protein kinases (MAPKs). Consistent with the activation of this growth factor signal transduction pathway by PKC isoforms was the finding that PKC-ε, when stably overexpressed in fibroblasts, functions as an oncogene (Cacace *et al.*, 1993). The action of this isoform in fibroblasts appears to be mediated by its activation of Raf-1 kinase since its oncogenic activity was blocked in fibroblasts which were transfected so as to express a dominant negative Raf-1 mutant, but not in clones which instead expressed a dominant negative Ras mutant (Cacace *et al.*, 1996). In contrast, in T-lymphocytes, PKC appears to activate the MAPK cascade by inhibiting GTPase-Activating Protein (GAP) and thus stimulating the activity of Ras (Downward *et al.*, 1990).

Other recent studies have, in addition, indicated the potential for inhibition of endothelial nitric oxide synthase (NOS) activity by the PKC-mediated phosphorylation of this enzyme (Hirata *et al.*, 1995) and the enhancement of the activity of G protein coupled receptor kinase upon the phosphorylation of the latter by PKC (Chuang *et al.*, 1995) (see section 1.4.5.3).

1.4.2. cAMP-dependent protein kinase (PKA)

1.4.2.1. The structure, regulation and distribution of PKAs

The cyclic AMP-dependent protein kinase (PKA), a serine/threonine kinase, exists in its inactive state as a tetrameric holoenzyme comprising two regulatory (R) and two catalytic (C) subunits (Taylor, 1989). Activation of the holoenzyme, consequent upon the binding of two cAMP molecules to each of the regulatory subunits, results in the dissociation of the latter from the catalytic subunits, thus permitting the free diffusion and activity of the C subunits (Krebs and Beavo, 1979; Taylor, 1989). Two major isoforms of PKA exist, type-I and type-II, classified according to the nature of their regulatory subunits. These subunits exist in two forms, RI and RII, which are distinguished by their amino acid sequence, molecular weights, immunoreactivity and affinity for cAMP analogues. In addition, two alternative isotypes of each of these R subunit isoforms exist, namely RI (α and β) and RII (α and β). Moreover, the catalytic subunit exists in three forms, C(α, β and γ) (Showers and Maurer, 1986; Uhler *et al.*, 1986a; Uhler *et al.*, 1986b; Beebe *et al.*, 1990). Of these, the α and β species are the mostly highly homologous, with 93% identity at the amino acid level (Showers and Maurer, 1986; Uhler *et al.*, 1986a; Uhler *et al.*, 1986b). The γ form exhibits only 83% amino acid identity to α, and 79% to β (Beebe *et al.*, 1990).

The different forms of these regulatory and catalytic subunits do appear to possess distinct properties. For example, while the RI isoform exhibits a wide tissue distribution, RII is only expressed in a limited number of tissues (Rubin *et al.*, 1972; Corbin *et al.*, 1977). The RI and RII isoforms also display differences with regard to their intracellular locations. Thus, while RI isoforms are found predominantly in the cytosolic compartment of the cell, the RII isoforms are generally associated with cellular structures, including the golgi apparatus and the cytoskeleton (Nigg *et al.*, 1985; Salavatori *et al.*, 1990). This localisation is, at least in part, mediated by A-kinase anchoring proteins, as discussed below.

The catalytic subunit isoforms, like the regulatory monomers, differ with respect to their tissue distribution, with the γ species being restricted to testis and the α and β forms being widely expressed (Beebe *et al.*, 1990). Furthermore, the α and β isoforms appear to differ from the γ isoform with respect to their substrate specificity. For instance, the latter, unlike the α and β isoforms, more readily phosphorylates histone than the heptapeptide substrate, Kemptide (Beebe *et al.*, 1992). In addition, the histone kinase activity of the γ catalytic isoform is inhibited by the protein kinase inhibitor (PKI) protein to a much lesser extent than is that of the α isoform (Beebe *et al.*, 1992).

1.4.2.2. A-kinase anchoring proteins (AKAPs)

It is now known that the type-II PKA holoenzyme is localised to specific subcellular regions by binding to specific A-kinase anchoring proteins (AKAPs) (Scott and Carr, 1992). The high affinity interaction of PKA-RII with these adaptor proteins requires the prior dimerisation of the RII monomer (Luo *et al.*, 1990; Scott *et al.*, 1990) and appears to be mediated by the NH_2-terminal 5 amino acids of each RII subunit, of which the isoleucines at positions 3 and 5 serve as the principal sites of contact (Hausken *et al.*, 1994).

AKAPs contain additional binding sites which mediate their interaction with cellular structures. Such domains, for example, target AKAP95 to the nucleus (Coghlan *et al.*, 1994), AKAP75 to the cytoskeleton (Glantz *et al.*, 1993) and AKAP79 to post-synaptic densities (Faux and Scott, 1996). Furthermore, it has been shown that AKAP79 binds not only PKAII, but also the Ca^{2+}/calmodulin-dependent protein phosphatase 2B, calcineurin (Coghlan *et al.*, 1995) in addition to PKC-α and PKC-β isoforms (Klauck *et al.*, 1996). The formation of such a signalling complex may permit the concerted action of the three co-localised multisubstrate enzymes, PKA, PKC and calcineurin, on individual substrate molecules, in response to the distinct second messenger signals: cAMP, Ca^{2+}/phospholipid and Ca^{2+}/calmodulin, respectively.

1.4.2.3. The substrate specificity of PKA

PKA is the prototype multisubstrate enzyme. This feature, which it shares with many other kinases including PKC and cascin kinase II, distinguishes it from such kinases as phosphorylase kinase, which exhibit a higher degree of selectivity with respect to their substrates (Walsh and Van Patten, 1994). The activation of PKA, therefore, results in the phosphorylation of numerous protein substrates. For example the mechanism by which an increase in the rate and force of cardiac contractility results from β-adrenergic stimulation of heart muscle, involves the PKA-mediated phosphorylation of several proteins, including troponin I, phospholamban, sarcolemma p27, phosphorylase kinase and glycogen synthase (England, 1975; England, 1976; Walsh *et al.*, 1979; Huggins and England, 1983; Angelos *et al.*, 1987).

The activity of PKA towards potential substrates is, however, influenced by several factors including not only the localisation of PKA and its substrate, but also the conformation of the substrate and the amino acid sequence surrounding the candidate phosphoacceptor residues. Thus most PKA substrates contain the amino acid motif RRxS*x, where S* represents the phosphoacceptor serine (Kemp and Pearson, 1990). A conformational change induced by covalent modification of a protein may affect its ability to undergo PKA-mediated phosphorylation. An example of such a phenomenon, termed hierarchical phosphorylation (Roach, 1991), is the requirement for phosphorylation of one site on phosphorylase kinase by PKA for the subsequent phosphorylation of another site on the same enzyme by PKA (Ramachandran *et al.*, 1987). The efficacy of substrate utilisation by PKA has been shown, by extensive studies of the phosphorylation of pyruvate kinase, to correlate with the occurrence of a pair of arginine residues at positions -2 and -3 relative to a potential phosphoacceptor serine (Kemp *et al.*, 1977; Feramisco *et al.*, 1980). In fact the heptapeptide, Kemptide (LRRASLG), is as efficacious a substrate for PKA as is pyruvate kinase, the protein from which it was derived (Pilkis *et al.*, 1980).

In addition to phosphorylation of enzymes which are involved in metabolic processes, PKA is known to phosphorylate both hormone receptors and DNA-binding

proteins. The catalytic subunits of PKA also possess the ability to enter the nucleus and thereby to modulate gene transcription. The mechanism by which this occurs involves the phosphorylation of transcription factors such as the cyclic AMP-responsive element binding protein (CREB), which are also known as members of the activation transcription factor (ATF) family (Karin and Smeal, 1992). Although such phosphorylation does not increase the ability of these BZip proteins to bind to cyclic AMP-responsive elements (CREs), it does potentiate their ability to promote transcriptional activation, by causing a conformational change that enhances the interaction of CREB/ATF with TFIID, the TATA-binding factor (Meek and Street, 1992). The ability of PKA to phosphorylate membrane-bound receptors will be discussed below.

1.4.3. G-protein coupled receptor desensitization by PKC and PKA

An important group of substrates of the second-messenger activated kinases, protein kinases A and C, are the G-protein coupled receptors (GPCRs). G-protein coupled receptors, upon phosphorylation by the action of these kinases, become functionally uncoupled from G-proteins. The phosphorylation of such receptors constitutes part of a negative feedback mechanism (Benovic *et al.*, 1985).

The prototypical receptor used in studies of this type of desensitization has been the β_2-adrenergic receptor, whose phosphorylation has been studied extensively (Sibley *et al.*, 1984; Benovic *et al.*, 1985; Bouvier *et al.*, 1987; Bouvier *et al.*, 1989; Hausdorff *et al.*, 1989; Johnson *et al.*, 1990; Okamoto *et al.*, 1991; Roth *et al.*, 1991; Pitcher *et al.*, 1992a; Chuang *et al.*, 1995). This receptor contains two consensus phosphorylation sites for PKA: one in the N-terminal part of the C-terminal tail region and one in the C-terminal region of the third intracellular loop. The latter is the preferred phosphorylation site (Bouvier *et al.*, 1989; Clark *et al.*, 1989) and its phosphorylation is sufficient to impair the activation of G_s by the receptor (Pitcher *et al.*, 1992a). PKC also phosphorylates the β_2-adrenergic receptor, at the same sites utilised by PKA, again with

a preference for the site in the C-terminus of the third intracellular loop (Bouvier *et al.*, 1987; Johnson *et al.*, 1990; Pitcher *et al.*, 1992a). Neither PKA nor PKC-mediated desensitization appears to involve the binding of β-arrestin, which interacts at an equally low level with β_2-adrenergic receptors phosphorylated by either of these kinases as it does with the control receptors (Lohse *et al.*, 1992; Pitcher *et al.*, 1992a).

The use of heparin and PKI to inhibit GRK2 and PKA, respectively, has permitted the analysis of the rate of desensitization of the β_2-AR that is mediated by each of these two kinases in human A431 carcinoma cells (Roth *et al.*, 1991). It was determined by this means that PKA-mediated desensitization was considerably less rapid than that mediated by GRK2. In these cells, in the presence of 10 μM isoproterenol, the $t_{1/2}$ for each process was 2 minutes and <15 seconds, respectively (Roth *et al.*, 1991). These values are, however, likely to differ in other cell types, where the expression levels of receptors, kinases and other proteins will be different.

While PKA mediates the desensitization of a number of other GPCRs such as the PGE1 receptor (Clark *et al.*, 1988) and the peripheral DA1-dopamine receptor (Bates *et al.*, 1991), the occurrence of such a process is not observed in other systems, such as the β_3-adrenergic receptor, which contains no PKA consensus phosphorylation site (Nantel *et al.*, 1993). In an analogous manner, many receptors which lead to the activation of PKC can undergo desensitization mediated by the latter kinase, in addition to GRK-mediated desensitization, which is discussed below. Such receptors include the H_1-histamine receptor (Leurs *et al.*, 1991; Smit *et al.*, 1992), the thrombin receptor (Brass, 1992), the $5HT_2$ -serotonin receptor (Kagaya *et al.*, 1990), the parathyroid hormone receptor (Dunlay and Hruska, 1990), the angiotensin II receptor (Sakuta *et al.*, 1991; Oppermann *et al.*, 1996) and the glucagon-like peptide-1 (GLP-1) receptor (Widmann *et al.*, 1996).

1.4.4. Protein kinase D

Protein kinase D (PKD) is a serine/threonine protein kinase with several similarities to PKC but which possesses, in addition, several distinct structural and functional characteristics which distinguish it from PKC isoforms (figure 1.3).

1.4.4.1. Cloning of PKD and its expression in mouse tissues

The use of PCR oligonucleotides specific for conserved regions within the coding sequences of tyrosine kinase family members (Wilks, 1989) led, fortuitously, to the identification of a product of a novel putative serine/threonine kinase. The entire coding sequence, elucidated from cDNA clones isolated from a mouse lung cDNA library was found to encode a protein of 918 amino acids with a predicted molecular weight of 102 kD (Valverde *et al.*, 1994). This protein was named protein kinase D (PKD) (Valverde *et al.*, 1994). Northern blotting of RNA extracted from mouse tissues demonstrated that the expression of PKD is highest in lung and brain, with intermediate levels of expression in skeletal muscle, testis, heart and kidney and low levels in liver and spleen (Valverde *et al.*, 1994). In addition, Western blotting was undertaken following the immunoprecipitation of the kinase from Swiss 3T3 cells and mouse embryo fibroblasts with a polyclonal antiserum raised against a synthetic 15-residue peptide corresponding to part of the C-terminal region of the protein. The results showed that PKD migrates as a single band with an apparent molecular weight of 110 kD (Valverde *et al.*, 1994).

1.4.4.2. Structural features of PKD

The protein possesses a modular structure, consisting of catalytic and regulatory domains (figure 1.3). The former encompasses the region between residues 589 and 845, which conforms to the protein kinase consensus composed of 11 distinct sub-domains (Hanks and Quinn, 1991; Hanks and Hunter, 1995). Sequence comparisons indicate that PKD is distantly related to Ca^{2+}-regulated kinases but is not a member of any of the recognised protein kinase sub-families (Rozengurt *et al.*, 1995). For example, the PKD amino acid sequence exhibits the unusual feature, of the substitution in PKD of a cysteine for the arginine residue that is highly conserved in many protein kinases in the

HRDL motif of subdomain VIB (Rozengurt *et al.*, 1995). The PKD catalytic domain is most highly homologous to that of myosin light-chain kinase (MLCK) of *Dictyostelium* (41% identity), but is also homologous to calcium/calmodulin dependent protein kinase (CaM kinase) types II and IV, PKA, and phosphorylase B kinase, in decreasing order of homology (Valverde *et al.*, 1994). It is notable, however, that the catalytic region of PKD exhibits only a low level of homology with the conserved regions of PKC isoforms, with marked sequence differences in subdomains I, II-III, VI and VII. Interestingly, several differences between PKD and a PKC consensus are apparent in a motif, xxDLKxxN/D, within subdomain VI, which has been shown to be invariant in all cPKC, nPKC and aPKC isoforms (Rozengurt *et al.*, 1995). This motif is believed to play an important role in orienting the peptide substrate in order to permit subsequent catalysis (Knighton *et al.*, 1991; Rozengurt *et al.*, 1995). An implication of these differences is that PKD may have a substrate specificity that is distinct from the members of the conventional, novel and atypical classes of PKC isoforms.

1.4.4.3. Functional differences between PKD and PKCs

It has been demonstrated that PKD isolated from transfected COS cells (Van Lint *et al.*, 1995) and a bacterially expressed fusion protein containing the catalytic domain of PKD (Valverde *et al.*, 1994), both display only low levels of kinase activity towards a variety of substrates utilised by PKCs, including histone, protamine and a synthetic peptide (ERMRPRKRQGSVRRRV) based upon the sequence of the PKC-ε pseudosubstrate region. PKD is also unable to phosphorylate either myelin basic protein, which is a substrate both of PKCs and of CaM II, or G peptide, which contains a PKA consensus site (Valverde *et al.*, 1994). In marked contrast, PKD catalyses a high level of phosphorylation of a synthetic peptide, syntide 2 (PLARTLSVAGLPGKK), which is phosphorylated by PKD exclusively on the serine residue (Valverde *et al.*, 1994). Intriguingly, PKD is unable to phosphorylate a syntide-2 variant (PLAATLSVAGLPGKK), which contains an alanine residue instead of the arginine at position 4 (Van Lint *et al.*, 1995). Thus it is possible that a requirement for

phosphorylation by PKD is a basic residue upstream of the phosphoacceptor serine. Additional requirements are likely to be important, however, since an arginine was present in the same relative position in the PKC-ε-derived pseudosubstrate peptide, which PKD was unable to phosphorylate (Valverde *et al.*, 1994; Van Lint *et al.*, 1995).

1.4.4.4. Regulatory domain of PKD

A prominent feature of the amino-terminal, regulatory domain of the deduced amino acid sequence of PKD is the presence of a cysteine-rich repeat sequence (H-X_{12}-C-X_2-C-X_{13}-C-X_2-C-X_4-H-X_2-C-X_7-C-). This sequence is homologous to the tandemly repeated zinc finger-like cysteine-rich sequence which is present in the regulatory region of all known classical and novel PKCs and which confers phorbol ester sensitivity on these iso-enzymes (Ono *et al.*, 1989; Gschwendt *et al.*, 1991; Hug and Sarre, 1993). However, the length of the intervening sequence between the cysteine-rich domains varies greatly. It ranges from 28 amino acids in conventional PKCs to 35-36 amino acids in the novel PKCs and as much as 95 residues in PKD. As in PKCs, the zinc finger-like domains confer high affinity phorbol ester binding on PKD (Valverde *et al.*, 1994). Furthermore, as with PKCs, the activity of immunopurified PKD can, *in vitro*, be stimulated by either DAG or phorbol ester in the presence of phosphatidylserine (Van Lint *et al.*, 1995). Very recent work in Rozengurt's laboratory (Zugaza *et al.*, 1996) has demonstrated that PKD activation can be effected by phorbol esters in a variety of cell types, namely transfected COS cells, Swiss 3T3, NIH3T3 and Rat-1 cells and in secondary mouse embryo fibroblasts.

Besides the spacing of the zinc finger-like domains, there are several other important structural differences in the NH_2-terminal domain of PKD which distinguish this kinase from members of the PKC family (figure 1.3.). These include the presence at the NH_2-terminus of a highly hydrophobic stretch of amino acids, constituting a putative transmembrane domain, and also a pleckstrin homology (PH) domain which is found between the cysteine-rich regions and the catalytic domain. PH domains are regions of approximately 100 amino acids which consist of a beta-barrel capped by an alpha helix

(Yoon *et al.*, 1994). These are present in a variety of signalling and cytoskeletal proteins (Gibson *et al.*, 1994) but are not found in PKCs. It has been shown that PH domains can bind both to PIP_2 and to Gβγ heterodimers (Pitcher *et al.*, 1995b). In fact it has been suggested that it is this binding which mediates the association of the β-adrenergic receptor kinase with the plasma membrane (Pitcher *et al.*, 1995b).

Especially conspicuous is the absence in PKD of any of the typical pseudosubstrate sequences, which are found on the NH_2-terminal side of the cysteine-rich domains in all known mammalian (House and Kemp, 1987; Hug and Sarre, 1993) and yeast (Toda *et al.*, 1993) PKCs. Such sequences consist of an alanine residue, instead of the substrate serine residue, flanked by basic amino acids. These mediate the intra-peptide regulation of PKCs by blocking the access of exogenous substrate to their active sites (Orr and Newton, 1994).

1.4.4.5. The similarity between PKD and PKC-μ

The 912-amino acid kinase termed PKC-μ was isolated from a human cDNA library and shown to be expressed constitutively at a relatively low level in normal tissues but at elevated levels in tumour cell lines (Johannes *et al.*, 1994). In each of the normal human tissues examined by Northern blotting the PKC-μ-specific transcript was present, albeit at very low levels in comparison to other genes. The highest steady state levels were detected in lung and kidney, followed by heart, liver, skeletal muscle, placenta, pancreas and brain (Johannes *et al.*, 1994).

The deduced amino acid sequence of PKC-μ displays a homology to PKD of 92% overall (extending to 98% in the catalytic domain) and exhibits very similar structural features. It was originally believed by Johannes et al. that PKC-μ exhibited neither phorbol ester binding nor phorbol ester-induced activation and it was thus concluded that it is a member of the atypical class of PKCs (Johannes *et al.*, 1994). These authors noted that the level of phorbol ester binding was only 1.2-fold greater in PKC-μ-transfected COS cells than in control cells. In apparent contrast, the corresponding increase in phorbol ester binding in PKD-transfected COS cells observed by Rozengurt's group was

4.8 fold (Van Lint *et al.*, 1995). It should be noted, however, that the experiments performed with PKC-μ used stable COS transfectants whereas those done with PKD utilised a transient transfection strategy. Thus the different levels of expression may have contributed to some extent to the apparent differences in phorbol ester binding. In fact, very recently, it has been shown, by *in vitro* studies using baculovirus-expressed PKC-μ, that this kinase is, under these conditions, activated by phorbol ester (Dieterich *et al.*, 1996). In conclusion, it is highly likely, that PKD and PKC-μ are murine and human functional homologues, and that on the basis of structural characteristics and their sensitivity to phorbol ester, neither of these kinases can be classed strictly as an atypical PKC isoform.

1.4.4.6. Implications of the properties of PKD for cellular signalling

The discovery (Valverde *et al.*, 1994; Van Lint *et al.*, 1995) that PKD is a novel kinase that can be activated by phorbol ester suggests that certain effects of phorbol esters are not mediated by PKC alone. That its activity can also be stimulated by DAG indicates that PKD may mediate some of the effects of extracellular stimuli which promote the hydrolysis of membrane phospholipids by phospholipase C. The activation of phospholipase C can be elicited through both the receptor tyrosine kinase-mediated activation of PLC-γ and through the activation of PLC-β by the GPCR-stimulated release of $G_q\alpha$ (Rhee and Choi, 1992). Thus there are many extracellular stimuli which could be envisaged as being capable of eliciting the activation of PKD.

1.4.5. G-protein coupled receptor kinases

The G-protein coupled receptor kinases (GRKs) constitute a group of kinases whose only known substrates are the GPCRs (Lohse, 1993). In contrast to the effector kinases which, as described above, are known to phosphorylate either the agonist-bound or the agonist-free forms of the receptor, the phosphorylation of a GPCR by a GRK requires agonist occupancy of the appropriate receptor itself (Hausdorff *et al.*, 1990).

Phosphorylation of GPCRs by GRKs occurs in response to a conformational change in the receptor that results from agonist-induced activation. Such a change has been proposed to involve a lateral rearrangement of the transmembrane helices brought about by a charge neutralisation resulting from ligand binding (Oliveira *et al.*, 1994).

The GRK-induced phosphorylation of a particular receptor causes an increase in the affinity of the receptor for a family of cytosolic inhibitor proteins, termed arrestins, which, upon binding to the phosphorylated receptor, cause the uncoupling of the receptors from the G proteins (Lohse *et al.*, 1992; Ranganathan and Stevens, 1995). The arrestin family comprises at least six members which include arrestin-1, arrestin-2 (β-arrestin), arrestin-3 and their splice variants (Premont *et al.*, 1995). Although multiple arrestin forms have been identified, the functional specificity of particular somatic (non-retinal) arrestins remains to be demonstrated.

Intriguingly, it has been shown very recently that the binding of β-arrestin to the βAR promotes an association of the receptor with the protein clathrin. Such an interaction is believed to facilitate the endocytosis of the receptor and thus the interaction of β-arrestin with the βAR may be pivotal not only for receptor-G protein uncoupling but also for receptor sequestration (Menard *et al.*, 1996).

1.4.5.1. The family of GRKs

Six G-protein coupled receptor kinases have now been cloned (Benovic *et al.*, 1989; Benovic *et al.*, 1991; Ambrose *et al.*, 1993; Benovic and Gomez, 1993; Inglese *et al.*, 1993; Kunapuli and Benovic, 1993). Based on their sequence and functional similarities, these can be grouped into three sub-families: (1) rhodopsin kinase (GRK1); (2) the β-adrenoceptor kinase sub-family comprising β-adrenoceptor kinase 1 and 2 (βARK-1 and 2, or GRK2 and GRK3) and (3) the GRK4 sub-family, consisting of GRK4 (IT11), GRK5 and GRK6. Rhodopsin kinase mRNA is found almost exclusively in the rod and cone photoreceptor cells (Lorenz *et al.*, 1991). In contrast to the limited distribution of this kinase, expression of the other GRKs has been demonstrated in a variety of tissues. This is particularly true of GRK2, GRK3 and

GRK6 (Benovic *et al.*, 1991; Benovic and Gomez, 1993), while GRK4 mRNA is found predominantly in testis (Ambrose *et al.*, 1993) and GRK5 mRNA levels are highest in heart, lung and retina (Kunapuli and Benovic, 1993; Premont *et al.*, 1994).

Although, *in vitro*, all six GRKs appear to possess the ability to phosphorylate the βAR, only two have been shown to regulate the function of this receptor, namely GRK2 and GRK3 (Inglese *et al.*, 1993).

1.4.5.2. Membrane attachment

All six GRKs cloned to date possess the same general structure, consisting of a central catalytic domain flanked by relatively well conserved amino and carboxy termini. The approximately 185 residue amino-terminal region is thought to be important for recognition of the activated receptor substrate (Premont *et al.*, 1995). An additional common feature is that the C terminal domain mediates membrane association (Inglese *et al.*, 1993; Pitcher *et al.*, 1995b), which is possibly a pre-requisite for substrate phosphorylation. However, the mechanism by which this region confers membrane attachment is one characteristic which distinguishes the members of the GRK family. Thus, while GRK1 (rhodopsin kinase) is isoprenylated at a CAAX motif at its C-terminus (Inglese *et al.*, 1993), which presumably confers membrane association, GRK2 and GRK3 both possess a C-terminal 125 amino acid region that contains a pleckstrin homology domain (Inglese *et al.*, 1993; Gibson *et al.*, 1994). This domain confers plasma membrane association upon these kinases by virtue of its ability to bind to G-βγ heterodimers and phosphatidylinositol 4,5-bisphosphate (PIP_2) (Pitcher *et al.*, 1995b). Thus, it is likely that the membrane translocation of GRK2 and GRK3 which occurs upon receptor activation is triggered by the release of the βγ heterodimers from the activated G proteins.

Recently, it has been shown that GRK2 exists not only in cytosolic and plasma membrane bound pools but also in association with internal microsomal membranes in a variety of tissues and cell lines (Garcia-Higuera *et al.*, 1994). Furthermore, the kinase is anchored to these internal membranes by both an interaction with G protein components

as described above, and by a postulated association with a membrane anchoring protein by the N-terminal region of the kinase (Murga *et al.*, 1996). The functional role of this pool of GRK2 remains to be determined.

Like GRK2 and GRK3, GRK5 is able to bind membrane phospholipids. However, for GRK5 this is proposed to occur through an interaction of its highly basic C-terminal 46 amino acids with head groups of acidic phospholipids (Premont *et al.*, 1994).

In contrast, GRK6 is targeted to membranes by the palmitoylation of a cluster of three C-terminal cysteines (Stoffel *et al.*, 1994).

GRK4, the only known GRK to exist in multiple splice forms, appears to be membrane associated, even in unstimulated cells (Premont *et al.*, 1995). The mechanism by which membrane attachment is conferred on this kinase, however, remains to be ascertained.

1.4.5.3. Regulation of GRK activity

In the vertebrate retina, the level of active rhodopsin kinase is regulated by the association of this kinase with a 24 kDa acylated protein known as recoverin (or S-modulin) (Dizhoor *et al.*, 1993). This protein, in the presence of Ca^{2+}, binds to rhodopsin kinase and prevents it from phosphorylating light-activated rhodopsin (Dizhoor *et al.*, 1993). Since the intracellular Ca^{2+} concentration is highest in the dark (Yarfitz and Hurley, 1994), this may constitute a molecular basis for dark adaptation, in that, under dim light conditions, recoverin-mediated inhibition of GRK1 would permit longer signalling by any light-activated rhodopsin (metarhodopsin II) molecules. In view of the existence of non-retinal recoverin-like proteins (Chen *et al.*, 1995a) it is conceivable that similar regulatory mechanisms modulate the activity of other GRKs.

As described above, the activities of GRK2 and GRK3 are enhanced by their membrane translocation, which is promoted by the release of Gβγ. It is now known, however, that a further mechanism by which the activity of GRK2 (and possibly GRK3) is regulated is the PKC-mediated phosphorylation and activation of this kinase both *in*

vitro and *in vivo* (Chuang *et al.*, 1995). Whether this phosphorylation potentiates the interaction of the GRK with G protein βγ or phospholipid remains to be ascertained.

1.4.5.4. Receptor specificity

Perhaps as a consequence of the technical difficulty of isolating pure receptor proteins, the ability of individual GRKs to phosphorylate specific G protein-coupled receptors has been assessed for only a small number of such receptors. Those that have been tested as GRK substrates have been members of the largest sub-family of GPCRs, β_2-adrenergic receptor-related proteins. While some members of the gastrointestinal hormone receptor sub-family such as the glucagon receptor (see section 1.5.4) and the glucagon-like peptide-1 (GLP-1) receptor (Widmann *et al.*, 1996) have been shown to undergo an agonist-dependent desensitization process, these receptors have not yet been tested as GRK substrates. From the rather limited studies which have been undertaken so far, it appears that there is no clear specificity of any individual GRK for any particular receptor or class of receptors. Thus, GRK2, the β-adrenergic kinase, has been found to phosphorylate not only the β_2-adrenergic receptor, but also the α_2-adrenoceptor when its cDNA was co-transfected with that encoding GRK2 into COS cells (Kurose and Lefkowitz, 1994). In addition, purified GRK2 has been shown to effect the phosphorylation of the rat substance P receptor (SPR) after the partial purification of the SPR from recombinant baculovirus-infected Sf9 cells and its reconstitution in phospholipid vesicles (Kwatra *et al.*, 1993). More recently, this kinase has also been found to phosphorylate the rat type IA angiotensin II receptor, expressed in transfected 293 cells (Oppermann *et al.*, 1996). It is possible, however, that differential tissue-specific expression of individual GRKs determines which GRKs catalyse the physiological phosphorylation of any particular receptor *in vivo*. While mRNAs encoding several GRKs have been found to be present in heart tissue (Inglese *et al.*, 1993), the levels of expression of the individual kinases in the distinct cell types which constitute that organ remain to be determined.

With regard to the nature of the phosphorylation sites on receptors that are phosphorylated by the GRKs, only in the cases of rhodopsin and the human β_2-adrenergic receptor have the precise sites of receptor phosphorylation been determined. Both *in vitro* and *in vivo*, GRK1 phosphorylates rhodopsin on specific residues located at the distal portion of the carboxy-terminal tail, principally Ser^{338} and Ser^{343} (Palczewski *et al.*, 1991). Although GRKs 1, 2, 3 and 5 all phosphorylate these two serines of rhodopsin, GRK1 and GRK5 preferentially phosphorylate Ser^{338} whereas GRK2 and GRK3 favour Ser^{343} (Palczewski *et al.*, 1995).

From studies undertaken with model peptide substrates, it appears that phosphorylation by GRK2 is favoured by the presence of several acidic residues on the amino-terminal side of the putative phospho-acceptor residue (Onorato *et al.*, 1991). At present, a meaningful GRK consensus phosphorylation site cannot be defined owing to the dearth of data regarding the phosphorylation sites in receptors other than rhodopsin. Very recently, however, the phosphorylation sites in the human β_2-adrenergic receptor have been identified (Fredericks *et al.*, 1996). In a manner analogous to GRK1-mediated phosphorylation of rhodopsin, it is clear that phosphorylation of this receptor by GRK2 and GRK5 takes place at multiple serine and threonine residues, all of which were found to be contained within a peptide comprising the carboxyl-terminal 40 amino acids of the β_2AR (Fredericks *et al.*, 1996). Moreover, in keeping with the implications of the peptide studies mentioned above, both rhodopsin and the β_2AR possess pairs of acidic residues located on the amino-terminal side of the most amino-terminal phosphorylated residue (Fredericks *et al.*, 1996).

1.5. The glucagon receptor

1.5.1. The glucagon receptor gene

Glucagon, a hormone that is secreted by the α-cells of the islets of Langerhans of the pancreas in response to hypoglycaemia, is a key regulator of hepatic glucose production (Sneader, 1996). The glucagon receptor (GR) is a member of the secretin sub-family of G protein-coupled receptors (GPCRs). The rat hepatic GR cDNA, was isolated by expression cloning (Jelinek *et al.*, 1993) and by low-stringency homology cloning using degenerate primers whose sequence was based on consensus cDNA sequences (Svoboda *et al.*, 1993a; Svoboda *et al.*, 1993b). The open reading frame of 1455 nucleotides encodes a 485 amino acid protein with the seven predominantly hydrophobic putative trans-membrane domains which are characteristic of the GPCR superfamily (Houslay, 1992). Interestingly, although the rat hepatic glucagon receptor shares no significant sequence homology with the majority of members of the GPCR super-family, the amino acid sequence of the receptor displays a 47-57% identity, in the core region that excludes the amino and carboxy-termini, with the deduced amino acid sequence of other members of the secretin receptor sub-family (Jelinek *et al.*, 1993; Svoboda *et al.*, 1993a; Svoboda *et al.*, 1993b).

More recently, the receptor has also been cloned from a human liver cDNA library. This was achieved both by the use of degenerate PCR primers (Lok *et al.*, 1994) and by cDNA hybridisation using the rat glucagon receptor cDNA as a probe followed by the technique of rapid amplification of cDNA ends (RACE) (MacNeil *et al.*, 1994). The isolated cDNA was found to encode a protein of 477 amino acids with 80% identity to the rat glucagon receptor (MacNeil *et al.*, 1994). In addition, as a prelude to future transgenic studies of glucagon receptor function, the murine glucagon receptor gene has now been sequenced (Burcelin *et al.*, 1995). Its cDNA, like that of the rat, encodes a 485 amino acid protein, and the two homologues share 93% identity at the amino acid level (Burcelin *et al.*, 1995).

Genomic DNA sequencing (Burcelin *et al.*, 1995) has revealed that while the murine GR promoter region lacks both TATA and CAAT boxes it does, however, possess putative binding sites for transcription factors AP1, AP2, SP1 and HNF1, the latter possibly conferring high transcriptional activity in liver (Xanthopoulos and Mirkovitch, 1993). In addition, the promoter region was noted to contain the c-fos transcriptional regulatory serum response element (c-fos-SRE) (Treisman, 1992). The transcriptional start site was mapped by the RNase protection assay to lie approximately 175 bp upstream of the start codon (Burcelin *et al.*, 1995). Similarly, the promoter region of the human GR which, like the murine gene, lacks TATA and CAAT boxes, contains binding sites for SP1 and AP2 in addition to the liver-specific LF-A1 factor (Buggy *et al.*, 1995a). Identification of the transcriptional start point of the human gene, by primer extension analysis, determined that it lies approximately 475 bases upstream of the start codon (Buggy *et al.*, 1995a).

In contrast to the genes which encode the adrenergic and muscarinic receptors, which are devoid of introns, the rat hepatic glucagon receptor gene contains 11 introns and stretches over 3.8 kb of genomic DNA (Svoboda *et al.*, 1993a; Christophe, 1995). Similarly, the human glucagon receptor gene spans a genomic region of 5.5 kb and is interrupted by some 12 introns (Lok *et al.*, 1994). Southern blot analysis of rat, murine and human genomic DNA revealed a hybridisation pattern that was consistent in each case with the existence of only a single copy of the glucagon receptor gene (Lok *et al.*, 1994; Burcelin *et al.*, 1995; Christophe, 1995).

Despite the existence of multiple exons in the glucagon receptor gene, Northern and rtPCR analyses of rat and murine tissue RNA, respectively, have detected the presence of only a single glucagon receptor-specific transcript of 2.3 kb with no evidence for alternative splicing (Yoo-Warren *et al.*, 1994; Burcelin *et al.*, 1995). Interestingly, Northern blot analysis of rat glucagon receptor expression indicated that the gene was expressed in both kidney and heart (Yoo-Warren *et al.*, 1994). Although no transcripts were detected in this particular study in fat, brain, lung, heart and muscle (Yoo-Warren *et al.*, 1994) the control, ribosomal RNA probe showed that the quantity of RNA loaded in

each lane was markedly different. In particular, significantly weaker control signals were detected in the fat and heart RNA samples, thus calling into question the authors' inferences of tissue specificity from these results.

1.5.2. Structural aspects

A notable feature of the rat hepatic glucagon receptor is the N-terminal region. This contains a hydrophobic sequence which constitutes a putative signal sequence and also four potential asparagine-linked glycosylation sites at positions 47, 60, 75 and 79. In fact, the hypothesis that the receptor is glycosylated is supported by the observation that the apparent molecular weight, on denaturing gels, of the intact glucagon receptor is 7 kDa greater than the predicted value of 54.9 kDa. It is also indicated by the finding that its size is reduced by treatment with endo-N-acetylglucosaminidase F (Iyengar and Herberg, 1984). Another feature of the primary sequence of the GR is the presence of eight cysteine residues (C-8, 20, 44, 59, 68, 82, 101 and 122) which could form intra-molecular disulphide bonds.

The regions of the glucagon receptor which determine glucagon binding specificity have recently been delineated by the analysis of a series of chimaeric receptor constructs formed by replacing various domains of the human glucagon receptor with the homologous regions of the glucagon-like peptide I receptor (Buggy *et al.*, 1995b). Binding studies performed following the expression of these mutants in COS-7 cells revealed that the ligand binding specificity is determined by the membrane-proximal half of the amino-terminal region, the sixth trans-membrane domain and one or more of the following regions: the first extracellular loop, and the third and fourth transmembrane domains (Buggy *et al.*, 1995b). A separate study has been undertaken since then in which chimaeras were constructed by the transposition of the amino-terminal domains of the human calcitonin and glucagon receptors. It was observed that neither of these mutant receptors, when stably transfected into BHK cells, led to specific glucagon binding or to a glucagon-induced elevation of intracellular cAMP (Stroop *et al.*, 1995). This lends further support to the implication that the amino-terminal extracellular region

of the glucagon receptor is necessary but not sufficient to confer specific glucagon binding upon the glucagon receptor.

More recently, polyclonal antisera have been raised against peptides corresponding to the first extracellular loop and the membrane-proximal region of the amino-terminal tail of the GR (Unson *et al.*, 1996). Each of these antisera, when incubated with rat liver membranes, was found to block the binding of glucagon to its receptor and to attenuate the hormone-stimulated adenylyl cyclase response (Unson *et al.*, 1996). These findings, which suggest that these two regions of the GR contain important determinants of glucagon binding, are thus consistent with the conclusions of the studies using chimaeric receptor constructs (Buggy *et al.*, 1995b) mentioned above.

In addition to the construction of chimaeric receptors, the delineation of the ligand binding site of the glucagon receptor has been approached by other techniques involving the mutagenesis of the receptor. Site-specific mutagenesis has thus been used to mutate Asp^{64} in the amino-terminal extracellular region of the rat glucagon receptor to Glu, Asn, Lys or Gly (Carruthers *et al.*, 1994). Asp^{64} was chosen on account of its alignment with Asp^{60} in the hypothalamic growth hormone releasing factor (GRF) receptor, which is mutated in the *little* mouse phenotype (Lin *et al.*, 1993). The finding that any of the above replacements of Asp^{64} in the rat glucagon receptor led to a complete inability of the receptor to bind glucagon, was interpreted as an indication that this residue may directly interact with the hormone (Carruthers *et al.*, 1994). The possibility, however, that such a mutation caused a conformational disruption of the extracellular domain of the receptor, can not be excluded.

Another method by which the determinants of the glucagon binding site of the receptor have been analysed is the creation of a series of deletion mutants of the glucagon receptor cDNA (Unson *et al.*, 1995). The deletion of a large part of the amino terminal region abrogated glucagon binding. In addition, however, deletion of any of the seven putative transmembrane segments led to a similar failure to bind glucagon (Unson *et al.*, 1995). It is conceivable, however, that any such deletion could have caused gross conformational changes in the tertiary structure of the receptor.

The cytoplasmic carboxyl terminus of the GR contains three cysteine residues, one of which may be palmitoylated and thus mediate the attachment of the C-terminal tail to the plasma membrane (Svoboda *et al.*, 1993a). Interestingly, a deletion of all but the proximal ten amino acids of the carboxy-terminal tail of the glucagon receptor failed to prevent glucagon-stimulated cAMP accumulation, implying that while the C-terminal tail may regulate the coupling efficiency, the greater part of this region is not directly necessary for G_s activation (Unson *et al.*, 1995). That the third intracellular loop is likely to play an important role in mediating the receptor-G_s interaction is supported by the presence, at the carboxy-terminal end of this loop, of the amino acid motif R-L-A-R, found in the rat and murine sequences (Jelinek *et al.*, 1993; Svoboda *et al.*, 1993b; Burcelin *et al.*, 1995) and R-L-A-K, found in the human sequence (Lok *et al.*, 1994). Such a motif, Basic-(L/A)-(L/A)-Basic, matches the sequence K-A-L-K which is present at this location in the β_2AR and whose basic residues have been shown to be important for coupling to $G_s\alpha$ (Okamoto *et al.*, 1991). It is unlikely however, that this short motif is the only region of the receptor that is required to effect coupling to G_s. In this respect it is notable that the C-terminal half of the seventh trans-membrane domain and the beginning of the intracellular C-terminal tail of the receptor conforms to the consensus sequence F-Q-G-Hydrophobic-Hydrophobic-V-A-X-Hydrophobic-Y-C-F-X-N-X-E-V-Q/R which is found at this position in all of the receptor sequences of the secretin receptor sub-family (Lok *et al.*, 1994).

Another region that is highly conserved among the members of this receptor sub-family is the first intracellular loop. At the time of writing, the structure-function relationships of these regions of the GR remain to be investigated. In the calcitonin receptor, however, it appears that the structure of the first intracellular loop is a determinant of whether the receptor is able to couple to $G_{q/11}$. The calcitonin receptor, which, like the GR, is a member of the secretin receptor sub-family, exists as two isoforms, calcitonin receptor 1 (CTR1) and calcitonin receptor 2 (CTR2) (Gorn *et al.*, 1992). While both isoforms can stimulate the production of cAMP, only CTR2 can also stimulate IP_3 production (Gorn *et al.*, 1992). It has therefore been suggested that a

supplementary stretch of 16 amino acids that is present in the first intracellular loop of CTR1, but not in that of CTR2, inhibits the formation of IP_3 production (Nussenzveig *et al.*, 1994). Indeed it has been found that the insertion of the nucleotide sequence encoding this stretch into the corresponding position in the CTR2-encoding cDNA results in the loss of the ability of this isoform to stimulate IP_3 production (Nussenzveig *et al.*, 1994). Like CTR2, the GR can stimulate the production of IP_3 (see section 1.5.3.). The absence of such a 16-residue stretch from the first intracellular loop of the GR (figure 1.4.) is thus consistent with the possible importance of this loop in the coupling of the GR to $G_{q/11}$.

1.5.3. Effector systems of the glucagon receptor

Glucagon binding causes the glucagon receptor to interact with G_s, the alpha subunit of which, upon dissociation from beta/gamma subunits, subsequently activates adenylate cyclase. The consequent elevation of intracellular cAMP leads, in liver cells, to the stimulation of both gluconeogenesis (Exton *et al.*, 1969; Johnson *et al.*, 1972) and glycogenolysis (Exton *et al.*, 1970). From analyses of the signalling consequences of glucagon exposure, however, evidence is accruing that glucagon may elicit cellular responses through the activation of at least two G protein-mediated signal pathways in hepatocytes. For example, like PTH (Abou-Samra *et al.*, 1992) and calcitonin (Chabre *et al.*, 1992), both of which bind to receptors closely related in structure to the glucagon receptor, glucagon has been demonstrated to cause not only an increase in cAMP production but also a significant increase in the level of intracellular calcium (Charest *et al.*, 1983; Mauger *et al.*, 1985; Sistare *et al.*, 1985; Blackmore and Exton, 1986; Mine *et al.*, 1988). The elevation of intracellular Ca^{2+} is likely, however, to be principally due to a cAMP-dependent calcium influx rather than a release of calcium from intracellular stores, as although glucagon has been shown to cause an increase in inositol phosphates in hepatocytes, the degree of elevation is small (Wakelam *et al.*, 1986; Whipps *et al.*, 1987; Unson *et al.*, 1989).

In addition to eliciting the elevation of intracellular calcium and inositol phosphates, glucagon has been demonstrated to elicit the generation of DAG (Boeckino *et al.*, 1985; Pittner and Fain, 1991) and to effect a stimulation of protein kinase C (Pittner and Fain, 1991; Tang and Houslay, 1992). Glucagon has also been shown to stimulate the hydrolysis of the membrane lipid phosphatidylcholine (Pittner and Fain, 1991). In such studies, in which rat hepatocytes were exposed to 10 nM glucagon, it was observed that the release of choline was greater than that of phosphocholine or of glycerophosphocholine, indicating a predominant phospholipase D activation with a possible additional activation of phospholipases C and A_2, albeit to a lesser extent (Pittner and Fain, 1991).

In conclusion, glucagon does not merely elicit an elevation in the level of intracellular cAMP. The hormone also stimulates the hydrolysis of both phosphatidylcholine and PIP_2 and the activity of PKC. In addition, the hormone is known to elicit an elevation in the levels of DAG and intracellular Ca^{2+}. It is likely, therefore, that the GR couples not only to $G_s\alpha$, but also to other G-proteins such as G_q or G_{11}.

1.5.4. Glucagon receptor desensitization

1.5.4.1. The timecourse for glucagon-stimulated intracellular cAMP accumulation and its implications

Glucagon binds to specific receptors on the external surface of hepatocytes (Rodbell *et al.*, 1971c; Sonne *et al.*, 1978) and exerts its cellular effects through the activation of adenylate cyclase (Birnbaumer *et al.*, 1971; Pohl *et al.*, 1971; Rodbell *et al.*, 1971a; Rodbell *et al.*, 1971c; Rodbell *et al.*, 1971b; Houslay *et al.*, 1980). Exposure of rat hepatocytes to glucagon causes an increase in intracellular cAMP accumulation which is rapid, reaching a maximum value of 10-20 fold above basal levels within 6 minutes (Johnson *et al.*, 1972; Pilkis *et al.*, 1975) and is dose-dependent with an EC_{50} for

glucagon of 1-2 nM in the presence of PDE inhibitors (Christoffersen and Berg, 1974; Sonne *et al.*, 1978; Heyworth *et al.*, 1983).

This increase in the intracellular level of cAMP is, however, short-lived. In the presence of the phosphodiesterase inhibitor, IBMX, a gradual decline in cAMP levels to approximately 50% of maximal stimulated values occurs subsequent to the peak of cAMP accumulation (Heyworth *et al.*, 1983; Murphy *et al.*, 1987). Similar results were also obtained in preparations of parenchymal liver cells in the presence of the PDE inhibitor, theophylline (Christoffersen and Berg, 1974). The decline in intracellular cAMP is not thought simply to reflect an increased rate of loss of cAMP to the extracellular environment as only a small proportion of the total cAMP is detectable in the incubation medium (Christoffersen and Berg, 1974; Heyworth *et al.*, 1983) and the rate of exit of cAMP actually decreases after the observed maximum intracellular cAMP accumulation (Pilkis *et al.*, 1975). Nor is it thought to be due to hormone degradation as further additions of glucagon could not overcome the desensitization (Murphy *et al.*, 1987). Rather it is believed that it reflects an underlying profound attenuation of the activity of adenylate cyclase (Heyworth and Houslay, 1983; Newlands and Houslay, 1991; Houslay, 1994).

In the absence of PDE inhibitors, although the cAMP accumulation is again maximal at 5-6 min after glucagon addition, the magnitude of the cAMP increase is reduced by 3-5 fold, and the cAMP level returns fully to basal levels within 20-30 min (Johnson *et al.*, 1972; Heyworth *et al.*, 1983). The additional decline observed when PDE inhibitors are absent reflects an increased metabolism of cAMP by phosphodiesterases and the activation of type III PDE by a protein kinase A mediated mechanism (Heyworth *et al.*, 1983; Houslay, 1994).

1.5.4.2. Glucagon receptor desensitization elicited by pre-exposure to the hormone

The glucagon-stimulated adenylate cyclase activity in membranes isolated from hepatocytes exhibits a profound inhibition of the response if the cells have been pre-

exposed to glucagon (Plas and Nunez, 1975; DeRubertis and Craven, 1976; Gurr and Ruh, 1980; Heyworth and Houslay, 1983; Noda *et al.*, 1984; Murphy *et al.*, 1987; Murphy and Houslay, 1988; Premont and Iyengar, 1988; Murphy *et al.*, 1989; Savage *et al.*, 1995). The desensitization was dose-dependent with an observed maximum reduction of 30-40% following a pre-treatment period of 5 minutes with a concentration of 10 nM glucagon (Heyworth and Houslay, 1983). Furthermore, treatment of hepatocytes with glucagon has been shown to reduce the elevation in intracellular cAMP that is elicited by the subsequent challenge of the intact cells with this hormone (Savage *et al.*, 1995). This supports the hypothesis that the transience of the glucagon-stimulated intracellular cAMP accumulation described in section 1.5.4.1 reflects the rapid desensitization of the response of adenylate cyclase to glucagon stimulation (Newlands and Houslay, 1991; Houslay, 1994).

Although the glucagon receptor was one of the first adenylate cyclase-linked GPCRs for which agonist-dependent receptor desensitization was described, there are several other receptors of this type for which the phenomenon has been documented. For example the desensitization of both the β-adrenergic receptor-stimulated adenylate cyclase system (Benovic *et al.*, 1989; Lefkowitz, 1996; Lohse *et al.*, 1996) and that of lutropin (luteinizing hormone) (Dix and Cooke, 1982; Dix *et al.*, 1982) have been studied in detail. Moreover, very recently, an agonist-dependent desensitization process affecting another member of the secretin receptor sub-family, the glucagon-like peptide-1 (GLP-1) receptor, has been described (Widmann *et al.*, 1996). In insulinoma cell lines, following the pre-exposure of the cells to GLP-1, the intracellular cAMP accumulation in response to GLP-1 was reduced by 20% and the dose-response curve shifted 3-5 fold rightward (Widmann *et al.*, 1996).

1.5.4.3. Evidence for phosphorylation of the receptor

Studies have been undertaken to investigate the mechanism by which the desensitization of the glucagon-stimulated adenylate cyclase response occurs. It is evident that phosphorylation of a membrane protein is pivotal to the process of

desensitisation as alkaline phosphatase treatment of membranes causes a striking reversal of the desensitization of glucagon-stimulated adenylate cyclase (Savage *et al.*, 1995). Earlier studies have also shown that in the desensitised state, neither NaF-stimulated adenylate cyclase activity, which depends upon G_s-cyclase coupling, nor ^{125}I-glucagon binding is reduced (Santos and Blazquez, 1982; Heyworth and Houslay, 1983). This implies that the mechanism of the desensitization involves the uncoupling of the glucagon receptor from the stimulatory G protein, G_s.

1.5.4.4. The potential role of PKC

In hepatocytes, a similar reduction in the ability of glucagon to stimulate adenylate cyclase activity to that which is induced by pre-exposure to glucagon, can be induced by treatment of the cells with a variety of other agents. Thus, in hepatocytes, activation of lipid signalling pathways by hormones such as vasopressin and angiotensin II (Murphy *et al.*, 1987) and challenge of cells with either the tumour promoting phorbol ester, PMA (Heyworth *et al.*, 1984; Garcia-Sainz *et al.*, 1985; Heyworth *et al.*, 1985; Murphy *et al.*, 1987; Refsnes *et al.*, 1989) or the synthetic diacylglycerols 1-oleoyl-2-acetyl glycerol (OAG) and dihexanoyl glycerol (DHG) (Newlands and Houslay, 1991), have been demonstrated to lead to a profound reduction in the ability of glucagon to stimulate adenylate cyclase activity.

In contrast to TPA, DAG and DAG-elevating hormones, the use of permeant cAMP analogues, such as dibutyryl cAMP, did not induce desensitisation, implying that the cAMP-dependent kinase (PKA) cannot mediate such an effect (Heyworth and Houslay, 1983). Similarly, the rate of the onset of desensitization by glucagon was not enhanced by IBMX, despite the fact that this PDE inhibitor greatly enhanced cAMP accumulation (Heyworth and Houslay, 1983). Furthermore, treatment of hepatocytes with the calcium ionophore A23187 was unable to elicit a reduction of either the adenylate cyclase activation or the intracellular cAMP accumulation stimulated by glucagon (Savage *et al.*, 1995), implying that calcium entry is, in itself, insufficient to cause desensitization.

As discussed above, glucagon has been demonstrated to elicit the elevation of not only intracellular cAMP but also the level of intracellular calcium (Charest *et al.*, 1983; Mauger *et al.*, 1985; Sistare *et al.*, 1985; Blackmore and Exton, 1986; Mine *et al.*, 1988). It has been shown, however, that the increase in intracellular calcium concentration is not necessary for the desensitization as the latter was blocked neither by treatment of cells with EGTA, to chelate calcium, nor by the addition of La^{2+}, which blocks the entry of calcium across the hepatocyte plasma membrane (Barritt and Hughes, 1991).

The ability of glucagon to stimulate the generation of DAG (Boeckino *et al.*, 1985; Pittner and Fain, 1991), together with the evidence described above that treatment of hepatocytes with DAG analogues or hormones which increase cellular DAG levels can attenuate the ability of glucagon to stimulate adenylate cyclase activity, suggests that the mechanism of glucagon and vasopressin-induced glucagon desensitization in hepatocytes is likely to involve a DAG-activated protein kinase. The obvious candidate to propose for such a kinase was protein kinase C (PKC). In this regard it is interesting that the dose-dependence observed for the glucagon-induced desensitization of glucagon-stimulated adenylate cyclase activity in hepatocyte membranes (Ka of 0.45 nM) (Heyworth and Houslay, 1983; Murphy *et al.*, 1987) closely matches that observed for the glucagon-induced stimulation of inositol phospholipid hydrolysis (Ka of 0.25 nM) (Wakelam *et al.*, 1986). Furthermore, glucagon-stimulated PKC activation has been demonstrated in rat hepatocytes (Pittner and Fain, 1991; Tang and Houslay, 1992) and compounds known to inhibit PKC such as chelerythrine, calphostin C and staurosporine have been found (albeit to differential extents) to block the vasopressin and glucagon-induced desensitization (Savage *et al.*, 1995). These observations lend additional support to the conclusion that a DAG-activated kinase, such as PKC, is likely to mediate the desensitization of glucagon-stimulated cAMP accumulation in hepatocytes.

The work described in this thesis aimed, using a model system in which the GR was transiently expressed in COS cells, to address the possibility that PKC does, in fact, trigger the desensitization of glucagon-stimulated cAMP accumulation.

Class	Members	Modifying toxin	Functions	Refs.
α_s	α_s, α_{olf}	Cholera	Stimulates AC	(a)
			Stimulates Ca^{2+} channels	(b)
α_i	α_{i1-3}, α_o, α_{gust}, α_z	Pertussis (except α_z)	Inhibits AC; Stimulates Ca^{2+} and K^+ channels; Stimulates PLC (α_o)	(a), (b), (c)
	α_{t1-2}	Pertussis	Activates cGMP PDE	(d)
α_q	α_q, α_{11}, α_{14-16}		Activates PLCβ (β1+β4 > β2 and β3)	(e), (f), (g)
α_{12}	α_{12}, α_{13}		Stimulates Na^+/K^+ exchange	(h)

Table 1.1. The functions of the G protein α subunits.

Cholera toxin and pertussis toxin catalyse the ADP-ribosylation of an Arg residue and a Cys residue, respectively, of the indicated α-subunits (Gilman, 1987; Birnbaumer *et al.*, 1990a). The references cited in the table are: (a) (Gilman, 1987), (b) (Birnbaumer *et al.*, 1990b), (c) (Moriarty *et al.*, 1990), (d) (Fung *et al.*, 1981), (e) (Cockcroft and Thomas, 1992), (f) (Sternweis and Smrcka, 1992), (g) (Grand *et al.*, 1996) and (h) (Voyno-Yasenetskaya *et al.*, 1994)

AC	First described	G_i inhib.	βγ effect	Stim by PKC	Ca^{2+} effect	Refs.
I	1989	Yes	Inhib.		Stim.	(a), (b), (c)
III	1990		No	No	Stim.	(d), (e)
VIII	1994			No	Stim.	(f)
II	1991	No	Stim.	Yes	No	(b), (c), (g), (h)
IV	1991		Stim.	No	No	(i)
VII	1994		Stim.	Yes	No	(j), (k)
V	1992	Yes	No	Yes	Inhib.	(l), (m), (n)
VI	1992	Yes	No	No	Inhib.	(m), (o), (p), (q)
IX	1995		No		No	(r), (s)

Table 1.2. The regulation of mammalian adenylyl cyclases.

The table shows the regulatory properties of the currently identified adenylyl cyclases as deduced largely from the study of the regulation of these isoforms following the expression of their cDNAs in intact HEK293 cells. The references cited in the table are: (a) (Krupinski *et al.*, 1989), (b) (Tang and Gilman, 1991), (c) (Taussig *et al.*, 1993), (d) (Bakalyar and Reed, 1990), (e) (Sunahara *et al.*, 1996), (f) (Cali *et al.*, 1994), (g) (Feinstein *et al.*, 1991), (h) (Lustig *et al.*, 1993), (i) (Gao and Gilman, 1991), (j) (Watson *et al.*, 1994), (k) (Völkel *et al.*, 1996), (l) (Ishikawa *et al.*, 1992), (m) (Premont *et al.*, 1992), (n) (Kawabe *et al.*, 1996), (o) (Yoshimura and Cooper, 1992), (p) (Debernardi *et al.*, 1993), (q) (Katsushika *et al.*, 1992), (r) (Paterson *et al.*, 1995), (s) (Premont *et al.*, 1996)

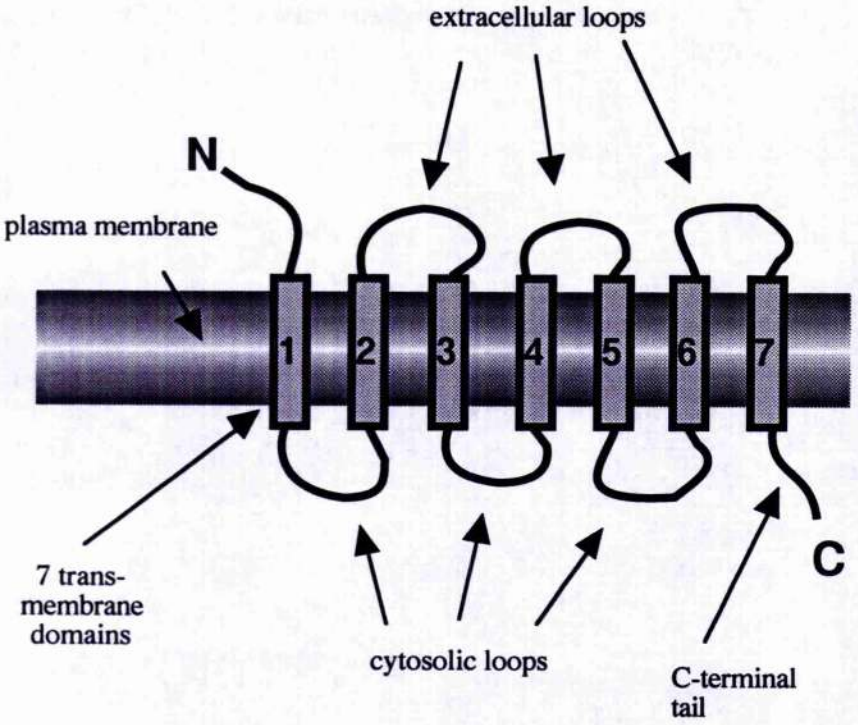

extracellular loops
N
plasma membrane
1
2
3
4
5
6
7
C
7 trans-
membrane
domains
cytosolic loops
C-terminal
tail

Figure 1.1. Structural features of G-protein-coupled receptors.

This model of the structure of a G-protein-coupled receptor (GPCR) is based on the presumed structural analogy between the GPCR, rhodopsin, and the photoprotein, bacteriorhodopsin, whose structure was elucidated by electron diffraction. It is assumed that other GPCRs, which share a high degree of primary sequence homology within their 7 hydrophobic regions with rhodopsin, adopt similar tertiary structures.

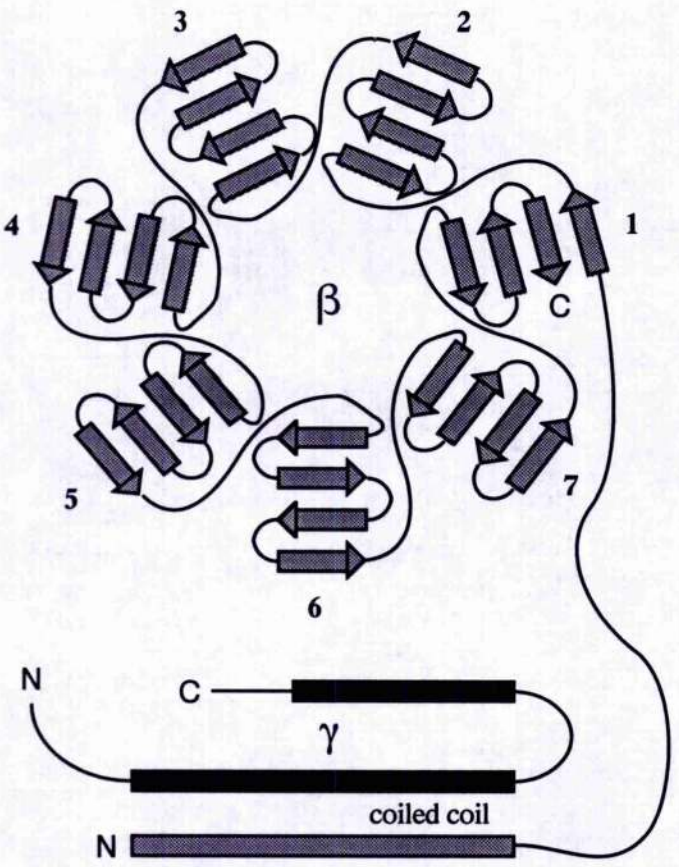

3
2
4
1
β
C
5
7
6
N
C
γ
coiled coil
N

Figure 1.2. Schematic illustration of the Gβγ heterodimer.

This diagram shows the overall structure of the G-protein βγ heterodimer, as determined by the recent crystallography of rod transducin (Sondek *et al.*, 1996). The β subunit (lighter shading) is composed of an amino-terminal α helix followed by a repeating module of seven similar β-sheets, each comprising four anti-parallel strands. The arrangement of the modules is such that each one forms a single "blade" of a propeller-like structure. The outer strand of each sheet, with the inner three strands of the following sheet, together constitute the structural unit that corresponds to the "WD" sequence (Neer *et al.*, 1994).

The γ subunit (darker shading) comprises two α helical regions, but unlike the β subunit possesses no inherent tertiary structure. The N-terminal helices of the β and γ subunits together form a coiled coil, whereas the remainder of the γ subunit interacts extensively with the β-propeller domain of the Gβ subunit.

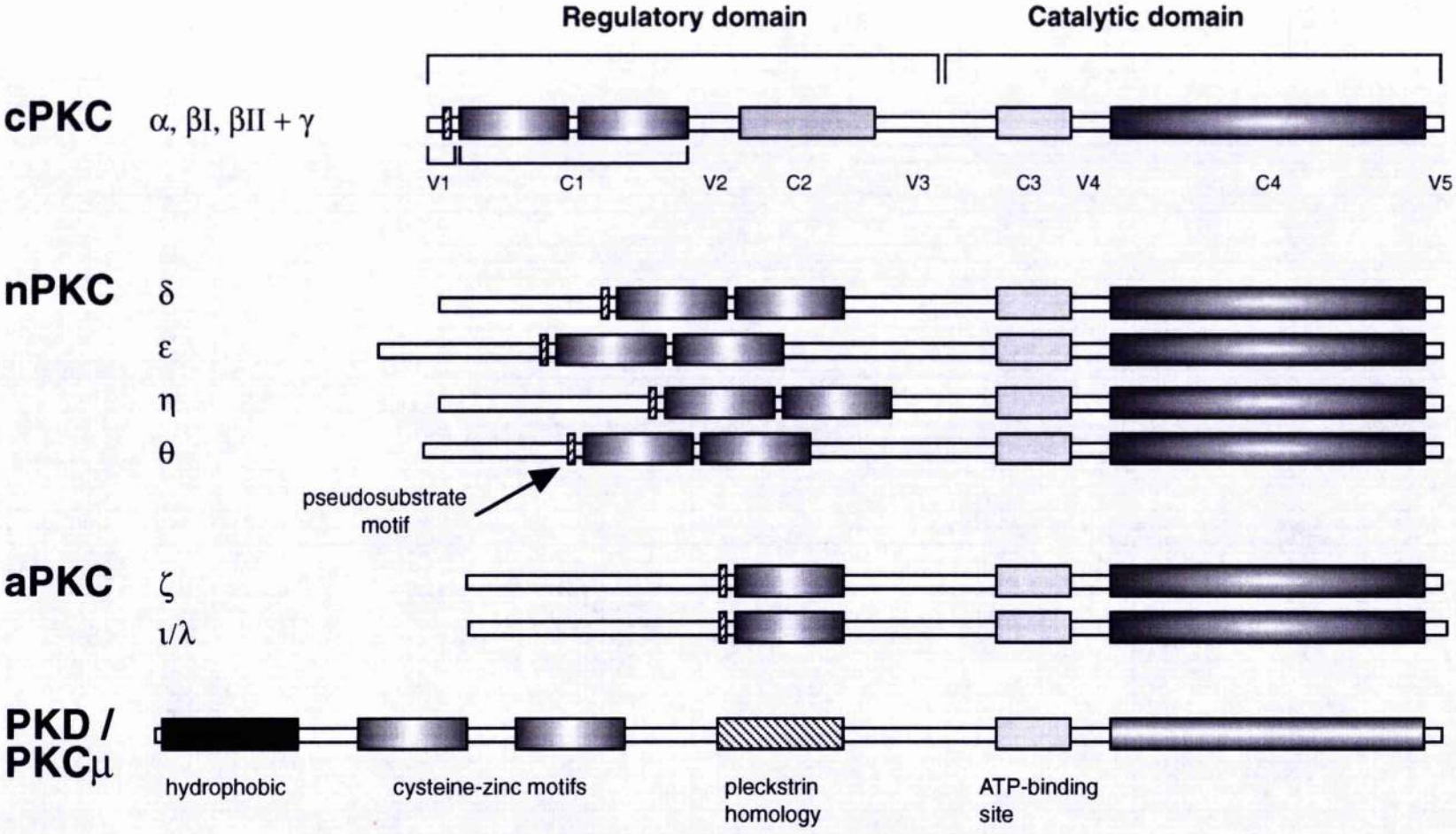

Regulatory domain
Catalytic domain
cPKC
α, βI, βII + γ
V1
C1
V2
C2
V3
C3
V4
C4
V5
nPKC
δ
ε
η
θ
pseudosubstrate motif
aPKC
ζ
ι/λ
PKD / PKCμ
hydrophobic
cysteine-zinc motifs
pleckstrin homology
ATP-binding site

Figure 1.3. Structural comparison of the members of the PKC family.

The isoforms are depicted in four groups: conventional (cPKCs), novel (nPKCs), atypical (aPKCs) and the more divergent PKD/PKCμ. The conserved (C1-C4) and variable (V1-V5) regions of PKC are indicated in the regulatory and catalytic domains. Conserved domain structure, where present, is indicated by identical shading.

Figure 1.4. Sequence comparison of the first intracellular loop of the glucagon receptor with that of the two calcitonin receptor isoforms.

In this figure, the primary sequence of the first intracellular loop of each of the two human isoforms of the calcitonin receptor (CTR1 and CTR2) has been aligned with the corresponding regions of the human and rat glucagon receptors (GRs). It is apparent that the 16-residue stretch that is present in the human CTR1 (Gorn *et al.*, 1992), and which is believed to inhibit coupling of the receptor to Gq/11 (Nussenzveig *et al.*, 1994), is absent from the CTR2 sequence and from that of the glucagon receptor.

RKLTTIFPLNWKYRKALSLGCQRVTLH	**human CTR1**
RSL----------GCQRVTLH	**human CTR2**
SKL----------HCTRNAIH	**human GR**
RKL----------HCTRNYIH	**rat GR**

CHAPTER 2

Materials and Methods

2.1. Materials

2.1.1. Radiochemicals

Amersham International	[8'-^{3}H] adenosine 3', 5'-cyclic monophosphate
Amersham,	[5', 8'-^{3}H] adenosine 3', 5'-cyclic monophosphate
Buckinghamshire, U.K.	(3-[^{125}I] iodotyrosyl^{10})glucagon
	[^{3}H]-palmitic acid
	D-myo [^{3}H] inositol 1, 4, 5-trisphosphate

2.1.2. General reagents

BDH, MERCK Ltd	Citric acid
Poole, Dorset, U.K.	Ethylenediaminetetra-acetic acid (EDTA)
	Glucose
	Glycine
	HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethane-sulphonic acid)
	Methanol
Gibco BRL	Tris(hydroxymethyl)aminomethane
Paisley, Scotland, UK	
Sigma	2,2,4-trimethylpentane
Poole, Dorset, U.K.	Acetic acid
	Butan-1-ol
	CHAPS (3-[(Cholamidopropyl)dimethylammonio]-1-propanc-sulfonate)
	Ethyl acetate

	Ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetra-acetic acid (EGTA)
	Triton X-100
	Tween-20
Fisons Scientific Equipment	$CaCl_2$
Loughborough,	Dimethyl sulphoxide (DMSO)
Leicestershire, U.K.	Glycerol
	KCl
	KH_2PO4
	$MgSO_4$
	$NaHCO_3$
	Sodium acetate
	Sodium citrate
	Sodium dodecyl sulphate (SDS)
	Sucrose
Boehringer Mannheim	Keyhole limpet haemocyanin (KLH)
Lewes, East Sussex, U.K.	Triethanolamine hydrochloride

2.1.3. Biochemical reagents

Rhone Merieux	Sagatal
Harlow, Essex, U.K.	
Worthington Biochemical	Collagenase
Freehold, New Jersey, U.S.A.	
Sigma	3-isobutyl-1-methylxanthine (IBMX)
Poole, Dorset, U.K.	12-O-tetradecanoylphorbol 13-acetate (TPA, PMA)
	Alkaline phosphatase

Antipain

Aprotinin

ATP (adenosine 5'-triphosphate)

β-mercaptoethanol

Benzamidine hydrochloride

Bromophenol blue

BSA (bovine serum albumin)

Cholera toxin

Collagen

Cyclic-AMP (adenosine 3':5'-cyclic monophosphate)

Cytochalasin B

Dowex1X8-400 (chloride form 200-400 mesh)

Glucagon

Glutathione

GppNHp (5'-guanylylimidodiphosphate)

GTP (guanosine 5'-triphosphate)

Insulin

Pepstatin A

Phosphatidic acid

PMSF (phenylmethylsulphonyl fluoride)

Ponceau stain

Snake venom (from Hannah ophiophagus)

Sodium pyruvate

Streptozotocin

TEMED (N,N,N',N'-tetramethylethylenediamine)

Theophylline

Peptide Research Foundation
London, U.K.

Leupeptin

Lipid products	Phosphatidyl butanol
Surrey, U.K.	
BDH, MERCK Ltd	Charcoal (Norit Gsx)
Poole, Dorset, U.K.	DTT (dithiothreitol)
	Universal indicator
Calbiochem-Novabiochem	Forskolin
Nottingham, U.K.	Inositol (1,4,5)-trisphosphate
	NP-40 (Nonidet-P40)
Boehringer Mannheim	Creatine kinase
Lewes, East Sussex, U.K.	Creatine phosphate
	Diabur-Test 5000 test strips
	Nicotinamide adenine dinucleotide (reduced form)
Miles Ltd	Dextrostix test strips
Slough, U.K.	
Bio-rad Laboratories	30% acrylamide/bis-acrylamide mix (29:1)
Hertfordshire, U.K.	Bradford reagent
Eli Lilly & Co. .	Humulin Lente (insulin zinc suspension)
Basingstoke, Hants, U.K.	
National Diagnostics	Ecoscint, scintillation fluid
Buckinghamshire, U.K.	
May & Baker	Ammonium persulphate
Dagenham, UK.	
Gibco BRL	Pre-stained molecular weight markers
Paisley, Scotland	

Amersham Life Science Amersham, Buckinghamshire, U.K.	ECL (enhanced chemiluminescence) reagents
SAPU Carluke, Lanarkshire, Scotland, U.K.	Horseradish peroxidase-conjugated anti-rabbit IgG
MERCK Darmstadt, Germany	Agar Peptone (tryptone) from casein (pancreatically digested) Yeast extract
Pierce Beijerland, Holland	Freund's complete adjuvant Freund's incomplete adjuvant Sulfo-MBS
Pharmacia Biotech Herts., U.K.	Glutathione sepharose 4B beads Protein-A-sepharose

2.1.4. Molecular biology reagents

Boehringer Mannheim Lewes, East Sussex, U.K.	Agarose
Pharmacia Biotech Herts., U.K.	First strand cDNA synthesis kit
National Diagnostics Buckinghamshire, U.K.	Sequagel-6 acrylamide mixture
Sigma Poole, Dorset, U.K.	Agarose (low melting point) DEAE (diethylaminoethyl)-dextran

	DEPC (Diethyl pyrocarbonate)
	Ethidium bromide
	Mineral oil
	Tri-reagent
Promega	Deoxyribonucleoside trisphosphates (dNTPs)
Southampton, U.K.	$MgCl_2$
	Molecular weight markers
	Restriction enzymes
	T4 DNA ligase
	Taq buffer
	Taq polymerase
	Wizard Mini, Maxi, PCR and Clean-up preps
Stratagene	"Perfect match" PCR additive
Cambridge, U.K.	
Invitrogen	MC1061/P3 strain of Escherichia coli
San Diego, CA, U.S.A.	
IBI	Ammonium persulphate (sequencing grade)
New Haven, CT, U.S.A.	

2.1.5. Plasmid DNA

Dr. M. Svoboda	cDNA encoding the rat hepatic GR (gift)
Brussels, Belgium	
Dr. P. J. Parker	cDNAs encoding PKC-α, βII, and ε (gifts)
London, England	

Dr. J. L. Benovic	cDNAs encoding the bovine GRK2 and GRK3
Philadelphia , U.S.A.	(gifts)
Dr. E. Rozengurt	cDNA encoding the wild-type and mutant murine
London, England	PKD (gift)
Dr. A. Katz	cDNAs encoding the G protein sub-units β1 and γ2
Pasadena, U.S.A.	(gifts)
Prof. G. Milligan	cDNA encoding the β2 and β3 adrenoceptors
Glasgow, Scotland	
BRL	pSV-Sport1 vector DNA
Bethesda, U.S.A.	

2.1.6. Tissue culture

2.1.6.1. Media

Gibco BRL	Dulbecco's modification of Eagle's medium
Paisley, Scotland, UK	(10x)
	Foetal calf serum
	L-Glutamine (200 mM)
	Penicillin/Streptomycin (10000 IU/ml)
	Sodium bicarbonate (7.5%)
	Trypsin/EDTA

2.1.6.2. Plastic ware

Costar UK	50 ml centrifuge tubes
High Wycombe,	Falcon tissue culture flasks: 75 cm^2
Buckinghamshire, U.K.	Filters (0.22μm)

Multi-well plates

Tissue culture pipettes

2.1.7. Animal resources

Male adult Sprague-Dawley rats were purchased from commercial sources with weights ranging between 200 and 250g. Euthanasia was effected by the intraperitoneal injection of a lethal dose of Sagatal, and was confirmed by testing for the absence of eye reflexes and respiratory movement.

2.1.8. Cell lines

COS-7 cells

These cells were derived by the transformation of simian CV-1 cells with an origin-defective SV40 genome (Gluzman, 1981). They constitutively express wild-type SV-40 large T antigen, and therefore permit the replication and expression of plasmids which contain an SV-40 origin and promoter region.

P9 cells

This is an SV40-immortalised hepatocyte-derived cell line described first by Livingstone et al. (1994). These cells express several enzyme activities that are characteristic of hepatocytes, including glucose-6-phosphatase, glycogen phosphorylase, and bilirubin glucuronyltransferase (Livingstone *et al.*, 1994).

HEK 293 cells

This cell line was derived from human primary embryonal kidney cells by transformation with sheared human adenovirus type 5 (Ad 5) DNA (Graham *et al.*, 1977; Harrison *et al.*, 1977; Graham *et al.*, 1978).

2.2. Methods for genetic manipulation

2.2.1. Water purification

The water used for work involving DNA or RNA was purified by reverse osmosis and subsequently autoclaved.

2.2.2. Plasmid purification

L-broth

171 mM NaCl	10.0 g/l
Bacto-tryptone (Casein/Pepsin)	10.0 g/l
Bacto-yeast extract	5.0 g/l
pH to 7.5 with NaOH, sterilise	

SOB medium

8.56 mM NaCl	0.5 g/l
Bacto-tryptone	20.0 g/l
Bacto-yeast extract	5.0 g/l
2.50 mM KCl	10 ml of 250 mM KCl
pH to 7.0 with NaOH, sterilise	
1 mM MgCl2 (added before use)	0.5 ml of sterile 2M solution

For the large-scale purification of plasmid DNA for cell transfection, a single colony on an agar plate containing antibiotic was used to inoculate 5 ml L-broth or SOB medium containing an appropriate antibiotic, which was incubated in an orbital shaker at 37 °C overnight. Following confirmation by plasmid mini-prep (see below) and restriction digest (see section 2.2.4.) that the plasmid of interest was indeed present in the cultured bacteria, a 2 litre conical flask containing 500 ml of culture medium was then inoculated with 500 µl of the mini-culture. An overnight incubation was performed as

above, and the bacteria were subsequently pelleted, resuspended (in 50 mM Tris-HCl pH 7.5, 10 mM EDTA, 100 µg/ml RNase A) , lysed (in 0.2 M NaOH, 1% SDS), and neutralised (in 1.32 M KAc pH 4.8). The DNA was then purified by a DNA-binding resin supplied in a Promega plasmid Maxi-Prep kit. All steps were performed according to the manufacturers' instructions except that 0.6 instead of 0.5 volumes of isopropanol were added, in order to improve yield. Additionally, following elution of the DNA in 1.5 ml water, phenol/chloroform extraction (see section 2.2.2.1.) and ethanol precipitation (see section 2.2.2.2.) was undertaken to remove any contaminating endonuclease co-purified from the *endA positive* MC1061/P3 strain of E. coli. The purity and concentration of the DNA solution was then assessed by UV absorbance as described in section 2.2.3.. The total yield from a 500 ml culture was typically 1-2 mg of plasmid DNA.

When a lower yield was acceptable (eg. less than 10 µg), a Promega Mini-prep kit was used to purify plasmid DNA from 3 ml of an overnight culture. Again, all steps were performed according to the manufacturers' instructions, and the composition of the resuspension, lysis and neutralisation solutions was exactly as described above for the Maxi-prep kit.

2.2.2.1. Phenol/chloroform extraction

An equal volume of phenol was added to the DNA solution in a microcentrifuge tube and vortexed briefly. Subsequent phase separation was achieved by centrifugation at 12,000 g_{av} in a benchtop microcentrifuge for 15 seconds. The aqueous (upper) phase, was then transferred to a new microcentrifuge tube leaving the interface layer containing the protein and the organic (lower) phase. Phenol addition and phase separation was repeated until no protein was visible at the interface. An equal volume of chloroform was then added and the tube vortexed briefly and centrifuged as above. The upper phase was again transferred to a fresh tube. Chloroform extraction was repeated once prior to ethanol extraction of the DNA.

2.2.2.2. Ethanol precipitation

The DNA solution was divided in order that the maximum volume did not exceed 400 µl per microcentrifuge tube. The solution was briefly vortexed and 0.11 volumes of 3M sodium acetate (pH 5.2) were added. The tube was vortexed and 2 volumes of ice-cold absolute ethanol (analytical grade) were added. Following mixing, the tube was stored at -70 °C for at least 30 minutes, to allow full precipitation of the DNA. The tube was then centrifuged at 12,000 g_{av} at 0 °C for 10 min, and the supernatant carefully discarded. In order to wash the pellet, 1 ml of 70% (v/v) ethanol was added, and the centrifugation performed at 12,000 g_{av} at 0 °C for 2 min. The supernatant was carefully removed and discarded, and the wash was repeated once. The tube was then stored open on the bench to allow evaporation of any remaining fluid. The DNA pellet was subsequently resuspended in purified water.

2.2.3. Determination of DNA concentration and purity

Using a Bio-Rad UV spectrophotometer, the absorbance, or optical density (OD), of a 5 µl sample of the DNA solution, diluted to 1 ml in H_2O, was determined at wavelengths of 260 nm and 280 nm in quartz cuvettes. Following the subtraction from these readings of the OD of H_2O alone, the purity of the DNA was determined by the division of the OD_{260} by the OD_{280}, yielding, ideally, a result of 1.8 to 2.0. The concentration was determined by the multiplication of the OD_{260} by the dilution factor (200) and by a constant (50 µg/ml). Alternatively, when measuring the concentration of a solution of oligonucleotide primer, the constant used was 33 µg/ml.

2.2.4. Restriction Digest

Restriction enzymes were stored at -20°C and were kept on ice during the preparation of digests. Typically, reactions were prepared in a microcentrifuge tube by adding 7 µl deionised water to 10 µl DNA solution (containing approximately 1 µg DNA), 2.0 µl 10x restriction enzyme buffer and 1 µl restriction enzyme. The buffer composition was chosen carefully in order to provide the optimum conditions for the activity of the individual restriction enzyme. The contents of the tube were mixed gently

and centrifuged briefly. Incubation was performed at 37 °C in a water bath for 2 hours. In some cases, the digestion of DNA with two enzymes simultaneously was performed, and in such cases the buffer was chosen so as best to suit both enzymes. Confirmation of digestion of the DNA was obtained by running a sample (at least 0.3 μg) on an agarose minigel.

2.2.5. Agarose gel electrophoresis

TAE (Tris-acetate/EDTA electrophoresis buffer)

40 mM Tris-acetate (in 1x)	242 g/l (for 50x stock)
5.71 % (v/v) glacial acetic acid	57.1 ml
1 mM EDTA, pH 8.0	100 ml 0.5 M EDTA, pH 8.0

DNA samples were separated by electrophoresis through an agarose gel of a selected concentration ranging from 0.8% to 2.0% (w/v), depending on the lengths of the DNA fragments to be resolved. Typically a 2% agarose minigel was used. Thus 0.4 g of agarose was mixed with 20 ml of TAE buffer and heated in a microwave oven until the agarose had dissolved completely. Subsequently, 1 μl of 10 mg/ml ethidium bromide was added, and, after allowing the gel to cool to about 60 °C, the gel mixture was poured into a casting tray assembly with casting blocks and an 8-well gel comb in place. Each DNA sample to be loaded was prepared by mixing a 10 μl DNA sample with 2 μl loading dye (Promega). In each minigel, one lane was also loaded with DNA markers, specifically λ/HindIII (Gibco BRL), ΦX174/HaeIII (Promega) or a 1 kb ladder (Gibco BRL). After allowing the gel to set at room temperature, the comb and casting blocks were removed and the gel and electrodes submerged in the electrophoresis tank in a volume of 200 ml TAE. The samples were then carefully loaded into the wells and the electrodes connected to a power supply. Electrophoresis towards the anode was then undertaken at a constant 75 volts at room temperature until the first dye front reached 2

cm from the end of the gel. Visualisation of the DNA was then performed using an ultraviolet light, and photographs were taken using a digitising camera.

2.2.6. Purification of DNA from an agarose gel

Where DNA was to be excised from an agarose gel, DNA fragments were separated on a gel consisting of low melting point agarose. Following electrophoresis, the fragment was visualised with ethidium bromide under ultraviolet light and a slice of the gel containing the entire DNA fragment excised with a scalpel blade. The agarose slice was subsequently melted at 70 °C for 2 min in the presence of 1 ml Promega PCR prep resin. The mixture was vortexed and the DNA purified on the columns supplied with the Promega PCR prep kit as described in the manufacturers' instructions.

2.2.7. Phosphatase treatment of DNA

Prior to ligation of an insert into a vector cut with a single restriction enzyme, the plasmid DNA was treated with calf intestinal alkaline phosphatase (CIAP). The CIAP removes the 5' phosphate groups and thus prevents recircularization of the vector during ligation. The following were added in a microcentrifuge tube: 2 µg vector DNA (equivalent to 2 pmol ends for a 3000 bp plasmid), 10 µl CIAP 10x buffer, 1 µl CIAP and H_2O to a total volume of 100 µl. The contents were mixed gently, centrifuged briefly and incubated for 1 hour at 37 °C. The reaction was terminated by the addition of 2 µl of 0.5 M EDTA. Prior to ligation of the phosphatase-treated vector to the insert DNA, removal of the CIAP was effected by the use of Promega's DNA Clean-up resin, as CIAP can interfere with efficiencies of ligation and transformation reactions.

2.2.8. DNA ligation

Prior to ligations, the vector DNA was digested (see section 2.2.4.), if necessary treated with alkaline phosphatase (see section 2.2.7.) and purified with Promega's Clean-up resin. The insert DNA, was prepared by digestion of either plasmid DNA or PCR DNA, and purified in the same way. Ligation reactions were prepared by the addition of

vector DNA and insert DNA, in varying ratios, prepared as above, to 0.5 µl T4 DNA ligase (3 units/µl) and 1 µl of 10x ligase buffer (300 mM Tris-HCl pH 7.8, 100 mM $MgCl_2$, 100 mM DTT, 10 mM ATP) in a total volume of 10 µl. Additional reactions, serving as controls, were also prepared and incubated, containing, in the absence of insert DNA, vector DNA which was either phosphatase-treated (testing the phosphatase efficiency) or not (testing the ligase efficiency) . The reactions were left overnight at room temperature and subsequently used to transform competent E. coli.

2.2.9. Preparation of competent E. coli

RF1 buffer

100 mM RbCl	20 ml/dl	0.5 M RbCl
50 mM $MnCl_2.4H_2O$	5.0 ml/dl	1.0 M $MnCl_2$
30 mM potassium acetate	3.0 ml/dl	1.0 M KAc
10 mM $CaCl_2$	1.0 ml/dl	1.0 M $CaCl_2$
15 % (v/v) glycerol	18.8 ml/dl	80% glycerol

pH to 5.8 with 0.2M acetic acid.

Filter sterilise (0.22 µm) and store at 4 °C.

RF2 buffer

10 mM RbCl	2.0 ml/dl	0.5 M RbCl
10 mM MOPS	2.0 ml/dl	0.5 M MOPS
75 mM $CaCl_2$	7.5 ml/dl	1.0 M $CaCl_2$
15 % (v/v) glycerol	18.8 ml/dl	80 % glycerol

pH to 6.8 with 0.2 M NaOH.

Filter sterilise (0.22 µm) and store at 4 °C.

Bacteria of either the JM109 or MC1061/P3 strain of E.Coli were grown overnight in an orbital shaker at 37 °C after inoculating 10 ml L broth or SOB medium (respectively) from a glycerol stock of E. coli. 5 ml of this culture was then used to

inoculate the 500 ml of the appropriate medium and this was incubated at 37 °C until the culture reached mid-log phase (OD_{550} = 0.5-0.55). It was then poured into 250 ml centrifuge containers and allowed to cool on ice for 30-60 min prior to harvesting the bacteria by centrifugation for 10 min at 7,500 g_{av} at 4 °C. From each 250 ml container the supernatant was decanted and the bacteria resuspended in 20 ml sterile ice cold RF1 solution. The suspension of cells was incubated on ice for 15 min and then centrifuged for 20 min at 1,500 g_{av} at 4 °C. The supernatant was again discarded and the pellet resuspended in 3.5 ml per tube of sterile ice cold RF2 buffer. The suspensions were pooled into one tube and again incubated on ice for 15 min. Aliquots of 225µl were then snap frozen in liquid N_2 in sterile microcentrifuge tubes and stored at -80°C.

2.2.10. Transformation

100 µl of competent cells were mixed with 5 µl of each of the ligation reactions, and incubated on ice for 30 min. For additional controls, aliquots of competent cells were incubated with undigested vector (testing transformation efficiency), digested but unligated vector (testing digestion efficiency), or no DNA (testing the ampicillin in the agar and confirming that the untransformed E. coli were not ampicillin-resistant). The bacteria were then placed in a 42 °C bath for exactly 2 min and returned to ice for 5 min. To each tube, 1 ml L-broth was then added and the mixture incubated, with shaking, at 37 °C for 1 hour. From each tube, 100 µl was then streaked onto selective 1.5% (w/v) agar plates containing 50 µg/ml ampicillin. In addition a plate of agar without antibiotic was streaked with untransformed E. coli to check the viability of the bacteria. Where the MC1061/P3 strain of E. coli was used instead of the JM109 strain, SOB medium was used in place of L-broth, and tetracycline at 7.5 µg/ml was added in addition to ampicillin. The culture plates were subsequently incubated at 37 °C overnight and examined for the presence of colonies.

2.2.11. *Transient transfection*

TE buffer

10 mM Tris.HCl (pH 7.4)	1.21 g/l	
1 mM EDTA (pH 8.0)	2 ml	0.5 M EDTA, pH 8.0

Exponentially growing COS-7 cells, 40-60% confluent, were transfected, where indicated, with expression vectors encoding the glucagon receptor (in pCDM8), PKC-α (in pMT2), PKC-βII (in pCO2), PKC-ε (in pMT2), βARK1 and βARK2 (each in pBC12BI), β_2-adrenoceptor (in pcDNA3), β_3-adrenoceptor (in pcDNA3-RSV), and both mutant and wild-type PKD (in pcDNA3) using the DEAE-dextran method as described previously by Al-Molish et al. (Al-Molish and Dubes, 1973). Briefly, for each 75 cm^2 flask to be transfected, 5 μg plasmid DNA was diluted with TE to a total volume of 250 μl and mixed with 200 μl of 10 mg/ml DEAE dextran solution. This was left at room temperature for 15 min and then added to 5 ml DMEM containing 10% (v/v) newborn calf serum and 100 μM chloroquine. The medium was aspirated from the COS cells and the above mixture added. Following an incubation period of 3 hours at 37 °C, the cells were shocked by replacing the medium with 5 ml dimethylsulphoxide (DMSO) 10% (v/v) in phosphate-buffered saline (PBS). This was aspirated after exactly 2 min and the cells washed with 10 ml PBS before 10 ml DMEM containing 10% (v/v) foetal calf serum (FCS) was added. After an incubation at 37 °C for 2 days the cells were split into 6-well plates and subsequently grown to confluence in DMEM supplemented as above. Cells were stimulated approximately 72 hours following transfection, as described in section 2.4.14.1..

2.2.12. *RNA extraction*

Total cellular RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987) from confluent monolayers of COS-7 cells, suspensions of isolated hepatocytes or from fresh samples of rat tissue. Briefly, approximately 100 mg tissue was homogenised in 1 ml Tri-reagent

using a sterilised glass homogeniser. Alternatively a confluent monolayer of COS cells in a 75 cm^2 flask was washed with sterile PBS (10 ml) and then harvested using a cell scraper into 1 ml Tri-reagent. Where hepatocytes were used, $5x10^6$ cells were pelleted by centrifugation at 1,000 g_{av} for 5 min followed by resuspension and lysis by repetitive pipetting in 1 ml Tri-reagent. In each case the lysate was stored at room temperature for 5 min and 1 ml subsequently transferred to autoclaved microcentrifuge tubes. In order to remove the cell membranes and high molecular weight DNA, the tubes were centrifuged at 12,000 g_{av} for 10 min at 4 °C and the supernatant transferred to fresh tubes. To separate the RNA from DNA and protein, 0.2 ml chloroform was added, and the tube vortex-mixed. After storing the tube at room temperature for 3 min, phase separation was performed by centrifugation at 12,000 g_{av} for 15 min at 4 °C, and the aqueous (upper) phase transferred to fresh tubes.

To precipitate the RNA, 0.5 ml of isopropanol was added. The mixture was then stored at room temperature for 5-10 min, and centrifuged at 12,000 g_{av} for 10 min at 4 °C. The supernatant was removed and the pellet washed by adding 1 ml of 75% (v/v) ethanol, vortexing for 15 sec and centrifuging at 7,500 g_{av} for 5 min at 4 °C. Finally, the RNA pellet was dried *in vacuo* and re-solubilised in di-ethyl pyrocarbonate (DEPC) treated water by repetitive pipetting and incubating for 10-15 min at 55-60 °C. The RNA concentration was then determined as described below (section 2.2.13.).

2.2.13. Determination of RNA concentration

Using a Bio-Rad UV spectrophotometer, the absorbance, or optical density (OD), of a 5 µl sample of the RNA solution, diluted to 1 ml in H_2O, was determined at a wavelength of 260 nm in a quartz cuvette. Following the subtraction from this reading of the OD of H_2O alone, the concentration was determined by the multiplication of the OD_{260} by the dilution factor (200) and by a constant (40 µg/ml).

2.2.14. First strand cDNA synthesis

First strand cDNA was prepared by the use of the Moloney Murine Leukaemia Virus (M-MuLV) reverse transcriptase and oligo-dT primer, provided with the Pharmacia First-strand cDNA Synthesis Kit. Briefly, 5 μg of RNA, as determined by its absorbance at 260 nm, was diluted in DEPC-treated water to a total volume of 20 μl and denatured by heating it for 10 min at 65 °C then chilling on ice. The solution was then added to 11 μl of "Bulk First-strand cDNA Reaction Mix", containing cloned M-MuLV reverse transcriptase, RNAguard, BSA, and dNTPs in an aqueous buffer. To this was then added 1 μl of 200 mM DTT, and 1 μl (0.2 μg) of the supplied poly (dT) primer (NotI-$d(T)_{18}$), the solution was mixed gently by pipetting, and the mixture incubated for 1h at 37 °C. In the complete reaction mixture the buffer and dNTP conditions were:

45 mM Tris (pH 8.3)

68 mM KCl

15 mM DTT

9 mM $MgCl_2$

0.08 mg/ml BSA

1.8 mM each dNTP

After synthesis, cDNA was used immediately as a template for PCR or stored at -80°C.

2.2.15. Polymerase chain reaction (PCR)

2.2.15.1. Design of oligonucleotide primers for PCR

Oligonucleotide PCR primers were designed using the GeneJockey II program. Typically primers were of approximately 20 bp in length, and members of each primer pair were designed so as to have approximately equal melting temperatures. The extreme 3' ends of each oligonucleotide contained at least two C or G nucleotides and were checked for complementarity with the other member of the primer pair in order to reduce the likelihood of "primer-dimer" formation. Sequences that could potentially form significant secondary structure or which contained stretches of polypurines or

polypyrimidines were avoided. To identify regions with homology to other sequences, sequence alignments were undertaken with the aid of the GeneJockey II and Lasergene software packages. In addition, prior to oligonucleotide synthesis, the specificity of each primer sequence was verified by performing a BLAST (Basic Local Alignment Search Tool) computer search of the Genbank nucleotide sequence database.

2.2.15.2. Preparation of PCR oligonucleotide primers

Synthesis of the primers was performed by Dr. Veer Math of the Institute of Biomedical and Life Sciences of the University of Glasgow. Synthesized primers were supplied in an aqueous solution of ammonium hydroxide and, before use, were purified by ethanol precipitation (see section 2.2.2.2.), quantified (see section 2.2.3.) and diluted to the required concentration in H_2O.

2.2.15.3. PCR conditions

PCR was performed, unless otherwise stated, in the presence of 1 x Taq buffer (50 mM KCl, 10 mM Tris.HCl, pH 9, 0.1% Triton X-100), 1.5 mM $MgCl_2$, 40 µM of each dNTP, 1.5 µM of each primer, 3 µl of first-strand cDNA synthesis reaction, 5 units of Taq polymerase and 0.5 units of Perfect Match® DNA polymerase enhancer in a total volume of 50 µl. 35 thermal cycles were undertaken, each consisting, typically, of a 1 min denaturation segment at 95 °C, a 2 min annealing step at 52.5 °C and a 3 min extension time at 72 °C. PCR products were resolved by electrophoresis on a 2% agarose gel with ethidium bromide and visualised under UV light. Prior to the use of PCR products as substrates for further enzymatic reactions, the DNA was purified from the other constituents of the PCR reaction by the use of Promega's PCR Prep resin, according to the manufacturers' instructions.

2.2.16. Site-directed mutagenesis

The strategy used to perform site-directed mutagenesis was essentially that of overlap extension PCR (Ho *et al.*, 1989). Briefly, two pairs of PCR oligonucleotide primers were designed with complementarity between the 3' primer of one pair and the 5'

primer of the other pair. Thus, PCR reactions were used to generate two DNA products with overlapping ends. The novel DNA sequence containing the desired specific nucleotide substitutions was incorporated into the region of overlap. In a subsequent 'fusion' reaction, the overlapping ends were permitted to anneal, allowing the 3' overlap of each strand to serve as a primer for extension, with the other member of the overlapping primer pair serving as template. The product of this fusion reaction was then amplified by successive rounds of PCR, thus incorporating the mutant sequence into all the resulting product DNA.

2.2.17. Automated DNA sequencing

The determination of DNA sequence was undertaken using an automated ABI sequencer linked to a Macintosh II computer running the SeqEd data collection and analysis program.

2.2.17.1. PCR reactions

DNA samples to be sequenced were first purified by mini-prep or maxi-prep. In addition, subsequent ethanol precipitation was found to improve markedly the quality of the data obtained. The purified DNA was used as a template for a PCR reaction containing dideoxyNTPs in order to generate a mixture of prematurely terminated single strand DNA products of varying lengths. Each PCR reaction was prepared as follows:

template DNA	8.5 µl	1.5 µg
primer (SP6 or T7)	2 µl	3.2 pmol
5x buffer	4 µl	
dNTP mix	1 µl	
dideoxy GTP	1 µl	
dideoxy CTP	1 µl	
dideoxy TTP	1 µl	
dideoxy ATP	1 µl	
AmpliTaq® DNA polymerase	0.5 µl	

The mixture was overlaid with 40 µl mineral oil and transferred to a thermocycler with the following preset programme, with the ramp rate set at 1 °C per second, for a total of 30 cycles:

Denaturation	96 °C	30 seconds
Annealing	45 °C	50 seconds
Extension	60 °C	4 minutes

2.2.17.2. Sequencing gel preparation

The glass plates were washed with the detergent, Alconox, and then rinsed with water followed by absolute ethanol. The spacer, casting comb, and sharkstooth comb were washed in a similar fashion but with the exclusion of ethanol. Once dry, the plates and spacers were assembled and the lower edge sealed with tape. Using Sequagel-6 (National Diagnostics) pre-mixed acrylamide solutions, 48 ml solution A was added to 12 ml solution B, and 480 µl of freshly prepared 10 % (w/v) ammonium persulphate was added. This mixture was then poured slowly into the gel assembly with the latter inclined at an angle of approximately 30°, taking care to avoid the introduction of air bubbles. When almost completely filled, the plates were laid flat and the casting comb inserted. After allowing the gel to cure for two hours, the tape was removed and the gel placed in the ABI sequencer. The upper and lower reservoirs were filled with running buffer and the casting comb removed. Prior to sample loading, the gel was scanned for contaminants and pre-run according to the instructions in the user manual.

2.2.17.3. Sequence sample purification and loading

The samples were removed from the thermocycler and 80 µl water was added beneath the layer of mineral oil. The aqueous (lower) phase was transferred to a fresh 0.5 ml tube, to which 100 µl phenol/chloroform/water was then added. After vortexing briefly, the mixture was centrifuged for 30 seconds at 12,000 g_{av} in a microfuge. The aqueous (upper) phase was then transferred to another tube and the phenol/chloroform extraction repeated. Ethanol precipitation was performed as described in section 2.2.2.2.

and the pellet dried by heating to 85 °C for 1 min. Finally, the pellet was resuspended in 4 µl deionised formamide, and, prior to loading onto the gel, the samples were heated to 90 °C for 3 min and placed on ice. With the sharkstooth comb in place, the samples were loaded and the sequencer switched on.

2.2.17.4. Data collection and analysis

Data from the sequencer was collected continuously during the electrophoresis of the samples and stored in a computer file by the SeqEd program. When electrophoresis was complete, the file was opened from within the GeneJockey II program and the mis-reading frequency examined (see fig. 2.1). All stretches of sequence with an error frequency of 10 % or more were discarded prior to exporting the sequence into a GeneJockey nucleotide file for subsequent analysis.

2.3. Methods for antiserum production

2.3.1. Generation of peptide antisera

Polyclonal antisera used for immunoblotting were raised in New Zealand White rabbits against synthetic peptides. Where the peptide sequence itself did not contain a cysteine, these were synthesised with an additional cysteine at the C-terminus in order to facilitate conjugation to keyhole limpet haemocyanin (KLH).

2.3.2. Coupling peptides to KLH

2.3.2.1. Solutions for coupling

Sodium phosphate

50 mM $NaH_2PO_4.H_2O$	6.85 g/l
0.04 M NaOH	40 ml/l 1M NaOH

N-ethylmaleimide

300 mM N-ethylmaleimide	38 mg/ml

Sodium phosphate/sodium chloride

20 mM $NaH_2PO_4.H_2O$	2.75 g/l
135 mM NaCl	7.85 g/l

pH to 5.6 and to 8.0 with 1 M NaOH

2.3.2.2. Coupling method

Prior to conjugation, the KLH was prepared as follows: 20 mg KLH was dissolved in 2 ml 50 mM sodium phosphate and dialysed overnight against this buffer to remove amines. To the dialysed KLH was then added 17 µl of 0.3 M N-ethylmaleimide (5 µmol). After 30 minutes 6.2 mg (15 µmol) sulfoMBS was added, and, after a further 30 minutes, the pH was adjusted to 6.0 by adding 1M HCl (approximately 50 µl). The solution was then dialysed overnight at 4 °C against 20 mM sodium phosphate,

135 mM NaCl pH 5.6 to remove unreacted or hydrolysed sulfo-MBS. Subsequently, an equal volume of 20 mM NaPO4/150 mM NaCl, pH 8, was added at room temperature. The pH was checked and adjusted to pH 6.6-6.8.

The peptide (9 mg) was then added immediately and dissolved by vigorous vortexing. Following re-adjustment of the pH to 6.7 with 1 M NaOH, the solution was incubated for 4 hours, at room temperature.

2.3.3. Expression and preparation of GST fusion protein

Protease inhibitors (1000x stock)

0.1 % (w/v) aprotinin

0.1 % (w/v) antipain

0.1 % (w/v) pepstatin

0.1 % (w/v) leupeptin

1 mM benzamidine

1 mM PMSF

solubilised in DMSO and stored in aliquots at -20°C

2.3.3.1. Induction of E. coli

Previously, using a pGEX vector, an in-frame GST fusion protein construct had been generated using PCR, that encoded GST linked to the C-terminal portion of the rat glucagon receptor. Glycerol stocks of bacteria transformed with the recombinant plasmid were frozen at -80 °C. In order to generate the fusion protein, 15 ml of L-broth (containing ampicillin) was inoculated with 20 µl of the glycerol stock, and the culture was incubated at 37 °C overnight. A 500 ml culture was then inoculated with the 15 ml overnight culture and incubated at 37 °C for 2 hours. Filter-sterilised IPTG was then added to a final concentration of 0.1 mM and the culture incubated for a further 5 hours. The bacteria were then pelleted by centrifugation at 4,000 g_{av} for 15 min at 4 °C and resuspended in 20 ml PBS containing protease inhibitors. The suspension of bacteria was then divided into aliquots of 1 ml and frozen at -80 °C until use.

2.3.3.2. GST fusion protein extraction

Aliquots of frozen bacteria were thawed and sonicated for 4 x 10 seconds or until the cells had lysed. Unlysed cells and debris were then pelleted by centrifugation in a microfuge for 5 min.

2.3.3.3. Verification of induction

In order to confirm that induction of expression of the fusion protein had taken place, following centrifugation, 10 µl from the supernatants of both the bacteria containing the fusion protein and of bacteria containing only the original GST vector, were boiled with 10 µl Laemmli (Laemmli, 1970) buffer and loaded onto an SDS polyacrylamide mini-gel. Following electrophoresis the gel was removed from the glass plates and stained for protein.

2.3.3.4. Gel staining, destaining and drying

This procedure was carried out as described previously (Sambrook *et al.*, 1989). Following electrophoresis, gels were soaked, with gentle shaking on a rotary shaker, for 1 h in 45% (v/v) methanol, 10% (v/v) acetic acid containing 0.25% (w/v) Coomassie Blue R-250. Gels were destained by washing with 45% (v/v) methanol, 10 % (v/v) acetic acid with frequent changes of the wash solution, and finally re-hydrated by immersion in water. Gels were subsequently dried down onto Whatman 3MM paper under vacuum at 60°C for two hours.

2.3.3.5. Fusion protein purification

Pre-swollen glutathione sepharose 4B beads were prepared by adding an equal volume of PBS containing protease inhibitors followed by pelleting for 10 sec in a microfuge. This wash was performed three times, and the beads subsequently resuspended to give a 50% (v/v) slurry. Binding was carried out by mixing 1 ml of supernatant containing the GST fusion protein (obtained as described in section 2.3.3.2. above) with 100 µl of the bead slurry, and incubating the mixture, end over end, for 60 min at room temperature or overnight at 4 °C. The beads were then subjected to three washes, each consisting of a 10 sec centrifugation, aspiration of the supernatant, and a

5 min incubation with 1 ml PBS containing protease inhibitors. At this stage an aliquot of beads was assayed for protein content by the method of Bradford (Bradford, 1976). In order to release the bound fusion protein, the washed beads, after pelleting, were incubated for 10 min with an equal volume of 10 mM glutathione in 50 mM Tris-HCl pH 8.0, centrifuged as above, and the supernatant removed and retained. This was performed three times and the retained supernatants combined.

2.3.4. Immunisation

Prior to immunisation, 200 µl of the solution, containing approximately 250 µl conjugated peptide, was mixed with 200 µl sterile PBS and 600 µl Freund's complete adjuvant. One month later the immunisation was boosted with the same peptide solution but mixed instead with Freund's incomplete adjuvant, and the first test bleed was performed after a further ten days.

2.3.5. Preparation of anti-serum

5-10 ml blood was obtained from the ear vein of rabbits and left overnight at 4°C to allow clot formation and contraction. The blood was then centrifuged at 1000 g for 5 min, the serum was removed and re-centrifuged at 1,000 g for 5 min. The supernatant was retained and then divided into 50 µl aliquots and stored at -80 °C.

2.4. Methods for biochemical analysis

2.4.1. SDS polyacrylamide electrophoresis

Resolving gel mix (8%) for two gels

27.8 ml water

16.0 ml acrylamide mixture

15.0 ml resolving gel buffer

0.6 ml 10% (w/v) SDS

0.6 ml 10% (w/v) ammonium persulphate

36 µl TEMED

Stacking gel mix (5%) for two gels

13.6 ml water

3.4 ml acrylamide mixture

2.5 ml stacking gel buffer

0.2 ml 10% (w/v) SDS

0.2 ml 10% (w/v) ammonium persulphate

20 µl TEMED

Resolving gel buffer

1.5 M Tris, pH 8.8 181.7 g/l

Stacking gel buffer

1.0 M Tris, pH 6.8 121.1 g/l

Electrophoresis buffer

191 mM glycine 14.4 g/l

25 mM Tris 3.0 g/l

0.1 % (w/v) SDS

Laemmli sample buffer (for 20 ml)

3.5 ml water

2.5 ml stacking gel buffer

5.0 ml glycerol

8.0 ml 10% (w/v) SDS

0.001 % (w/v) bromophenol blue

1.0 ml β-mercaptoethanol

The gel apparatus, consisting of two glass plates (measuring 160 mm x 180 mm) separated by 1.5 mm plastic spacers was assembled and clamped in place in a casting stand, after the plates and spacers had been thoroughly cleaned with ethanol and H_2O. The assembly was subsequently checked, by filling with distilled water, for leakage. Following the addition of the TEMED and 10% (w/v) APS to initiate polymerisation, 25 ml of 8% resolving gel mixture (see above) was introduced between the plates, overlaid gently with distilled water, and allowed to set. The water was then poured off, and, with a 10-well gel comb in place, the stacking gel mixture was added, taking care not to introduce air bubbles. After polymerisation of the stacking gel, the comb was removed and the assembly transferred from the casting stand to an electrophoresis tank, containing the anode submersed in 4.5 litres of running buffer. The remaining running buffer was poured into the upper reservoir, thus immersing the cathode. Samples were prepared by adding to each an equal volume of freshly prepared 2x sample buffer and an appropriate volume of H_2O to bring the total volume of each sample to 50 µl, unless otherwise stated. All samples were then placed in a boiling water bath for 3 minutes, and loaded into the appropriate wells. Pre-stained molecular weight protein standards (see section 2.4.2.) were loaded in one well for comparison. Electrophoresis towards the anode was performed at a constant current of 7 mA overnight or 60 mA for approximately 3 hours with constant cooling, until the bromophenol blue dye front reached 1 cm from the lower edge of the plates.

2.4.2. *Protein molecular weight markers*

Pre-stained protein molecular weight standards comprising myosin H-chain (200 kDa), phosphorylase B (97.4 kDa), BSA (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), β-lactoglobulin (18.4 kDa) and lysozyme (14.3 kDa) were purchased from Gibco and were made up as described by the manufacturers. Briefly, 500 µl 1 mM dithiothreitol (DTT) was added to the vial of standards. The contents were boiled for 5 minutes, vortexed and then aliquoted into 20 µl aliquots and stored at -20 °C. When required, an aliquot was thawed and an equal volume of sample buffer added. The vial was boiled for 3 minutes with the rest of the samples, then 20 µl was applied to the gel.

2.4.3. *Western blotting*

Electro-blotting buffer

191	mM glycine	14.4 g/l
25	mM Tris	3.0 g/l
20	% (v/v) methanol	

Tris-buffered Saline (TBS)

0.5	M NaCl	29.2 g/l
20	mM Tris, pH 7.5	2.42 g/l

Protein transfer from the SDS gel to nitrocellulose was performed in electro-blotting buffer in a Transblot apparatus (Hoefer Instruments) using a 1 h transfer time at a constant current of 1 amp. The blots were blocked for 2h at room temperature in 5% (w/v) skimmed milk powder in TBS, to prevent any non-specific antibody binding. After washing twice with TBS/0.05% (v/v) Nonidet P-40 and twice with TBS, each for 5 minutes, the nitrocellulose was incubated with the designated antiserum, diluted 1:100 in TBS containing 1% (w/v) skimmed milk powder, for 2 h at room temperature. The blots were then washed four times for 10 min each with TBS/0.05% (v/v) Nonidet P-40

and probed with horse-radish peroxidase-linked anti-rabbit IgG antiserum at a dilution of 1:500 in TBS containing 1% (w/v) skimmed milk powder, for 30 min at room temperature. Following a further four washes for 10 min each with TBS/0.05% Nonidet P-40, the blots were finally rinsed in TBS and immuno-detection carried out using an Amersham enhanced chemiluminescence (ECL) kit. This procedure was undertaken as per the manufacturers' instructions, using both photosensitive Amersham film and X-ray film with various exposure times.

The isoform-specific PKC antisera were generated and characterised previously in our laboratory (Tang *et al.*, 1993; Spence *et al.*, 1995). The PKD-specific polyclonal antiserum (PA-1), raised against a 15-residue peptide contained within the C-terminal region of mouse lung protein kinase D (Valverde *et al.*, 1994), was a kind gift from Dr E. Rozengurt.

2.4.4. De-hybridisation of antibodies from nitrocellulose membranes

De-hybridisation solution

100 mM β-mercapto-ethanol	0.781 % (v/v)
2 % (w/v) SDS	
62.5 mM Tris-HCl pH 6.7	62.5 ml 1M Tris pH 6.7 per litre

The nitrocellulose was incubated at 50°C in the pre-warmed de-hybridisation solution for 30 min, with agitation every 10 min. Subsequently, the membrane was washed 6 times for 5 min with TBS/0.05% NP40, and then processed again with the ECL immuno-detection kit to verify the complete absence of signal prior to re-blocking and re-probing.

2.4.5. Immunoprecipitation

The source material for the specific immunoprecipitation of [^{125}I]-glucagon binding activity was a hepatocyte membrane fraction (1.75 mg of starting material) solubilised by

CHAPS detergent. The solubilisation mixture comprised 25 mM HEPES pH 7.5; 137 mM NaCl; 2 mM EDTA pH 8.0; 6 mM CHAPS; and 1x protease inhibitor "cocktail" (see section 2.3.3.) . After a period of 30 min on ice during which the constituents were agitated every 10 min, any unsolubilised membranes were pelleted by centrifugation at 245,000 g_{av} for 30 min at 4°C. Antiserum was added to each sample of the supernatant at a dilution of 1:50 and the samples briefly vortexed before being left overnight at 4°C in order to allow conjugation to occur. Pansorbin (2% w/v final) was then added and the resultant suspension gently mixed at 4°C for 2h. The preparations were then centrifuged at 14,000g for 2 min and the pellets were resuspended in the immunoprecipitation buffer (25 mM HEPES pH 7.5; 137 mM NaCl; 2 mM EDTA pH 8.0; and 1x protease inhibitor "cocktail") before being recentrifuged as before. The resultant pellets were then washed twice more. The final pellet was resuspended in binding assay buffer and assayed immediately.

2.4.6. Induction and treatment of diabetes in rats

Adult male Sprague-Dawley rats were injected, by the intraperitoneal route, with 80 mg/Kg streptozotocin dissolved in 0.3 ml 0.1M sodium citrate, pH 4.5. After 4 days, the presence of glucose in the urine was tested using Diabur-Test 5000 (Boehringer Mannheim) test strips, to confirm the induction of diabetes mellitus. Animals which were treated with insulin, received 10 units of a long-acting insulin preparation (Humulin Lente), every 12 hours, for 7 days. Urine was checked as above to confirm the absence of glucose. At the time of dissection of killed rats, the level of blood glucose, determined using Dextrostix, was found to be 15-20 mM for untreated diabetic rats and to be approximately 5 mM both for control animals and for those diabetic rats which had been treated with insulin.

2.4.7. [^{125}I]-glucagon binding

This was done using a modification of a procedure described previously (Houslay *et al.*, 1977). Briefly, membranes were incubated at a final concentration of 100-200µg

protein/ml in a final reaction volume of 100 µl containing 0.1% (w/v) BSA, 1 mM EDTA, 20 mM Tris-HCl pH 7.5 (binding buffer), and 0.2 nM [^{125}I]-glucagon. Non-specific binding was determined by the addition of unlabelled glucagon to a final concentration of 1 µM. Incubations were done for 20 min at 32°C. The reaction was stopped by the addition of 3 ml ice-cold binding buffer and the diluted samples immediately filtered through 0.45-µm cellulose acetate filters which had been soaked in 10% BSA overnight and washed with 2 ml ice-cold binding buffer. The tubes were rinsed twice with 3 ml ice-cold binding buffer and the rinses filtered. The filters were then finally washed with 6 ml ice-cold binding buffer and the radioactivity bound to them was measured using a γ counter. Results were expressed in fmol [^{125}I]-glucagon-specific binding per mg membrane protein.

2.4.8. Protein determination

Protein was routinely measured by the method of Bradford (Bradford, 1976) with BSA as a standard. Briefly, in order to construct a standard curve, a set of tubes were prepared containing, in duplicate, 0, 1, 2, 5, 10, 15 and 20 µg BSA dissolved in 800 µl distilled water. To each was then added 200 µl of Bio-Rad reagent, the tubes were vortexed and the absorbance (A_{595}) read against a blank cuvette containing no protein, at a wavelength of 595 nm. The protein concentrations of the unknown samples were determined, in duplicate, in a similar manner, diluting 5 µl of the sample in 800 µl distilled water, adding Bio-Rad reagent and reading the absorbance in the spectrophotometer as before. Protein concentrations were determined by plotting the standard curve, with the protein concentration (mg/ml) represented on the y axis and the A_{595} on the x axis. Using the Cricket Graph software package, the equation of the best fit line was obtained and subsequently used to calculate the protein concentration by substituting the absorbance reading for "x" (see fig. 2.2).

2.4.9. *Preparation of hepatocytes*

Hepatocytes were kindly prepared by Dr. L. Zeng by a modification of the method of Berry and Friend (Berry and Friend, 1969) as described previously (Heyworth and Houslay, 1983) from 220 - 250 g fed male Sprague-Dawley rats.

2.4.10. *Isolation of adipocytes by collagenase digestion*

Low-phosphate Krebs

114 mM NaCl	6.92 g/l
4.7 mM KCl	0.32 g/l
1.18 mM $MgSO_4.7H_2O$	0.29 g/l
50 µM KH_2PO_4	6.8 mg/l
25 mM $NaHCO_3$	2.10 g/l
25 mM HEPES	5.96 g/l
3 % (w/v) BSA	30.0 g/l
1.0 mM $CaCl_2$	1 ml of 1 M $CaCl_2$ per litre

adjust to pH 7.4

Incubation buffer

Add 20 mg collagenase to 10 ml of low-phosphate Krebs buffer.

Epididymal fat pads were dissected and excised, rinsed with low-phosphate Krebs buffer at 37°C and cut into small pieces which were then washed using a tea strainer. Subsequently, the pieces were incubated in a shaking water bath at 37°C in 10 ml incubation buffer in a siliconised conical flask for approximately 1 h with continuous gassing with air. The adipocytes were then washed 5 times by centrifuging the suspension at 600 rpm (72 g_{av}) for 2 min, aspirating the buffer below the floating adipocytes, and adding 20 ml low-phosphate Krebs buffer (minus the BSA). Finally, the buffer was aspirated, and the adipocyte layer was either fractionated as described in

section 2.4.11., or added to an equal volume of Laemmli sample buffer and then placed in a boiling water bath for 4 min.

2.4.11. Preparation of membrane and cytosol fractions

Cells were homogenised in 1 mM EDTA, 20 mM Tris-HCl pH 7.5 buffer containing a 'cocktail' of freshly added protease inhibitors diluted from a 1000-fold stock (see section 2.3.3.). The cell suspension was homogenised in a glass dounce homogeniser, on ice, using 50 strokes and finally passed through a 25G syringe needle 10 times. The homogenate was subsequently centrifuged at 1,000 g_{av} for 10 min at 4°C to remove nuclei and intact cells, after which the supernatant was spun at 100,000 g_{av} for 40 min at 4°C. The pellet, representing the membrane fraction, was then resuspended in homogenisation buffer, while the resulting supernatant, containing the cytosol, was divided into aliquots and stored at -80 °C until subsequent use.

2.4.12. Lactate dehydrogenase (LDH) assay

This assay was used to provide a measure of the effectiveness of homogenisation. The level of LDH was ascertained by measuring the rate of oxidation of NADH. As NADH absorbs light of a wavelength of 340 nM, the rate of decrease of optical density at 340 nM is a measure of enzyme activity.

Free LDH in the homogenate was measured first, then Triton X-100 was added to a final concentration of 2% v/v in order to lyse any intact cells and the total LDH measured. The difference between the total and the free LDH levels gives the occluded LDH in the cells under investigation. Each reaction comprised 1.35 ml Tris-HCl (0.15M) pH 7.4, 50 µl sodium pyruvate (10 mM), 50 µl tissue sample (diluted so as to yield a measurable rate of change), in addition to 50 µl β-NADH (2 mM), added as substrate. The rate of change in A_{340}, at 30°C, was determined before and after the addition of Triton, reflecting free and total LDH activity, respectively.

% Cells broken = Free LDH/Total LDH x100

2.4.13. *Membrane cyclase assay*

Adenylate cyclase activity was determined by a modification of an assay described previously (Houslay *et al.*, 1976). Briefly, this involved using an assay mixture containing 10 µl of membranes (10-20 µg protein) and 50 µl of buffer/ATP regenerating system with final concentrations of 1.5 mM ATP, 5 mM $MgSO_4$, 10 mM theophylline, 1 mM EDTA, 1 mM dithiothreitol, 25 mM triethanolamine hydrochloride/KOH (pH7.4), 7.4 mg/ml phosphocreatine, 0.2 mg/ml creatine kinase and 0.8 mg/ml BSA. Where indicated, 10 µl of GTP (100 µM final concentration) or 10 µl of glucagon (10 nM final concentration) was added. The volume of each incubation mixture was made up to 100 µl with water. The membranes were incubated at 30 °C for 10 minutes prior to the addition of an equal volume of 4% PCA to stop the reaction. The samples were then centrifuged, the supernatants neutralised, and the cAMP content determined as described in section 2.4.14.6..

2.4.14. *Determination of intracellular cAMP production*

This was done using a modification of the procedure described previously (Savage *et al.*, 1995). The assay involves the measurement of intracellular cAMP production by the use of the cAMP binding protein, cyclic AMP-dependent protein kinase (Brown *et al.*, 1972). While the details of the assay are given in sections 2.4.14.1.-2.4.14.8., the basic outline is summarised below.

Briefly, following stimulation of the cells for the desired time, the cells were lysed, thus releasing the cytosolic cyclic nucleotides. After neutralising the extract, an aliquot was incubated with fixed quantities of [5', 8'-^{3}H] cyclic AMP and cyclic AMP binding protein, allowing competition of labelled and unlabelled cyclic AMP for a limited number of binding sites on the binding protein. Activated charcoal was then added to the sample to bind any free cyclic nucleotide and the charcoal pelleted by a brief centrifugation step. The radioactivity of a proportion of the supernatant was then determined, serving as an indication of the labelled nucleotide that was not displaced from the cAMP binding protein by the unlabelled cAMP. A standard curve was constructed by incubating a range

of known concentrations of unlabelled cyclic AMP with the fixed amounts of binding protein and radioactive cyclic AMP. Thus it was possible, thereafter, to determine unknown cyclic AMP concentrations from the radioactivity counted by reference to the standard curve.

2.4.14.1. COS cell stimulation

When confluent, approximately 72 hours following transfection, cells (approximately 5 x 10^5) in individual wells of a 6-well culture plate were incubated at 37°C with serum-free DMEM containing 1 mM-IBMX (unless stated otherwise), and, where appropriate, ligands were added to the indicated final concentrations after a further 15 min. When PMA was present, it was added to a concentration of 1 μM in the pre-incubation medium.

2.4.14.2. Extraction of cyclic AMP

After the indicated period of incubation with ligands, incubations were stopped by the aspiration of the medium from the cells and the addition of 500 μl of 2% perchloric acid (PCA). Following incubation for 15 min on ice, the cells were then scraped and the precipitated protein pelleted by centrifuging the samples in a microfuge at 13,000 g_{av} for 2 min at room temperature.

2.4.14.3. Neutralisation of the sample

To the supernatant 5 μl universal indicator solution was added, which turned pink. Neutralisation was achieved by the gradual addition of approximately 100 μl 2M KOH/0.5 M triethanolamine until the solution turned green, and the potassium perchlorate precipitate sedimented by a further centrifugation for 2 min at 13,000 g_{av}. The cyclic AMP content of the supernatant was determined using a cyclic AMP-binding protein prepared from bovine adrenal glands as described below.

2.4.14.4. Preparation of cAMP binding protein

Adrenal cortex homogenisation buffer

0.25 M sucrose	85.6 g/l
50 mM Tris, pH 7.4	6.06 g/l
25 mM KCl	1.86 g/l
5 mM $MgCl_2.6H_2O$	1.02 g/l

The cAMP binding protein was prepared by the method of Brown et al. (Brown *et al.*, 1972) from bovine adrenal glands. All steps were performed at 4 °C. From 30 fresh adrenal glands, collected from a local abattoir and kept on ice, the surrounding fat was dissected and the gland hemisected to expose the pale inner medulla and dark outer cortex. Using a scalpel, the medulla was removed and discarded. The cortex was then scraped from the adrenal capsule using a scalpel blade and placed in beaker of buffer. One volume of tissue was homogenised in one and a half volumes of homogenisation buffer in a Waring blender. The homogenate was filtered through muslin and the muslin washed with a small volume of buffer. The filtrate was then centrifuged at 27,000 g_{av} (eg. at 15,000 rpm in a JA20 rotor) for 15 minutes at 4°C. The supernatant was subsequently decanted through filter paper, and the filtrate was aliquoted into 0.25 ml fractions which were stored at -20°C. Separate samples were thawed and diluted for each assay.

2.4.14.5. Reagent preparation for the cAMP binding assay

The following solutions were prepared freshly on the day of the assay:

cAMP assay buffer

50 mM Tris 6.07 g/l

4 mM EDTA 1.49 g/l

pH to 7.4 and store at 4 °C

Binding protein: the binding protein was prepared as described in section 2.4.14.4., then diluted 30-fold in cAMP assay buffer, and stored at 4°C until required for use.

[5', 8'-^{3}H] cyclic AMP: 13 µCi (13 µl) [5', 8'-^{3}H] adenosine 3', 5'-cyclic monophosphate was added to 15 ml cAMP assay buffer, and stored at 4°C until required for use.

Charcoal solution: the required volume, consisting of 2% (w/v) activated charcoal and 1% (w/v) bovine serum albumin in assay buffer, was stirred, on ice, for at least 15 minutes before it was used.

cAMP standards: these were obtained by making a series of two-fold dilutions from the most concentrated standard, 16 pmol/50 µl. The latter was obtained by diluting an aliquot of 32 µM cAMP by 100 fold in assay buffer, to a concentration of 320 nM (16 pmol/50 µl).

2.4.14.6. Assay procedure

A standard curve was obtained for each assay performed by including a set of tubes containing, in duplicate, quantities of cyclic AMP varying between 0.0625 and 16 pmol. These tubes, together with the 50 µl experimental samples were set up as follows:

Tube no.	cAMP (pmol/50µl)	Buffer (µl)	[^{3}H]-cAMP (µl)	Binding protein (µl)
1, 2	-	200	100	-
3, 4	-	100	100	100
5, 6	0.0625	50	100	100
7, 8	0.125	50	100	100
9, 10	0.250	50	100	100
11, 12	0.500	50	100	100
13, 14	1.00	50	100	100
15, 16	2.00	50	100	100
17, 18	4.00	50	100	100
19, 20	8.00	50	100	100
21, 22	16.0	50	100	100
23-	samples	50	100	100

The first two tubes, to which no binding protein was added, were included to determine the quantity of cyclic AMP that remained after nucleotide binding by charcoal. The results from tubes 3 and 4 indicated the maximum cyclic AMP bound in the absence of any unlabelled cAMP and tubes 5-22 were prepared with a range of known cyclic AMP quantities in order to construct the standard curve, thus permitting the subsequent calculation of unknown cyclic AMP values to be calculated.

The tubes were set up as described above on ice, adding binding protein to the tubes last. The tubes were then vortexed and incubated at 4 °C for 2 hours. After this time, 250 µl charcoal solution was added, and the tubes were immediately vortexed and spun in a microfuge at 12000 g_{av} for 5 minutes. An aliquot of 300 µl was removed from the resulting supernatant, added to 2 ml Ecoscint, and the radio-activity determined by counting for 3 min in a scintillation counter. Curve-fitting software was used to calculate pmol unlabelled cAMP per vial.

2.4.14.7. Calculation of cyclic AMP levels

After correction for the sample volume and for the cell number per well (see section 2.4.14.8.) the results were expressed as mean pmol cyclic AMP produced/10^6 cells ±SEM.

2.4.14.8. Determination of cell number and viability

For each experiment, COS cells were divided into 3 additional wells which were then incubated together with the other 6-well plates of cells, but were reserved for cell counting. From these wells, the cells were trypsinised, pelleted and resuspended in 500 µl DMEM. Subsequently, an aliquot of 100 µl was mixed with an equal volume of Trypan Blue solution (0.4%) and the number of viable cells was determined. This was performed by viewing the cells under a light microscope in a heamocytometer and counting those cells that excluded the Trypan Blue dye; that is the cells whose membranes remained intact, thus remaining white under the microscope. The cell number was calculated by using the grid on the slide, which ensured that the same area was counted

for each determination. The number of cells per ml suspension was calculated by multiplying the mean number of intact cells in the central grid by 1 x 10^4.

2.4.15. Cyclic AMP phosphodiesterase assay

Cyclic AMP phosphodiesterase activity was determined by a modification (Marchmont and Houslay, 1980) of the two-step procedure of Thompson and Appleman (1971) (Thompson and Appleman, 1971). Briefly, [8-^{3}H] - adenosine-3',5'- cyclic monophosphate was hydrolysed by the PDE to form labelled nucleotide mono-phosphate which was then completely converted to the corresponding labelled nucleoside by the 5'-nucleotidase activity of snake venom. Unhydrolysed cyclic nucleotide was subsequently separated from the nucleoside by incubation of the mixture with Dowex-1-chloride, which binds charged nucleotides but not uncharged nucleosides.

2.4.15.1. Stimulation and lysis of cells for the PDE assay

Incomplete KHEM buffer

50 mM KCl	3.73 g/l
10 mM EGTA	3.80 g/l
50 mM HEPES, pH 7.2	13.0 g/l
1.9 mM $MgCl_2.6H_2O$	0.390 g/l

Complete KHEM buffer

Add, immediately prior to use, to incomplete KHEM buffer:

1 mM DTT	0.1 % (v/v) of 1 M stock
protease inhibitor mix	0.1 % (v/v) of 1000x stock
20 µg/ml cytochalasin B	0.1 % (v/v) of 20 mg/ml stock

TEA/KCl

150 mM KCl	11.2 g/l
10 mM triethanolamine	
pH to 7.2 with HCl	

Transfected COS cells, grown in 75 cm^2 flasks, were incubated at 37 °C with serum-free DMEM containing either 1 μM PMA or 0.1% DMSO for 15 min. Glucagon was then added to a final concentration of 10 nM and the incubation continued for a further 10 min. The flasks were then placed on ice, and the culture medium aspirated and discarded. 2 ml complete KHEM (ice-cold) was then added and the flasks incubated at 4 °C for 45 min. All subsequent steps were carried out at 4 °C. The KHEM was then removed and 5 ml ice-cold TEA/KCl added . After 10 min, the TEA/KCl was aspirated and the cells washed with incomplete KHEM. The latter was then replaced by 2 ml complete KHEM, which, after a further 2 min, was removed. Using a cell scraper, the cells were then harvested and the suspension was transferred to a glass on glass dounce homogeniser. Following homogenisation with 20 strokes, the homogenate was centrifuged at 4 °C for 5 min at 1500 rpm in a benchtop refrigerated microfuge to pellet nuclei and cell debris. The supernatant was then divided into aliquots of 10 μl, 'snap' frozen in liquid nitrogen and stored in the -80°C freezer until required.

2.4.15.2. PDE assay reagent preparation

3',5'-cyclic AMP was prepared at a concentration of 1 mM in 20 mM Tris-HCl, pH7.4, containing 5 mM $MgCl_2$ and frozen as stock. The concentration required for the assay was obtained by diluting the stock on the day of each assay. 3-isobutyl 1-methylxanthine (IBMX) was freshly prepared on the day of the assay as a 500 mM solution in DMSO and subsequently diluted to the desired concentration in 20 mM Tris-HCl / 5 mM $MgCl_2$ buffer, pH7.4. A maximum concentration of 0.2% DMSO was employed in the assays, and such levels of DMSO did not significantly affect the PDE activities. Snake venom was prepared by dissolving the venom of *Hannah ophiophagus* in distilled water at a concentration of 10 mg/ml. This stock solution was frozen down in aliquots and was diluted to 1 mg/ml in distilled water for use in the assay. Dowex 1X8-400-chloride was prepared as described previously (Thompson *et al.*, 1979). Briefly, at 4°C, 400g of Dowex 1-chloride was washed with 4 litres of 1M NaOH for 15 minutes.

The resin was then washed repeatedly with distilled water, until the pH of the eluate fell to 7.0. Subsequently, the resin was washed with 4 litres of 1M HCl for 15 minutes, followed by further washes in distilled water until the eluate pH rose to 3.0. The resin was stored at 4°C, mixed with distilled H_2O (1:1). For use in the assay, the Dowex:water mix was further diluted with ethanol, to a final ratio of Dowex:water:ethanol of 1:1:1.

2.4.15.3. Assay procedure

A 10 µl sample of cell extract was added, in microcentrifuge tubes on ice, to 40 µl of 20 mM Tris-HCl, pH7.4, 5 mM $MgCl_2$, containing, where indicated, either DMSO or IBMX at 2.5 times the desired concentration. Blanks were included with every experiment done, replacing the cell extract with 10 µl KHEM buffer (see section 2.4.15.1.). Following the addition of 50 µl of 2 µM cAMP containing 0.15 µCi cAMP, and mixing the tubes by vortexing, the tubes were placed in a 30°C waterbath and incubated for 10 minutes, unless otherwise indicated. After this time the tubes with placed in a boiling water bath for 2 minutes to terminate the phosphodiesterase activity. The samples were then allowed to cool on ice. 25 µg snake venom was added to the tubes and they were incubated for a further 10 min in a 30°C waterbath. The tubes were then placed on ice, and to each tube, 400 µl freshly prepared slurry of Dowex:ethanol:water (1:1:1) was added. During this addition, the Dowex was stirred gently, in order to ensure that a homogeneous suspension was being added. The tubes were vortexed twice over a 15 minute period, and then centrifuged at 12,000 g_{av} to pellet the Dowex resin. An aliquot of 150 µl supernatant was removed and mixed with 2 ml Ecoscint, before being counted in a liquid scintillation counter for 1 minute per sample.

2.4.15.4. Calculations

The cpm values obtained with the blank for each condition was subtracted from each cpm result to give the corrected cpm per sample. Calculation of specific activity (pmol cAMP hydrolysed/min/mg ± SEM) was performed after determining the protein content (see section 2.4.8.) of each sample.

2.4.16. *COS and 293 cell culture*

Stock cultures of COS-7 cells were maintained in DMEM supplemented with penicillin (100 units/ml), streptomycin (100 µg/ml), 2 mM glutamine and 10% (v/v) foetal bovine serum. Cells were grown in an atmosphere containing 5% CO_2 at 37 °C in a humidified incubator. Cells were routinely split 1:3 by trypsinisation and confluence was attained within 3-4 days.

2.4.17. *PtdCho-PLC and PtdCho-PLD measurement*

The measurement of PtdCho-PLD and PtdCho-PLC activities was performed using a modification of a method described previously (Cook *et al.*, 1991). COS-7 cells were transfected with the GR-encoding plasmid and subsequently grown in 24-well plates. When 80% confluent, the culture medium was replaced with 0.5 ml DMEM containing 1% (v/v) foetal calf serum (FCS) and 2 µCi [^{3}H]-palmitic acid/ml. After an incubation period of 24 hours the cells were then washed in 0.25 ml serum-free DMEM at 37 °C for 30 minutes prior to incubation for a further 5 min in 0.25 ml of serum-free DMEM containing 0.3% (v/v) butan-1-ol. Incubations were commenced by the addition of 28 µl glucagon in DMEM/0.3% (v/v) butan-1-ol giving the desired final concentration and were continued at 37 °C for a further 10 minutes unless otherwise stated. Incubations were terminated by aspiration of the medium and the addition of 0.2 ml of ice-cold methanol to each well. Samples were harvested on ice and transferred to glass vials. Each well was then rinsed with a further 0.2 ml methanol which was combined with the first. Lipids were extracted for at least 15 min at room temperature with 0.4 ml of chloroform. The solvent mixture was evaporated *in vacuo* and the lipids re-dissolved in 100 µl of chloroform/methanol (19:1, v/v) by vortex-mixing. Subsequently, 10 µl of the sample was transferred to a scintillation vial for counting in order to measure total lipid labelling and allow normalisation of sample size. The remainder of the sample was applied to a Whatman LK5D TLC plate which was developed in the organic phase of 2,2,4-trimethylpentane/ethyl acetate/acetic acid/water (5:11:2:10, by vol.) using an unlined chromatography tank, until the solvent reached 1 cm from the top of the plate. [^{3}H]-

PtdBut and [^{3}H]-PtdOH, identified, upon iodine-staining, by their co-migration with authentic PtdBut and PtdOH standards, were excised from the plates and their radioactivity determined by scintillation counting.

2.4.18. Measurement of inositol phospholipid hydrolysis

Transfected COS cells, grown for 48 hours in DMEM containing 10% (v/v) FCS were incubated in 0.5 ml DMEM containing 1% (v/v) FCS for a further 24 hours. The cells were then washed in 0.25 ml serum-free DMEM at 37 °C for 30 minutes. Stimulations were performed by the addition of 11 µl glucagon diluted appropriately in DMEM to give the desired final concentrations. Reactions were terminated after 10 seconds by the addition of 25 µl ice-cold 10% (v/v) perchloric acid. Samples were harvested on ice and neutralised in the presence of a trace of Universal Indicator by the addition of approximately 25-30 µl 1.5 M KOH/60 mM HEPES. A sample of 110 µl was taken for Ins(1,4,5)P_3 mass measurement.

2.4.18.1. Ins(1,4,5)P_3 measurement by binding assay

Ins(1,4,5)P_3 assay incubation buffer

4 mM EGTA	1.52 g/l
4 mM EDTA	1.17 g/l
0.4% (w/v) BSA	
100 mM Tris	12.1 g/l
pH to 9.0	

Ins(1,4,5)P_3 mass was quantified using a stereospecific radioligand binding assay (Palmer and Wakelam, 1990). All procedures were undertaken at 4°C. 110 µl neutralised cell extract was added to 110 µl incubation buffer, 110 µl [^{3}H]-Ins(1,4,5)P_3 (approximately 7,770 dpm) diluted with distilled water, and 110 µl of binding protein (approximately 3 mg), prepared from bovine adrenal cortex as described in section

2.4.14.4.. A standard curve using 0-256 pmol Ins(1,4,5)P_3 (diluted in DMEM, to which PCA, KOH, HEPES and Universal Indicator had been added) was conducted in parallel (fig. 2.3.).

Samples were incubated on ice for 30 min, then centrifuged at 12,000 rpm at 4 °C for 3 min, and the supernatant aspirated. Each pellet was mixed with 1 ml of scintillant and the associated radioactivity determined by scintillation counting. As a precaution, samples taken from the 1 μM and 30 nM dilutions, and from water, were diluted to 110 μl without DMEM to determine whether the phenol red contained within the medium interfered with binding. However, a comparison of these results with those obtained from the standards in which DMEM had been used to dilute the samples, indicated that no such interference took place.

Figure 2.1. Analysis of DNA sequencing error frequency

A typical analysis of the mis-reading frequency, within an 842 bp DNA sequence file, imported from an automated ABI DNA sequencer into the GeneJockey II software package. The error frequency is indicated on the vertical axis, while the horizontal axis represents the position within the entire length of the sequence. Only the region with an error frequency of 10 % or less, corresponding to nucleotides 42 to 425 of the sequence, was extracted for subsequent analysis.

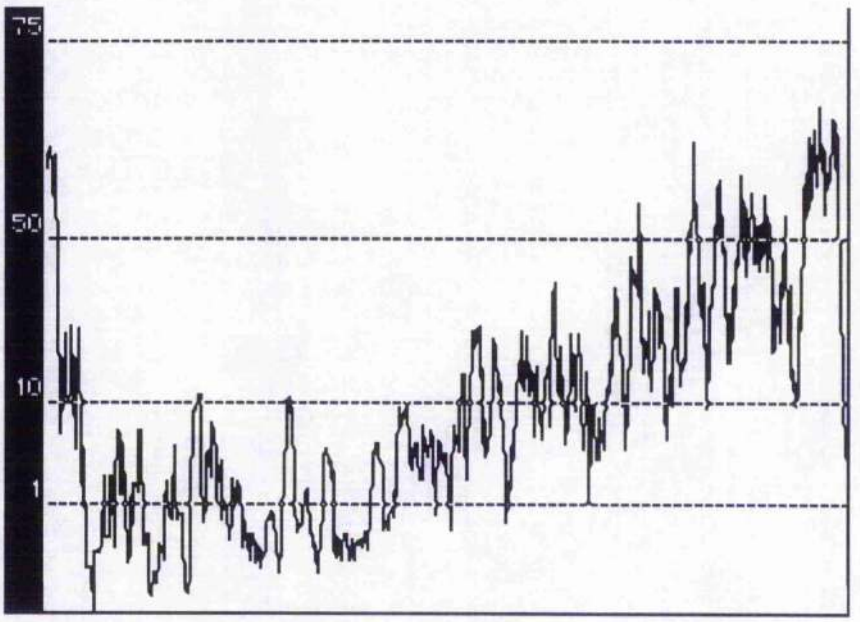


Figure 2.2. Standard curve for Bradford protein assay

The samples for the standard curve were prepared as described in section 2.4.8. BSA concentrations of 0-20 µg were used as protein standards as indicated, and the absorbance was read at 595 nm after zeroing the spectrophotometer using the sample to which no protein was added. Each point represents the mean of duplicate samples. This experiment has been carried out at least twenty times.

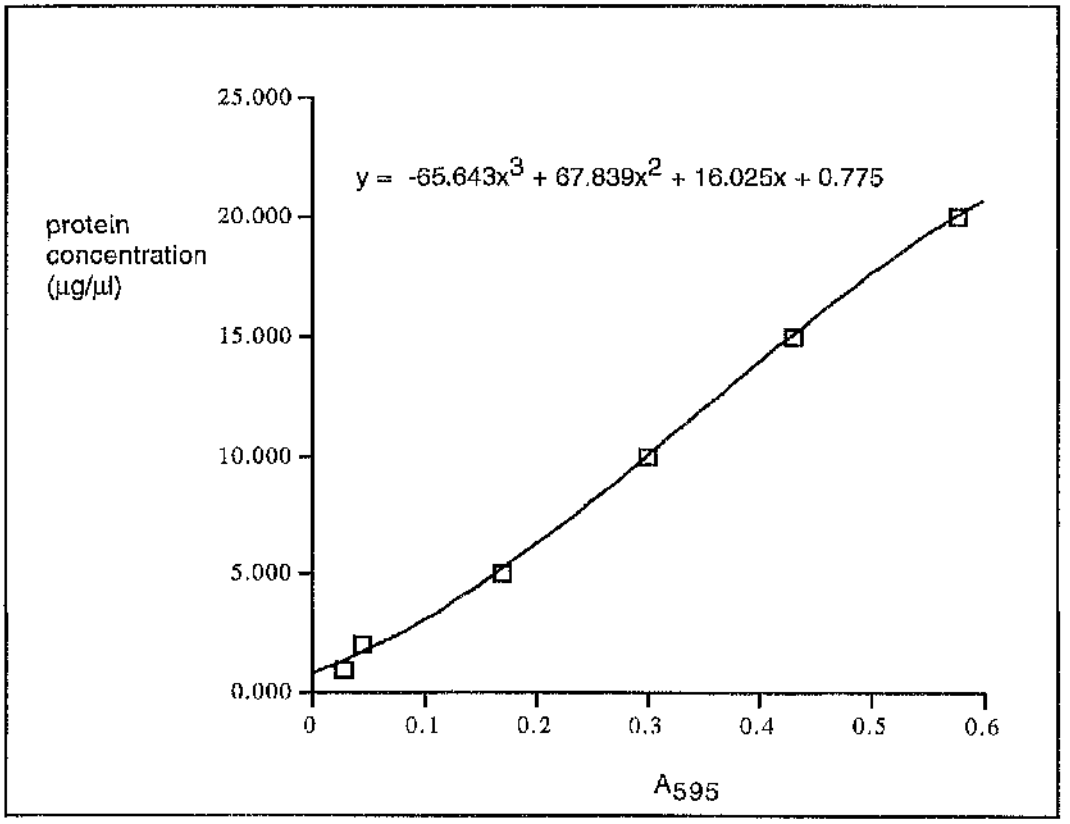
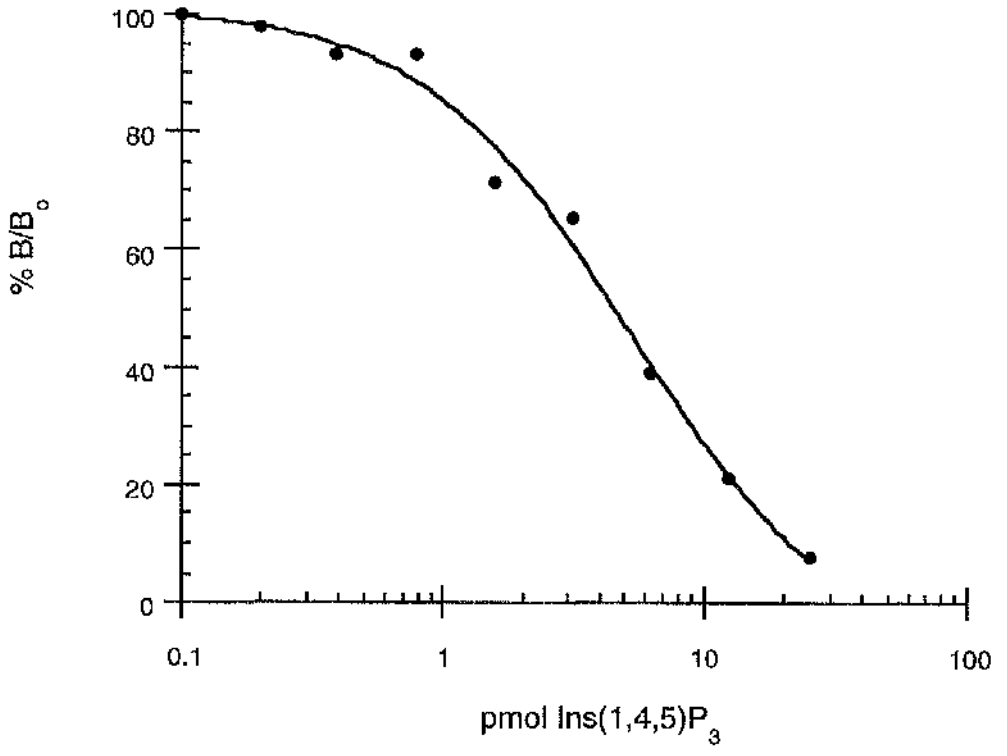


Figure 2.3. Standard curve for the Ins (1,4,5) P_3 assay

This standard curve represents the specific binding of [^{3}H]-Ins(1,4,5)P_3, using a range of concentrations of unlabelled Ins(1,4,5)P_3 as the competing ligand. The standard curve is plotted as %B/B_0 versus pmol/sample of unlabelled Ins(1,4,5)P_3. B represents the specific binding (i.e. dpm bound - NSB) at a given concentration of unlabelled Ins(1,4,5)P_3; B_0 is the maximum specific binding i.e. dpm bound in the absence of any unlabelled Ins(1,4,5)P_3 - NSB; and NSB is non-specific binding. Similar calculations were performed to determine the specific binding for the unknown samples. Thus, from the standard curve, the value of pmol Ins(1,4,5)P_3 present in each sample was determined. This assay is sensitive to changes in Ins(1,4,5)P_3 mass of approximately 1-30 pmol.



CHAPTER 3

Approaches towards the construction of a full-length cDNA encoding the glucagon receptor, and the incorporation of an epitope tag at its amino-terminus.

3.1. Introduction

A pre-requisite for the intended study, in transfected COS cells, of the mechanism by which the glucagon receptor (GR) is regulated, was the acquisition of the full-length cDNA encoding this protein. Unfortunately, we had hitherto been unable to obtain this cDNA from the authors of the two papers in which the cloning and sequencing of the receptor cDNA had been described (Jelinek *et al.*, 1993; Svoboda *et al.*, 1993b). It was deemed necessary therefore to obtain the receptor-encoding cDNA by another means. In fact, it appeared that another researcher in our laboratory, Dr. S. Griffiths, had previously isolated from a rat hepatocyte cDNA library, a partial cDNA for the GR, corresponding to the C-terminal region (nucleotides 505-1458 of the coding sequence). This cDNA species was isolated by screening the library with a 517 bp RT-PCR product that had been generated using PCR primers specific for regions 119-142 and 612-635 of the published rat GR nucleotide sequence. I therefore decided to use RT-PCR (utilising a polymerase possessing 3'-5' exonuclease proof-reading activity) to amplify the cDNA encoding the amino-terminal region of the GR, with sufficient overlap to permit the subsequent digestion of the product and its ligation to the existing C-terminal cDNA. The PCR and the subsequent analyses of the GR-encoding cDNAs are described in this chapter.

In addition, with the intention of examining the expression, intracellular distribution, disease-related changes in expression, and phosphorylation of the GR, it was considered necessary to develop a means by which the receptor could be both immunoblotted and immunoprecipitated. In previous years, when GR-specific antisera were not yet available, the identification of the receptor protein was undertaken by techniques such as electrophoresis of proteins following the binding and cross-linking of radio-labelled iodo-glucagon (Iyengar and Herberg, 1984). Such studies showed the receptor to migrate under denaturing conditions with an apparent mobility of 63 kD. Later, the partial purification of the rat hepatic receptor led to the development of GR-specific monoclonal antibodies (Iwanij and Vincent, 1990) which recognised both protein

and carbohydrate antigenic determinants. Such antibodies detected an immunoreactive species migrating with a Mwt of 62 kD (Iwanij and Vincent, 1990).

In our laboratory, using the amino acid sequence of the rat hepatic GR deduced from the cloning and sequencing of the cDNA encoding the receptor (Jelinek *et al.*, 1993; Svoboda *et al.*, 1993b), five peptides corresponding to various extracellular or intracellular regions of the receptor were synthesised. These peptides were individually coupled to KLH and used to immunise a series of rabbits.

An alternative strategy for the generation of polyclonal antisera involves the prior construction, and expression in bacteria, of a plasmid encoding a fusion protein comprising a hapten (the peptide sequence of interest) fused, in frame, to a carrier molecule such as glutathione S-transferase (GST). This method had been used previously in our laboratory (Huston *et al.*, 1996) to permit the generation of phosphodiesterase-specific antisera. Such a strategy, in addition to that of peptide immunisation, was employed here with the aim of raising an antiserum specific for the carboxy-terminus of the GR.

The testing of the antisera subsequently obtained from both the KLH-coupled peptide and the GST fusion protein techniques is described in the Appendix. Unfortunately, none of the antisera appeared to be able to detect the transfected glucagon receptor.

An additional method used to permit the immunodetection of a protein is to mutagenize the cDNA which encodes it by the technique known as epitope tagging. In this method, a foreign epitope, or epitope tag, for which a specific monoclonal antibody is available, is incorporated into the amino (N) or carboxy (C)-terminus of the protein of interest. This is achieved by the insertion of the appropriate nucleotide sequence, in frame, immediately 5' to the termination codon or immediately 3' to the start codon of the cDNA which encodes it. The incorporation, by a PCR-based strategy, of such a sequence into the N-terminal region of the cDNA encoding the GR is described in this chapter. This technique has been undertaken in a number of instances, using as an epitope, peptides such as substance P (Munro and Pelham, 1984; Albers and Fuchs,

1987) or c-Myc (Munro and Pelham, 1986; Munro and Pelham, 1987; Pelham *et al.*, 1988). However, since such epitopes are derived from cellular proteins, such as neuro-transmitters or proto-oncogenes, there is a significant chance that the resulting antisera will cross-react with a cellular protein other than the one of interest. To minimise this risk, the epitope tag chosen for the tagging described in this chapter, was composed of a viral epitope, corresponding to the peptide sequence of part of the C-terminus of the vesicular stomatitis virus G protein (Kreis, 1986; Soldati and Perriard, 1991).

3.2. Results

3.2.1. RT-PCR of the 5' region of the cDNA encoding the GR

The first step of the strategy outlined in figure 3.1. was to amplify the coding sequence corresponding to the N-terminal region of the glucagon receptor. In order to amplify this cDNA with sufficient overlap with the existing C-terminal fragment to permit subsequent digestion and ligation, PCR oligonucleotides were designed so as to amplify a region corresponding to nucleotides 1 to 932 of the open reading frame (ORF) of the published receptor sequence. The antisense primer matched a sequence within the ORF that contained a unique BamH I restriction site, while the 5' oligonucleotide was designed so as to anneal to the first 12 bases of the ORF, with in addition, a 9 bp 5' extension containing an Spe 1 site. As no Spe 1 site was present in the entire coding sequence of the GR, as deposited in Genbank, it was included in the 5' primer in order to facilitate subsequent ligation of the 5' end of the GR cDNA to the Spe 1-cut cloning site of the expression vector, pSV-Sport 1. The primer pair designed was, therefore:-

5' sense oligonucleotide ET-GR-s1; GCGACTAGTATGCTCCTCACC (Spe I site underlined)

3' antisense oligonucleotide ET-GR-as1; GGGATACGCAGGATCCACC. (BamH I site underlined)

Such primers would be predicted to amplify a specific 941 bp product corresponding to nucleotides 1-932 of the open reading frame of the rat hepatic glucagon receptor (Genbank accession no. M96674) with the additional 9 bases incorporated at the extreme 5' end.

The reaction conditions chosen were designed with an initial low annealing temperature cycle, in order to permit the 12 complementary bases of the 5' primer to anneal effectively. Thus, the first cycle consisted of a denaturation step of 95°C for 2 min, an annealing segment of 37°C for 2 min, and an elongation step of 55°C for 2 min. This was followed by 35 cycles, each comprising a denaturation step of 95°C for 1 min,

an annealing segment of 55°C for 2 min, and an elongation step of 72°C for 3 min. A final elongation step of 60°C for 7 min was also included.

RNA was extracted from rat hepatocytes by the method described in section 2.2.12. and subjected to reverse transcription and PCR using the primers described above. In addition, serving as a positive control, a PCR reaction was undertaken using cDNA and a pair of primers known to amplify a sequence of approximately 400 bp.

It was found that although no RT-PCR product could be detected, the control reaction yielded a DNA product of the predicted size (figure 3.2.A). It was concluded, therefore, that the reverse transcription reaction, itself, must have been ineffective, perhaps as a result of a defective reverse transcriptase enzyme. When this was replaced in a subsequent experiment with fresh enzyme, the RT-PCR did indeed yield a product of the predicted 941 bp size (figure 3.2.B). An additional DNA product of approximately 1030 bp was also visible, however, which may have reflected the amplification, by RT-PCR, of an incompletely spliced transcript.

In order to confirm that the species of higher mobility was indeed that resulting from amplification of the desired fully spliced GR transcript, this product was purified from an agarose gel and digested with the restriction enzyme Pst 1. This restriction digest resulted in two products (figures 3.3.A and 3.3.B), with mobilities corresponding exactly to the predicted lengths of 683 and 258 bp, respectively (figure 3.3.C).

3.2.2. Preparation and analysis of cDNA fragments of GR prior to ligation

In order to permit the subsequent ligation of the 5' DNA fragment prepared as described in section 3.2.1 to the previously isolated 3' fragment, and the insertion of the product into pSV-Sport 1 (figure 3.1.), it was necessary to subject all three DNA species to double restriction digestion. The 3' fragment, inserted in pUC-18, was thus digested with both BamH I and HinD III. It was calculated that the product of such a double restriction digest would be a 2.7 kb linearised vector fragment and an excised insert of

858 bp (figure 3.4.A). Surprisingly, however, the products of this reaction, comprised the 2.7 kb DNA fragment corresponding to the linearised pUC18 vector, and, instead of a single 858 bp insert, two fragments of approximately 560 bp and 600 bp, respectively, (figure 3.4.B.). I considered that such digestion products might have been obtained if the insert had contained an additional region of DNA, of approximately 300 bp, and if such a region was, itself, to contain a BamH I or a HinD III restriction site.

Intriguingly, the published genomic rat hepatic GR sequence (Svoboda *et al.*, 1993a), includes 4 introns, the most 3' of which, is located 1221 bp downstream of the start of the open reading frame, and thus 311 bp from the exonic BamH I site. In addition, this intron is 300 bp in length, and does indeed contain an additional BamH I restriction site, at a position 250 bp from its 5' end. If it is assumed that this intron is indeed present within the 3' GR DNA fragment then, based upon this data, the species predicted to result from a BamH I / HinD III double restriction digest would be 561 bp and 597 bp, respectively (figure 3.5.), in addition to the 2.7 kb vector. In view of the proximity of these predicted sizes to those actually observed, it was considered that the presence of this intronic DNA was a likely explanation for the aberrant digestion products. This hypothesis was tested by digesting the DNA with either BamH I or HinD III, independently. The resulting fragments were found to correspond to the predicted species of 3703 bp and 561 bp (BamH I) and 4264 bp (HinD III) (figure 3.6.).

3.2.3. Design of PCR oligonucleotides for the amplification of the entire GR coding sequence

It has been reported that the transfection of GR-encoding cDNA which contains one or more introns does not lead to the expression of functional receptors (Svoboda *et al.*, 1993a). Therefore, in view of the apparent presence of intronic DNA within the 3' GR fragment, to which it had been planned to ligate the 5' fragment obtained by RT-PCR, it was considered that a preferable approach would be to use RT-PCR to amplify the entire

GR coding sequence. The successful utilisation of such a strategy has been described previously (Tung *et al.*, 1989) with respect to the isolation of the entire coding sequence of a South American bat salivary protein. I thus designed oligonucleotide primers which would direct the amplification of the entire coding sequence of the glucagon receptor and, in addition, would possess the appropriate restriction enzyme sites to permit the subsequent insertion of the product into an expression vector. The choice of restriction enzyme was governed by the following criteria: such restriction sites should be absent from the coding sequence of the receptor itself, and should occur only once in the entire expression vector sequence, at the multiple cloning site.

As the 5' (sense) oligonucleotide primer described above had already been shown to direct the amplification of GR cDNA effectively, and as it contained a suitable restriction site, this primer was used again as the 5' sense PCR primer. The 3' oligonucleotide was designed so as to anneal to the GR-encoding sequence, downstream of the termination codon at the position 1496-1507 relative to the start of the open reading frame. A HinD III site (underlined) was incorporated into the 5' end of this antisense primer. The sequence of this 3' antisense oligonucleotide, ET-GR-as2, was: 5'-GCGTGAAGCTTTCTTGTGAGGCC-3'.

As the sequence to be amplified was relatively long (1527 bp), a proof-reading Pfu polymerase was used, to reduce the rate of errors. Unfortunately, no product of this size resulted from the RT-PCR, although it remained possible to amplify the 941 bp product with the primers ET-GR-s1 and ET-GR-as1, indicating that the Pfu polymerase, its buffer, and the first-strand cDNA preparation were functional (figure 3.7.). The desired PCR product was not obtained in subsequent reactions, despite varying the annealing temperature, using the "hot start" technique (Chou *et al.*, 1992), increasing the primer concentrations, preparing fresh RNA and, in addition, employing the following alternative 3' antisense oligonucleotide sequences:

Name	Sequence	Position in ORF
ET-GR-as3	5'-GCATGAAGCTTTTGGCTGGAGTC	1469-1480
ET-GR-as4	5'-CGGAAGCTTCTTCAGCCTTGGC	1528-1540
ET-GR-as5	5'-CGGAAGCTTTGCTGCTTTGCC	1542-1555
ET-GR-as6	5'-CGGAAGCTTCAGTTGAGGAAACAG	1203-1215 (truncating)

ET-GR-as6 was designed so as to effect a C-terminal truncation of the GR and therefore represents the complement of a sequence located within the ORF. The other oligonucleotides were designed so as to anneal to sequences located downstream of the 1458 coding sequence and should, therefore, have led to the amplification of a product encompassing the entire ORF.

Fortunately, we subsequently received the kind gift of an intron-free, full length GR-encoding cDNA (Svoboda *et al.*, 1993b) which was used successfully for the transfection studies described in chapters 4 and 5, and as a template for the epitope tagging PCR described in section 3.2.4..

3.2.4. Epitope tagging the glucagon receptor

In view of the difficulty in obtaining a GR-specific antiserum (see Appendix 1), it was considered worthwhile to attempt to engineer an epitope tag onto the receptor molecule. The tag chosen was the 12-residue epitope sequence of the VSV G protein C terminus, the successful use of which had been reported previously (Soldati and Perriard, 1991). It is recognised by the monoclonal antibody, P5D4. As Soldati and Perriard reported that it was not possible to truncate the epitope without preventing immunoprecipitation of the tagged protein by the monoclonal antibody, the full sequence was used here to tag the glucagon receptor.

As the addition of an amino acid sequence such as an epitope tag within the receptor sequence might be envisaged to cause a conformational change in the receptor, it was decided to place the tag at one or other of the termini of the molecule. It was considered,

however, that its addition to the C-terminus might interfere with the coupling of the receptor to Gs. Furthermore, tagging of the C-terminus could reduce any propensity of this region for phosphorylation, an effect which it was intended to analyse subsequently using the tagged receptor. Thus it was considered expedient to introduce the tag at the amino terminus of the protein. A PCR-based strategy was employed (figure 3.8.), in which the nucleotide sequence encoding the 36-base tag was incorporated into a 5' sense oligonucleotide primer, immediately 3' to the start codon. Both this sense primer and the antisense primer were so designed as to permit their subsequent cleavage by restriction enzymes and the ligation of the product into the appropriately cut vector containing the wild-type GR sequence. The sense primer, therefore, comprised a HinD III site, close to its 5' end, followed by the sequence ACCATG, serving as an optimal Kozak sequence (Kozak, 1986) and start codon, which, in turn, was followed by the tag-encoding sequence and the coding sequence of the amino-terminus of the GR. The 3' primer was designed so as to anneal to a region of the GR sequence that contained the unique restriction site, Esp I.

The PCR conditions, which included an initial low-temperature annealing cycle to permit the 3' portion of the sense oligonucleotide to anneal to its complementary sequence, were as follows:

1 cycle comprising:

95°C 2 min
62°C 2 min
70°C 8 min

35 cycles, each comprising:

94°C 1 min
71°C 8 min

1 cycle comprising:

72°C 10 min

The Pfu polymerase with 3'-5' proof-reading exonuclease activity was used in the reactions in order to minimise the error rate.

The PCR reaction gave rise to the predicted 832 bp product (figure 3.9.A, lane 4). This species was excised and purified from the gel. Following restriction digestion with

HinD III and Esp I, attempts were made to ligate it into the vector pCDM8-GR which had been similarly digested and purified. Notably, the fragment excised from this vector by the HinD III/ Esp I digestion (figure 3.9.A, lane 1) was considerably larger (at 950 bp) than the PCR product which was to be used to replace it, indicating the presence of additional 5' untranslated sequence in the vector, located between the HinD III site and the start of the sequence encoding the GR. Following ligation and transformation, several colonies were identified on the selective plates. In order to verify that these colonies had resulted from the desired ligation, their plasmid DNA was isolated and PCR was performed with the GR-specific primers ET-TAG-s1 and ET-TAG-as1. The cycling parameters were set so as to exclude the initial low-annealing temperature cycle, in order to minimise amplification from unmodified pCDM8-GR plasmid. The results from the analysis of 5 typical colonies are shown in figure 3.9.B.

Unfortunately, these PCR reactions failed to discriminate between unmodified and recombinant pCDM8-GR plasmid (figure 3.9.B, lanes 1-6). In addition, however, HinD III/ Esp I double digests were undertaken on the same DNA samples and on unmodified plasmid DNA as a control. It was subsequently found, by these restriction digests (figure 3.9.B, lanes 8-13), and by subsequent DNA sequencing reactions, that the plasmid DNA isolated from these colonies did not contain the tagged receptor sequence. Thus, the digests yielded either no detectable excised fragment or only the 950 bp fragment corresponding to the unmodified pCDM8-GR construct (figure 3.9.B). The confirmatory sequencing reactions were performed using as a sense oligonucleotide not only the T7 primer, complementary to the T7 bacteriophage sequence present immediately 5' to the multiple cloning site of pCDM8, but also using an additional sense primer designed for this study so as to anneal further upstream. This was done to permit the sequence determination of the putative tag region with a reduced error frequency. Such sequencing reactions indicated, however, that several of the colonies arose from bacteria which contained no vector DNA, while others were derived from E. coli that contained only the original, unmodified pCDM8-GR vector, suggesting re-circularisation of the plasmid.

3.3. Discussion

3.3.1. The attempted construction of the entire coding sequence of the rat hepatic glucagon receptor

The products resulting from the BamH I / HinD III double restriction digest of the previously isolated C-terminal cDNA fragment led me to predict that this cDNA may contain the 300 bp intron 4 of the glucagon receptor. This was confirmed by subsequent single digests of this cDNA. It is likely, therefore, that although this cDNA species was isolated from a cDNA library formed from poly-adenylated mRNA, a number of the mRNA molecules had not been fully spliced prior to polyadenylation. In fact, this phenomenon has been described previously for the glucagon receptor (Lewin, 1985; Svoboda *et al.*, 1993a). The rat hepatic glucagon receptor gene is known to contain four small introns of lengths 95, 89, 85 and 300 bp, respectively (Svoboda *et al.*, 1993a). The persistence of introns in some poly-adenylated mRNA species is unlikely to be due to anomalous splicing signals, as the 5' and 3' splice sites of each of the four introns present in the GR genomic sequence do conform to the consensus dinucleotide sequences GT and AG, respectively (Ohshima and Gotoh, 1987). Interestingly, a number of other receptors of the secretin receptor sub-family of GPCRs possess at least four small introns, including the calcitonin receptor (Lin *et al.*, 1991), the secretin receptor (Ishihara *et al.*, 1991), the vasoactive intestinal polypeptide receptor (Ishihara *et al.*, 1992), the parathyroid hormone receptor (Juppner *et al.*, 1991) and the glucagon-like peptide 1 receptor (Thorens, 1992).

It is possible that the DNA product of approximately 1040 bp which was visible in addition to the predicted 941 bp N-terminal GR RT-PCR product may have reflected the amplification, by RT-PCR, of a partially spliced transcript. According to the reported genomic sequence of the GR gene (Svoboda *et al.*, 1993a) such a transcript, containing intron 1, intron 2, or intron 3, would, indeed, be predicted to be of 1036, 1030, or 1026 bp in length, respectively. Alternative explanations for the appearance of this extra

product include the non-specific amplification from a related sequence and RT-PCR from a mature mRNA of a genuine splice variant. It is unlikely that the RT-PCR product of approximately 1040 bp was due to the presence of a mature mRNA from a splice variant of the GR as there is no documented evidence for the existence of more than one splice variant of the glucagon receptor. Particularly notable is that Northern blotting, using a GR cDNA probe, of RNA isolated from a variety of tissues, revealed only a single band (Yoo-Warren *et al.*, 1994).

The subsequent inability to amplify the entire 1527 bp sequence may reflect a limitation of the first-strand cDNA preparation, perhaps with sufficient integrity of the cDNA species to permit the amplification of the 941 bp product but not that of the longer sequence. Alternatively, the amplification by PCR of the long target sequence may have been compromised by the preferential amplification of shorter non-specific products. In this respect, the lengths of the designed primers are unlikely to have been the cause of such a difficulty, as they were designed so as to have balanced melting temperatures of approximately 65°C. This is considered to be sufficiently high to ensure the level of reaction specificity that is required for long PCR (Cheng *et al.*, 1994).

3.3.2. Incorporation by PCR of an N-terminal epitope tag into the coding sequence of the glucagon receptor

Although the 832 bp PCR product was amplified as predicted using the primers designed so as to incorporate an amino terminal epitope tag, it was not possible to then insert this fragment into the expression vector containing the remainder of the coding sequence of the GR. This difficulty may have been due to a failure of efficient restriction digestion of one or both extremities of the PCR product. Such incomplete digestion, which in the case of the 5' end could occur as the result of the proximity of the restriction site to the end of the DNA, would be difficult to detect using agarose gel electrophoresis. It is known that restriction enzymes cut less efficiently when a restriction site is located at the extreme terminus of a DNA sequence (Doyle, 1996). In fact, it was for this reason that an additional six nucleotides were incorporated into the design of the sense

oligonucleotide on the 5' side of the HinD III restriction site. It is, however, conceivable that the exonuclease activity of the proof-reading polymerase may have caused subsequent restriction (and therefore ligation) difficulties by degrading the ends of the amplified products during PCR.

Another possible explanation for the difficulty encountered in ligating the product into the vector was that the cut vector may have re-circularised without the inclusion of the insert. This is less likely to occur when employing a strategy such as that used here in which the vector is cut with two different restriction enzymes, than when the vector is only cut with a single enzyme. The possibility remains, however, that one of the two enzymes failed to cut efficiently, potentially leaving cohesive ends. To combat this, following restriction digestion, the plasmid DNA was treated with phosphatase, in order to remove the 5' terminal phosphates and thus prevent re-circularisation. In addition, following phosphatase-treatment, the cut vector was separated from uncut vector by electrophoresis on a low melting point agarose gel, and was subsequently excised and purified prior to ligation to the cut PCR product. It appears from subsequent digests and DNA sequencing, however, that despite these precautions the original unmodified GR vector resulted from the ligation process.

The genetic manipulations were hampered by the additional difficulties created as a result of the nature of the vector containing the GR sequence. This vector, pCDM8, is one which necessitates the use of the MC1061/P3 strain of E. coli (Seed, 1987). As this strain is Endo A positive, it contains an endonuclease which makes purification, and subsequent manipulations difficult. In addition, it depends on an antibiotic selection process involving the suppression by a plasmid-encoded suppressor tRNA of premature stop-codons in the antibiotic resistance genes of the P3 episome. This system, however, may yield false-positive colonies (F. McCallum, personal communication), possibly due to read-through of the amber non-sense mutations (Lewin, 1985). Furthermore, very few unique restriction sites were available at the 5' and 3' ends of the GR DNA sequence.

Consequently, in order to facilitate further studies, it would be desirable to sub-clone the coding region of the glucagon receptor from pCDM8 into a more easily manipulated mammalian expression vector such as pc DNA3 or pSV-Sport1.

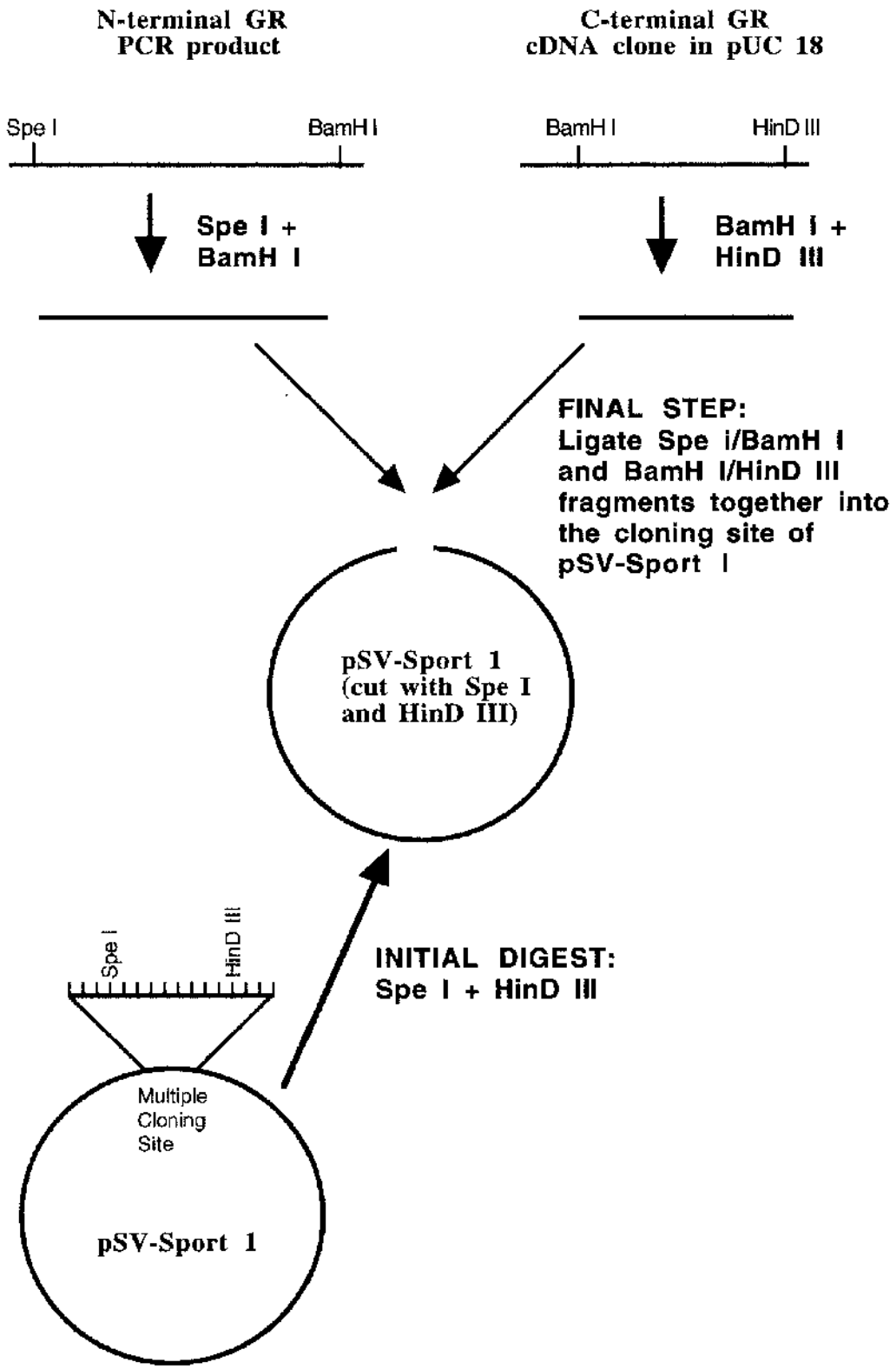
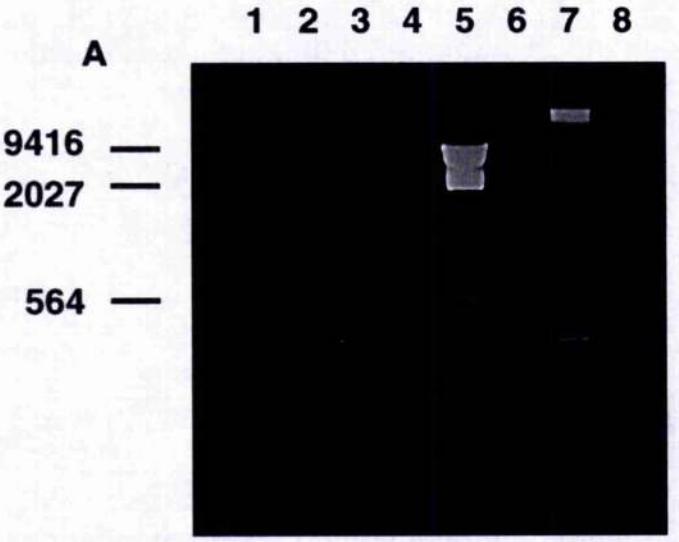

N-terminal GR
PCR product
C-terminal GR
cDNA clone in pUC 18
Spe I
BamH I
BamH I
HinD III
Spe I +
BamH I
BamH I +
HinD III
FINAL STEP:
Ligate Spe I/BamH I
and BamH I/HinD III
fragments together into
the cloning site of
pSV-Sport I
pSV-Sport 1
(cut with Spe I
and HinD III)
Spe I
HinD III
INITIAL DIGEST:
Spe I + HinD III
Multiple
Cloning
Site
pSV-Sport 1

Figure 3.1. Strategy for the construction of the full length glucagon receptor coding sequence

It was intended to obtain the full glucagon receptor coding sequence by digesting the 941 bp RT-PCR product with Spe I and BamH I, and the pre-existing cDNA clone with BamH I and HinD III. These digestions would have permitted the subsequent ligation of these N-terminal and C-terminal coding regions, respectively, together into the multiple cloning site of the mammalian expression vector, pSV-Sport I following the excision of the Spe I/HinD III stuffer fragment.


A
1 2 3 4 5 6 7 8
9416
2027
564

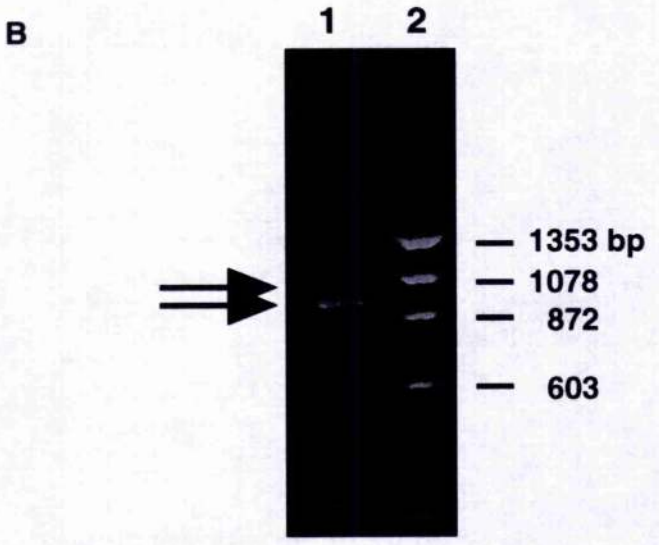
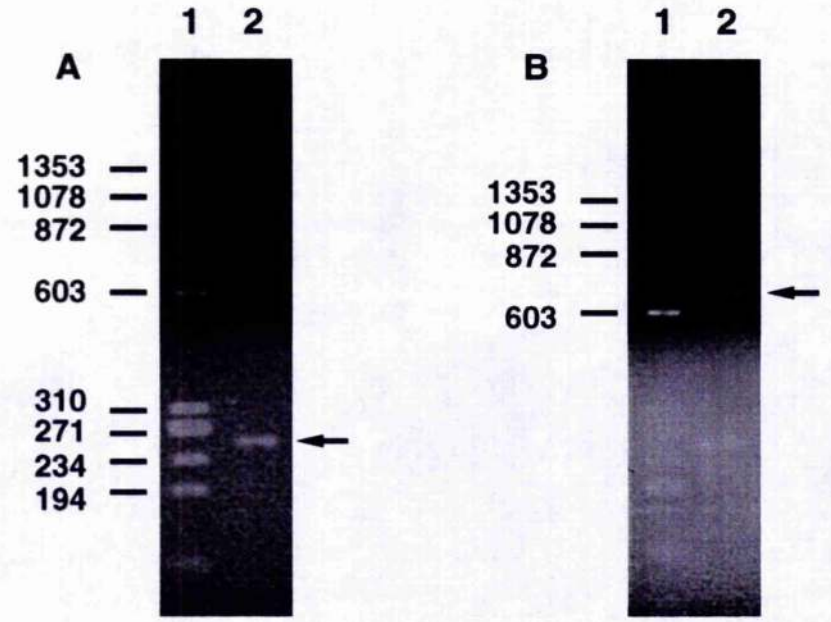

B
1 2
1353 bp
1078
872
603

Figure 3.2. The use of RT-PCR to amplify the 5' region of the sequence encoding the rat hepatic glucagon receptor

(A) Ethidium bromide stained 1.2% agarose gel electrophoresis of the RT-PCR amplification products from 2.5 μg (lane 1) and 5 μg (lane 2) hepatocyte RNA with the GR-specific primers ET-GR-s1 and ET-GR-as1; 5 μg positive control RNA with appropriate primers (lane 3); and 5 μg of a different hepatocyte RNA preparation with the GR-specific primers (lane 8). Lane 7 represents PCR of positive control cDNA with appropriate control primers and lane 5 contained lambda / HinD III DNA markers. The sizes are indicated in bp. Lanes 4 and 6 were not loaded. The predicted amplification product was detected only in lane 7, suggesting that defective first strand synthesis was the cause of the RT-PCR failure.

(B) Ethidium bromide stained 1.2% agarose gel electrophoresis of the RT-PCR amplification products of 5 μg rat hepatocyte RNA after reverse transcription and PCR with the GR-specific primers ET-GR-s1 and ET-GR-as1(lane 1), with lane 2 containing ϕX174 / Hae III DNA markers. While the PCR reagents were as used in the reactions represented in figure 3.2A, the reverse transcriptase was taken from a fresh stock. The 941 bp predicted product was detected, in addition to a weaker signal of approximately 1030 bp, which is discussed in the text.


1 2
A
1353
1078
872
603
310
271
234
194
1 2
B
1353
1078
872
603

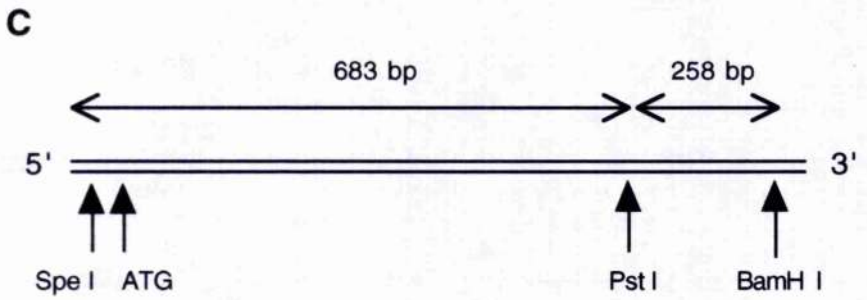
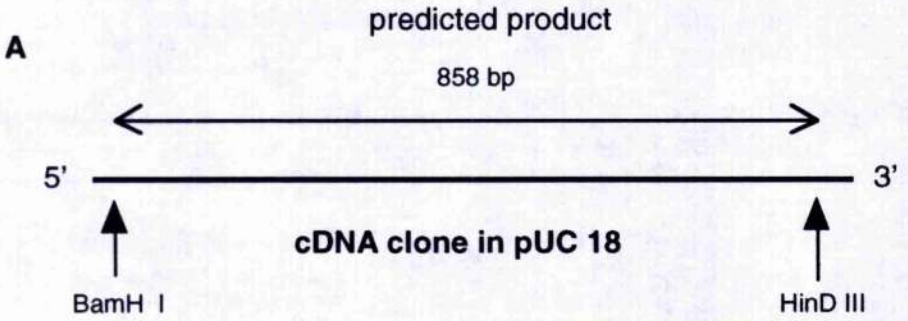

C
683 bp
258 bp
5'
3'
Spe I
ATG
Pst I
BamH I

Figure 3.3. Pst I digestion of the 941 bp RT-PCR product.

(A) and (B) Ethidium bromide stained 1.2% agarose gel electrophoresis of the Pst I digestion products (lane 2) of the 941 bp RT-PCR product, following the excision and purification of this fragment from a low-melting point agarose gel. Lane 1 contains the ϕX174 / Hae III DNA markers. The marker fragment sizes are indicated in bp. The gel was photographed with two alternative exposures (A and B) so as to reveal the 683 bp fragment (arrow in A) and the 258 bp fragment (arrow in B).

(C) This shows the predicted Pst I digestion pattern of the 941 bp PCR product. Amplification of the 5' region of the cDNA encoding the rat hepatic glucagon receptor by RT-PCR using the primers ET-GR-s1 and ET-GR-as1 containing Spe I and BamH I sites, respectively, was predicted to yield a product of 941 bp. Excision and purification of this fragment from a low-melting point agarose gel and subsequent Pst I digestion should yield the two products of the lengths indicated. The translation initiation codon is separated from the 5' end of the 941 bp cDNA by 9 bp, containing the Spe I restriction site, ACTAGT, incorporated into the product by the sense primer. The Pst I and BamH I sites are located within the sequence which encodes the glucagon receptor.


A
predicted product
858 bp
5'
3'
cDNA clone in pUC 18
BamH I
HinD III

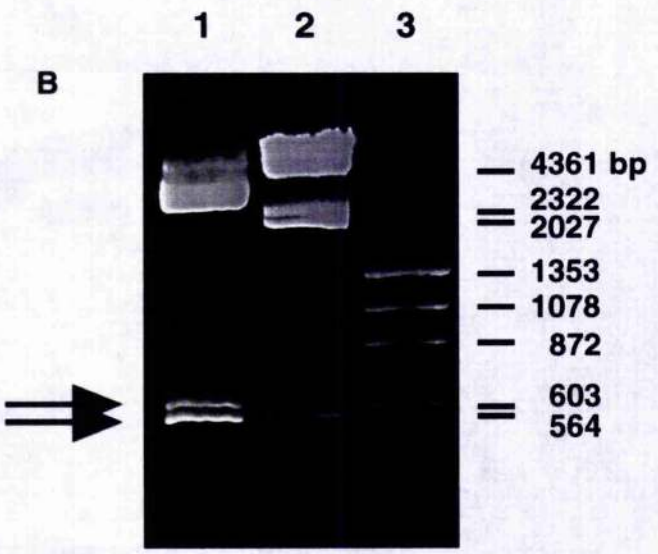
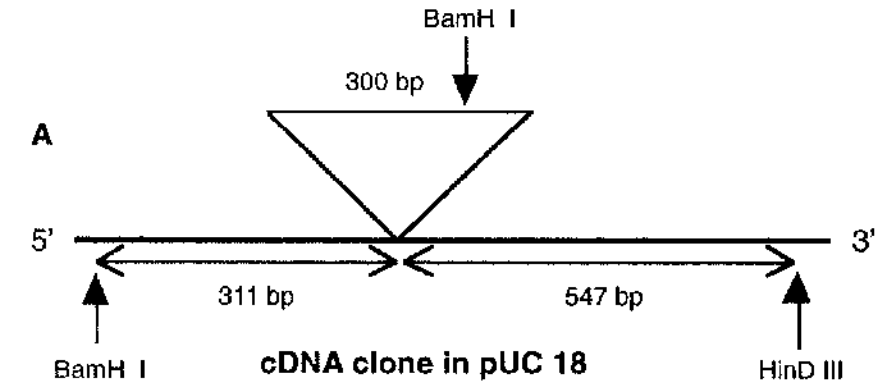

1
2
3
B
4361 bp
2322
2027
1353
1078
872
603
564

Figure 3.4. Restriction digestion of the cDNA clone encoding the C-terminal region of the glucagon receptor

(A) The cDNA clone corresponding to the C-terminal region of the glucagon receptor had been previously isolated and inserted into the cloning site of the vector pUC 18. Double digestion of this construct with BamH I and HinD III was predicted to yield a single 858 bp product in addition to the cut vector.

(B) Ethidium bromide stained 1.2% agarose gel electrophoresis of the BamH I/HinD III double digestion products (lane 1) of the C-terminal glucagon receptor cDNA clone. To permit more precise determination of the size of these products, two different sets of DNA markers were used: lambda/HinD III (lane 2) and ϕX 174 / Hae III (lane 3). The marker fragment sizes are indicated in bp. The digest resulted in two fragments of approximately 560 bp and 600 bp, respectively, rather than the predicted single 858 bp product.


BamH I
300 bp
A
5'
3'
311 bp
547 bp
BamH I
cDNA clone in pUC 18
HinD III

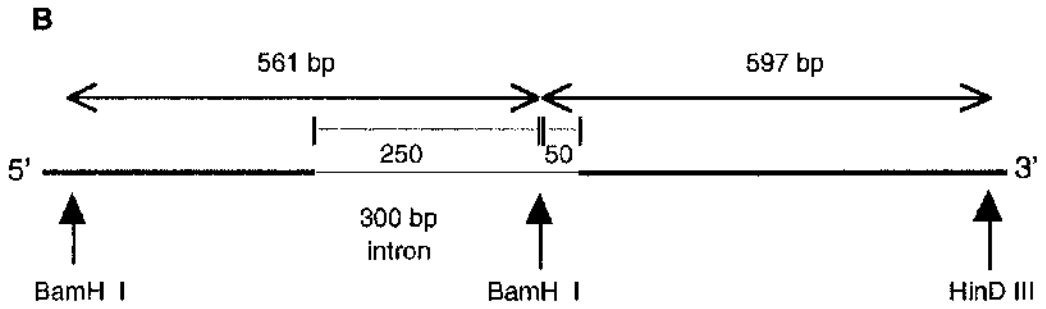
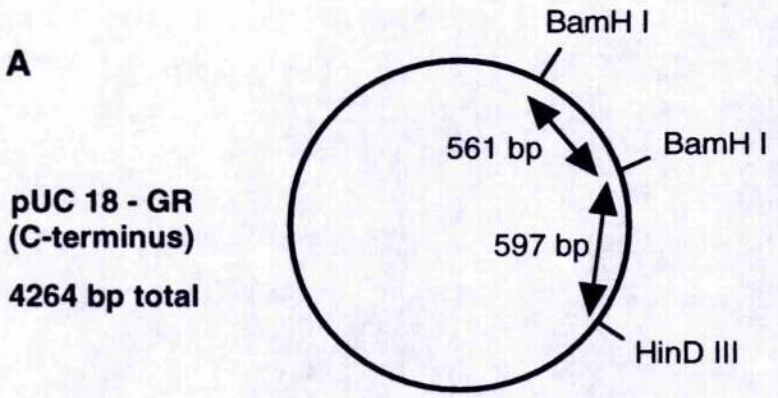

B
561 bp
597 bp
250
50
5'
3'
300 bp
intron
BamH I
BamH I
HinD III

Figure 3.5. Hypothesis for the rationalisation of the digestion products of the C-terminal GR cDNA clone

The finding of two digest products, with a total length of approximately 1160 bp, led the author to speculate on the presence of additional sequence within the cDNA clone, such as an intron. In fact, intron 4 of the rat hepatic glucagon receptor genomic sequence is exactly 300 bp in length (Svoboda *et al.*, 1993a). In addition, it contains a single BamH I site at 250 bp from its 5' end and no HinD III sites (A) . The presence of such an intron, located at 311 bp downstream of the exonic BamH I site would, indeed, be predicted to give rise to two products, of 561 bp and 597 bp, respectively, upon digestion with BamH I and HinD III (B).


A
BamH I
561 bp
BamH I
pUC 18 - GR
(C-terminus)
597 bp
4264 bp total
HinD III

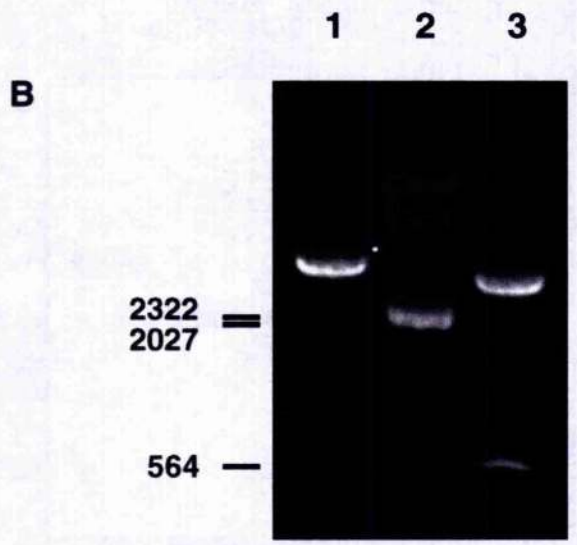

1
2
3
B
2322
2027
564

Figure 3.6. Confirmation of the presence of intronic DNA by individual BamH I and HinD III digests of the pUC 18 – GR construct

(A) The two BamH I sites which would be present if the cDNA did contain an intron are separated by 561 bp. A BamH I digest would therefore be predicted to yield this fragment in addition to a 3703 bp vector fragment. A HinD III digest, however, would only give rise to a single fragment of 4264 bp, corresponding to the entire linearised vector.

(B) Ethidium bromide stained 1.2% agarose gel electrophoresis of the HinD III (lane 1) and BamH I (lane 3) single digestion products of the vector containing the C-terminal glucagon receptor cDNA clone. The DNA markers used were lambda/HinD III (lane 2). The marker fragment sizes are indicated in bp.

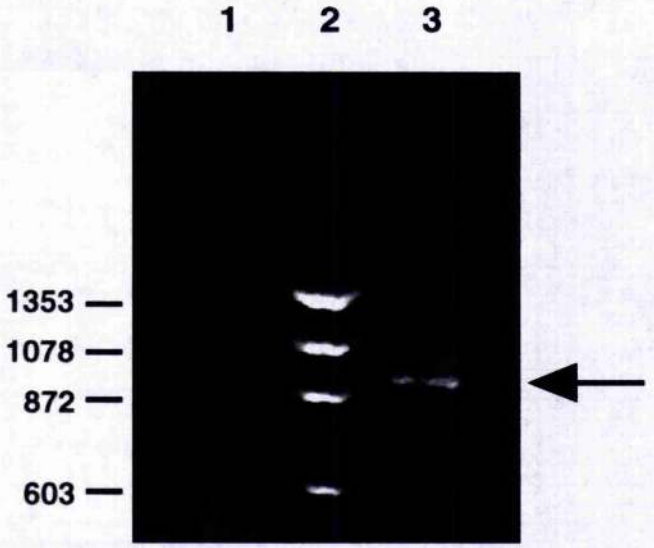

1
2
3
1353
1078
872
603

Figure 3.7. Attempted RT-PCR of the full-length cDNA encoding the glucagon receptor

Ethidium bromide stained 1.5% agarose gel electrophoresis of the reaction products following the synthesis of first strand cDNA from rat hepatocyte RNA and the subsequent PCR using the primers ET-GR-s1 and ET-GR-as2 (lane 1). The predicted amplification product was 1527 bp in length. Despite the use of the proof-reading Pfu polymerase, and, in addition, a number of reaction modifications including the use, in subsequent PCR reactions, of the alternative anti-sense primers ET-GR-as3, 4, 5 and 6, no product of this length could be detected. The DNA markers used were ϕX 174 / Hae III (lane 2) and the fragment sizes are indicated in bp. As a control, the pair of primers shown previously to yield a 941 bp product, ET-GR-s1 and ET-GR-as1, were used in a parallel reaction in order to test the PCR reaction constituents (lane 3).

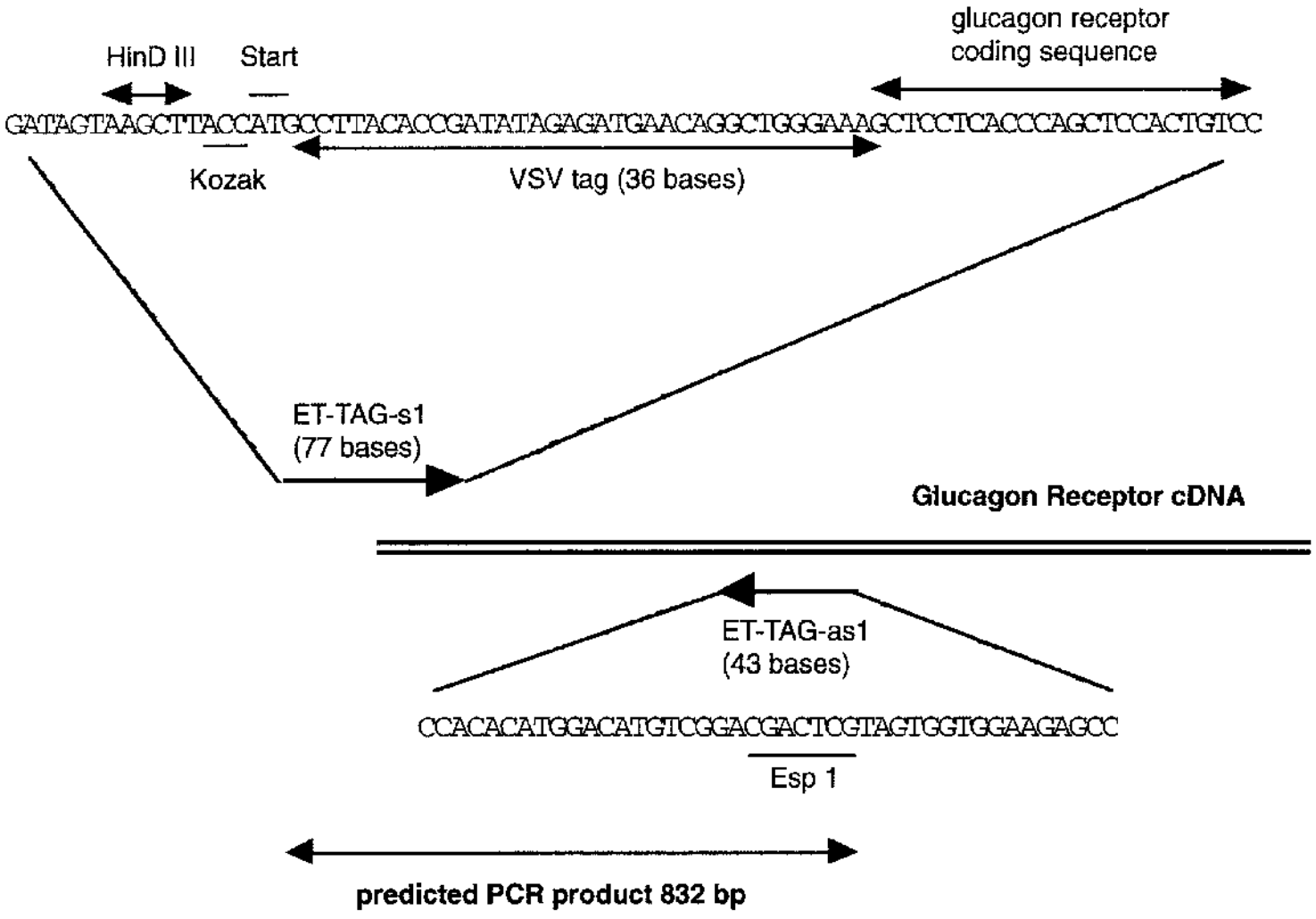
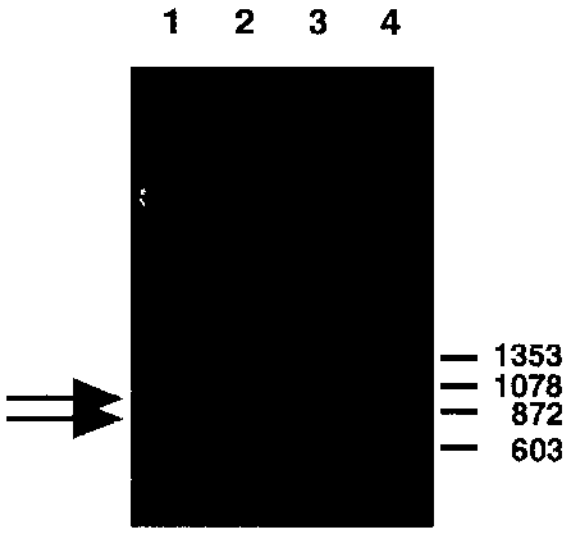

HinD III
Start
glucagon receptor
coding sequence
GATAGTAAGCTTACCATGCCTTACACCGATATAGAGATGAACAGGCTGGGAAAGCTCCTCACCCAGCTCCACTGTCC
Kozak
VSV tag (36 bases)
ET-TAG-s1
(77 bases)
Glucagon Receptor cDNA
ET-TAG-as1
(43 bases)
CCACACATGGACATGTCGGACGACTCGTAGTGGTGGAAGAGCC
Esp 1
predicted PCR product 832 bp

Figure 3.8. Strategy for the incorporation of a VSV epitope tag into the coding sequence of the glucagon receptor by PCR

The oligonucleotide primers were designed so as to add the tag onto the amino terminus of the protein. In the PCR-based strategy employed, the nucleotide sequence encoding the 36-base VSV tag was incorporated into a 5' sense oligonucleotide primer, immediately 3' to the start codon. Both this sense primer, ET-TAG-s1, and the antisense primer, ET-TAG-as1, were so designed as to permit the subsequent cleavage of the amplified product by restriction enzymes and the ligation of the fragment into the appropriately digested vector containing the wild-type GR sequence. The sense primer, thus, comprised a HinD III site, close to its 5' end, followed by the sequence ACCATG, serving as an optimal Kozak sequence (Kozak, 1986) and start codon, which was then followed by the tag sequence and the amino-terminal GR sequence. The 3' primer was designed so as to anneal to a region of the GR sequence that contained the unique restriction site, Esp I. The predicted length of the product amplified by these primers was 832 bp.

A



B

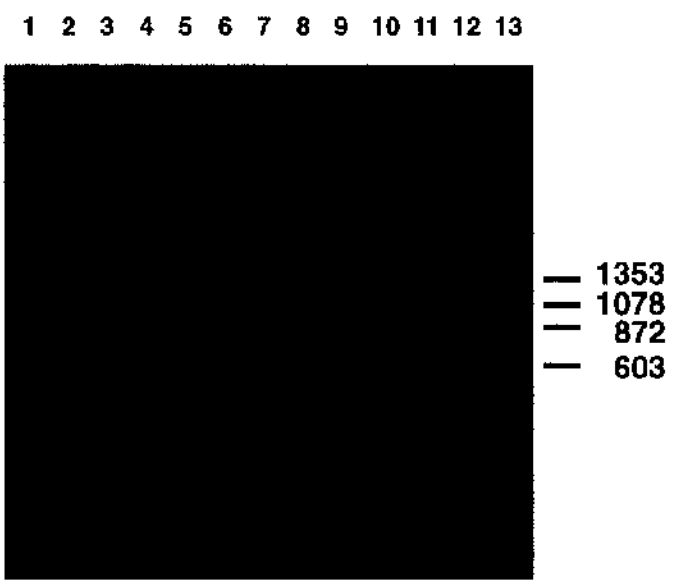


Figure 3.9. Analysis of epitope tag incorporation by PCR amplification and restriction digestion

(A) Ethidium bromide stained 0.9% agarose gel electrophoresis of the PCR amplification products following PCR from glucagon receptor-encoding vector cDNA template (lane 4) or, as a control, in the absence of DNA template (lane 3), with the GR-specific primers ET-TAG-s1 and ET-TAG-as1. The DNA markers were ϕX 174 / Hae III (lane 2) and the fragment sizes are indicated in bp. Lane 1 shows the products of a HinD III/ Esp I digestion of the unmodified pCDM8-GR construct. Notably, this digestion excised a fragment of 950 bp, whereas the PCR product that was to be inserted in its place was only 832 bp.

(B) Ethidium bromide stained 1.5% agarose gel electrophoresis following PCR amplification using primers ET-TAG-s1 and ET-TAG-as1, from the DNA of 5 of the colonies which appeared after the ligation (lanes 2-6), and HinD III/ Esp I double digests of these samples (lanes 9-13). As a control, the same PCR and restriction digests were performed on unmodified pCDM8-GR plasmid (lanes 1 and 8, respectively). It was observed that with none of the DNA samples did the HinD III/ Esp I digest excise a fragment of a length corresponding to the PCR-generated insert. Rather no detectable product was excised (lanes 11 and 13), or the digest yielded only a product corresponding to that obtained from digestion of unmodified plasmid (lanes 9, 10 and 12).

CHAPTER 4

Glucagon stimulation of COS-7 cells transfected with cDNA encoding the glucagon receptor and PKC isoforms

4.1. Introduction

Glucagon, a hormone that is secreted by the pancreas in response to hypoglycaemia, is a key regulator of hepatic glucose production. Glucagon binding causes the glucagon receptor (GR) to interact with the stimulatory G protein, Gs, the alpha subunit of which, upon dissociation from beta/gamma subunits, subsequently activates adenylate cyclase. The consequent elevation of intracellular cAMP leads, in liver cells, to gluconeogenesis (Exton *et al.*, 1969; Johnson *et al.*, 1972) and glycogenolysis (Exton *et al.*, 1970). The glucagon receptor, however, like several other hormone receptors, undergoes a rapid regulatory process during continuous exposure to an agonist, that attenuates its response to subsequent hormonal challenge. This phenomenon is known as desensitisation.

4.1.1. Evidence for rapid glucagon receptor desensitization

Subsequent to the binding of glucagon to specific receptors on the hepatocyte surface (Rodbell *et al.*, 1971c; Sonne *et al.*, 1978), an activation of adenylate cyclase ensues which results in the rapid accumulation of intracellular cAMP, reaching a maximum value of 10-20 fold above basal levels within 6 minutes (Johnson *et al.*, 1972; Pilkis *et al.*, 1975). This response is dose-dependent with an EC_{50} for glucagon of 1-2 nM in the presence of phosphodiesterase (PDE) inhibitors (Christoffersen and Berg, 1974; Sonne *et al.*, 1978; Heyworth *et al.*, 1983).

The increase in the intracellular level of cAMP is, however, transient, as following the peak cAMP accumulation at 6 min after glucagon addition, a gradual decline in cAMP levels, to approximately 50% of maximal stimulated values, is observed in the presence of the PDE inhibitor, IBMX (Heyworth *et al.*, 1983; Murphy *et al.*, 1987). Similar results, have also been observed in preparations of parenchymal liver cells in the presence of the PDE inhibitor, theophylline (Christoffersen and Berg, 1974). The decline in intracellular cAMP is unlikely to simply reflect an increased rate of loss of cAMP to the extracellular environment as only a small proportion of the total cAMP is detectable in the

incubation medium (Christoffersen and Berg, 1974), and the rate of exit of cAMP actually decreases after the observed maximum intracellular cAMP accumulation (Pilkis *et al.*, 1975). Nor is it likely to be due to hormone degradation since the desensitization can not be overcome by further additions of glucagon (Murphy *et al.*, 1987). Rather it is believed that it reflects an underlying profound attenuation of the activity of adenylate cyclase (Newlands and Houslay, 1991; Houslay, 1994).

In the absence of PDE inhibitors, the decline in intracellular cAMP is even more pronounced. Thus, although the magnitude of the peak cAMP increase is reduced by 3-5 fold, the cAMP level returns fully to basal levels within 20-30 min (Johnson *et al.*, 1972; Heyworth *et al.*, 1983). This accentuated decline, observed when PDE inhibitors are absent, is believed to reflect an increased metabolism of cAMP by phosphodiesterases and the activation of type III PDE by a protein kinase A mediated mechanism (Heyworth *et al.*, 1983; Houslay, 1994).

4.1.2. Glucagon receptor desensitization observed upon repeated glucagon administration

It is well established that the glucagon-stimulated adenylate cyclase activity in membranes isolated from hepatocytes is profoundly inhibited if the cells have been pre-exposed to glucagon (Plas and Nunez, 1975; DeRubertis and Craven, 1976; Gurr and Ruh, 1980; Heyworth and Houslay, 1983; Noda *et al.*, 1984; Murphy *et al.*, 1987; Murphy and Houslay, 1988; Premont and Iyengar, 1988; Murphy *et al.*, 1989; Savage *et al.*, 1995). This desensitization displays a maximum reduction of 30-40% following a 5-minute pre-treatment with 10 nM glucagon (Heyworth and Houslay, 1983). Furthermore, treatment of intact hepatocytes with glucagon has been shown to desensitize the ability of a subsequent challenge with the hormone to elevate the level of intracellular cAMP (Savage *et al.*, 1995).

These observations support the premise that the rapid desensitization of the response of adenylate cyclase to glucagon stimulation underlies the transience of the

glucagon-stimulated intracellular cAMP accumulation described above (Newlands and Houslay, 1991; Houslay, 1994).

4.1.3. The potential involvement of PKC in the mechanism of desensitization

It is evident that phosphorylation of a membrane protein is pivotal to the process by which the desensitization of the glucagon-stimulated adenylate cyclase response occurs, as alkaline phosphatase treatment of membranes causes a striking reversal of the desensitization of glucagon-stimulated adenylate cyclase (Savage *et al.*, 1995). Moreover, since in the desensitised state, neither ^{125}I-glucagon binding nor NaF-stimulated cyclase activity, which depends upon Gs-cyclase coupling, is reduced (Santos and Blazquez, 1982; Heyworth and Houslay, 1983), the mechanism of the desensitization is likely to involve the uncoupling of the glucagon receptor from the stimulatory G protein, Gs.

In hepatocytes, a similar degree of inhibition of glucagon-induced adenylate cyclase stimulation to that which is induced by pre-exposure to glucagon, can be elicited by treatment of the cells with a variety of other agents. Thus, a profound reduction in the ability of glucagon to stimulate adenylate cyclase activity has been demonstrated following the activation of lipid signalling pathways by hormones such as vasopressin and angiotensin II (Murphy *et al.*, 1987) and after challenge of these cells with either the tumour promoting phorbol ester, PMA (Heyworth *et al.*, 1984; Garcia-Sainz *et al.*, 1985; Heyworth *et al.*, 1985; Murphy *et al.*, 1987; Refsnes *et al.*, 1989) or the synthetic diacylglycerols 1-oleoyl-2-acetyl glycerol (OAG) and dihexanoyl glycerol (DHG) (Newlands and Houslay, 1991).

In marked distinction to the agents which mimic the action of DAG, permeant cAMP analogues such as dibutyryl cAMP do not induce desensitisation, implying that the cAMP-dependent kinase (PKA) cannot mediate such an effect (Heyworth and Houslay, 1983). Similarly, calcium entry appears, in itself, insufficient to cause desensitization since treatment of hepatocytes with the calcium ionophore A23187 was unable to elicit a

reduction of either the adenylate cyclase activation or intracellular cAMP accumulation stimulated by glucagon (Savage *et al.*, 1995).

It has become apparent that glucagon is able to stimulate the production of multiple second messengers, potentially leading to the activation of PKC. For instance, like other hormones which bind to receptors in the secretin receptor sub-family, such as parathyroid hormone (PTH) (Abou-Samra *et al.*, 1992) and calcitonin (Chabre *et al.*, 1992), glucagon has been shown to cause not only an increase in cAMP production but also a significant increase in the level of intracellular calcium (Charest *et al.*, 1983; Mauger *et al.*, 1985; Sistare *et al.*, 1985; Blackmore and Exton, 1986; Mine *et al.*, 1988). In addition, glucagon has been shown to stimulate the hydrolysis of the membrane lipid phosphatidylcholine (PtdCho) by phospholipases C and D (Pittner and Fain, 1991). It has been shown that the increase in intracellular calcium concentration, is not, in itself, necessary for the desensitization as this was blocked neither by treatment of cells with EGTA, which chelates calcium, nor by the addition of La^{2+}, which blocks the entry of calcium across the hepatocyte plasma membrane (Barritt and Hughes, 1991).

The ability of glucagon to lead to the generation of DAG (Bocckino *et al.*, 1985; Pittner and Fain, 1991), together with the evidence, described above, that treatment of hepatocytes with DAG analogues or hormones which increase cellular DAG levels can attenuate the ability of glucagon to stimulate adenylate cyclase activity, suggests that the mechanism of glucagon and vasopressin-induced glucagon desensitization in hepatocytes may involve protein kinase C. In this regard it is interesting that the dose-dependence observed for the glucagon-induced desensitization of glucagon-stimulated adenylate cyclase activity in hepatocyte membranes (Ka of 0.45 nM) (Heyworth and Houslay, 1983; Murphy *et al.*, 1987) is similar to that observed for the glucagon-induced stimulation of inositol phospholipid hydrolysis (Ka of 0.25 nM) (Wakelam *et al.*, 1986). Furthermore, glucagon-stimulated PKC activation has been demonstrated in rat hepatocytes (Pittner and Fain, 1991; Tang and Houslay, 1992), and compounds known to inhibit PKC such as chelerythrine, calphostin C and staurosporine were found (albeit to differential extents) to block the vasopressin and glucagon-induced desensitization

(Savage *et al.*, 1995). These observations lend additional support to the conclusion that a DAG-activated kinase such as protein kinase C (PKC) is likely to mediate the desensitization of glucagon-stimulated cAMP accumulation in hepatocytes.

4.1.4. Aims and Objectives

The work described here was undertaken with the aim of gaining further insight into the nature of the protein kinase species which confers phorbol ester-mediated inhibitory effects on glucagon-stimulated adenylate cyclase activity. The initial objective therefore was to obtain a model system for glucagon signalling by transfecting COS-7 cells so as to effect the transient overexpression of glucagon receptors. In these cells, the dose and time-dependence of the glucagon-stimulated cAMP accumulation was then characterised and the activation of phospholipases investigated. Attempts were made to obtain a membrane assay for glucagon-stimulated adenylate cyclase activity. Thereafter, the aim was to re-create in transfected COS cells the PMA-mediated inhibition of glucagon-stimulated cAMP accumulation previously reported in hepatocytes. COS cells were thus co-transfected with the glucagon receptor and individual PKC isoforms that were shown to be present in hepatocytes and that therefore could be responsible for the PMA-mediated inhibition in these cells. Expression of these transfected PKC isoforms was confirmed and assays undertaken to determine whether their expression could confer PMA-induced inhibition upon the glucagon-stimulated accumulation of cAMP.

4.2. Results

4.2.1. The transient transfection of glucagon receptor cDNA

The cells chosen for transient expression of the glucagon receptor were COS-7 cells. Due to their constitutive expression of the SV-40 large T antigen, they permit both the replication to a very high copy number and the expression of plasmids which contain an SV-40 origin and promoter region (Gluzman, 1981). These cells were transiently transfected with the mammalian expression vector pCDM8 containing the cDNA encoding the rat hepatic glucagon receptor (fig. 4.1.), as described in the Materials and Methods chapter.

4.2.1.1. The cAMP phosphodiesterase activity in GR-transfected COS cells and the effect of IBMX

In the experiments which follow, cAMP production by adenylate cyclase was gauged by the measurement of intracellular cAMP using the method described in the Materials and Methods chapter. The level of intracellular cAMP, however, is not governed merely by the rate of cAMP production by adenylate cyclase, but also by the rate at which the cyclic nucleotide is converted to the mono-phosphate by cAMP phosphodiesterase iso-enzymes. Thus, in order to obtain an indication of the activity of adenylate cyclase, isobutylmethylxanthine (IBMX), a non-selective inhibitor of PDE isoforms (Beavo and Reifsnyder, 1990; Hoey and Houslay, 1990), was used to block cAMP degradation by these cAMP phosphodiesterase enzymes. In order to confirm that in glucagon-transfected cells IBMX is indeed an effective general PDE inhibitor, GR-transfected cells were homogenised and the extracts assayed for cAMP PDE activity in the presence and absence of IBMX at a concentration of 1 mM. The assay was performed as described in the Materials and Methods chapter with control incubations performed in the presence of buffer alone or buffer containing DMSO at a concentration equal to that present in the IBMX-treated samples. It was observed that in these transfected cells, this concentration of IBMX inhibited the detectable cAMP PDE activity

by greater than 96% (n=3). Notably, DMSO alone did not affect the total PDE activity. Unless otherwise indicated, IBMX was included, thereafter, at a final concentration of 1 mM, in all stimulations of COS cells where cAMP accumulation was measured.

4.2.1.2. Dose-response experiments in GR-transfected COS cells

To determine whether transfection of these cells by this method would successfully confer expression of functional glucagon receptors leading to a dose-dependent accumulation of intracellular cAMP in response to glucagon, dose-response experiments were carried out on untransfected and transfected COS cells (fig. 4.2.). In the untransfected cells a relatively small accumulation of intracellular cAMP was observed, and this was only apparent at the highest concentrations of glucagon tested. It was observed that in the GR-transfected COS cells, at levels of the hormone of up to 100 pM, there was no detectable change in the intracellular cAMP level. At higher concentrations, however, the level of intracellular cAMP was seen to rise dramatically to a maximum level, observed at a concentration of 100 nM glucagon, that was approximately 10-fold greater than basal. The glucagon-stimulated increase in intracellular cAMP level occurred in a dose-dependent fashion with an EC_{50} value of 1.8 ± 0.4 nM (mean ± SD for 3 separate transfection experiments). The level of intracellular cAMP began to decline (by approximately 20%) at the higher concentration of 1 μm glucagon, possibly indicative of non-selective or even toxic effects of the hormone at this concentration.

In experiments performed thereafter on GR-transfected cells the concentration of glucagon utilised was that of the sub-maximal 10 nM.

4.2.1.3. Timecourse of glucagon-stimulated cAMP accumulation in GR-transfected COS cells

In order to ascertain the rate at which cAMP accumulated in response to glucagon in the transfected cells, GR-transfected COS cells were stimulated for durations ranging from 0 to 30 minutes in the presence and absence of 10 nM glucagon. Furthermore, to evaluate the extent to which PDE activity governs the level of cAMP in these cells, the

experiments were, in addition, performed in the presence and absence of 1 mM IBMX. The results shown in fig. 4.3. show that, in the presence of IBMX, the maximal cAMP level was reached after 15 min of stimulation with glucagon, while in the absence of the hormone very little accumulation of intracellular cAMP was observed, thus indicating a low level of basal adenylate cyclase activity. In the absence of IBMX, the glucagon-stimulated accumulation of intracellular cAMP was only 14% of that observed in the presence of the PDE inhibitor, suggesting that the cAMP phosphodiesterase activity in these cells is far greater than that of adenylate cyclase.

4.2.1.4. Glucagon-stimulated PtdCho and PIP_2 hydrolysis

In order to ascertain whether, in GR-transfected COS cells, glucagon treatment leads to activation of phospholipases C or D, as has been shown previously in hepatocytes (Wakelam *et al.*, 1986; Pittner and Fain, 1991; Bygrave and Benedetti, 1993; Bygrave *et al.*, 1993), transfected COS cells were stimulated with glucagon and assayed for the activity of PC-PLC and PC-PLD, or for that of PI-PLC.

4.2.1.4.1. Determination of PtdCho hydrolysis

In order to measure hydrolysis of PtdCho, cells were incubated with [^{3}H]-palmitic acid, the majority of which is incorporated into PtdCho (Cook *et al.*, 1991). Determination of PtdCho-PLD and PtdCho-PLC activities was by measurement of the formation of [^{3}H]-PtdBut and [^{3}H]-PtdOH, respectively, in the presence of 0.3% (v/v) butan-1-ol. As butanol is a better nucleophilic acceptor than water, hydrolysis of PtdCho by PLD results in the formation of phosphatidylbutanol (PtdBut) instead of PtdOH. Unlike the latter species, PtdBut cannot be further hydrolysed to diacylglycerol by phosphatidate phosphohydrolase (Pettitt *et al.*, 1994). Diacylglycerol formed by PLC-catalysed hydrolysis of PtdCho, is converted into PtdOH by the action of diacylglyerol kinase.

Incubation of untransfected COS-7 cells with glucagon for 10 min at concentrations ranging from 0 to 1000 nM was observed to have no significant effect on the production of [^{3}H]-PtdOH or that of [^{3}H]-PtdBut (figs. 4.4. A and B). In GR-transfected COS

cells, however, incubation with 1 and 10 nM glucagon led to a statistically significant rise (by 25%) in the level of [^{3}H]-PtdOH (fig. 4.4. A). Again, however, no statistically significant change in the level of [^{3}H]-PtdBut was observed (fig. 4.4. B). In a timecourse experiment (fig 4.5. A and B), in these cells, glucagon at a concentration of 10 nM caused a steady rise in the level of [^{3}H]-PtdOH which was significant (p=0.001) at 20 min, while the level of [^{3}H]-PtdBut was unchanged.

4.2.1.4.2. Determination of PIP_2 hydrolysis

Intracellular IP_3 production was measured as described in the Materials and Methods chapter. The level of IP_3 production in mock or transfected COS cells did not change significantly upon stimulation with 10 nM glucagon for 10 seconds (fig 4.6.).

4.2.1.5. Determination of adenylate cyclase activity in plasma membranes

In addition to the determination of cAMP accumulation as a measure of glucagon-stimulated adenylate cyclase activity, it was considered desirable to determine glucagon-stimulated adenylate cyclase activity in isolated membranes. Thus glucagon receptor-transfected COS cells were homogenised and membranes prepared as described in the Materials and Methods section 2.4.11. The membranes were then incubated with GTP at the concentration, 100 μM, used previously to permit glucagon-stimulated adenylate cyclase activation (Heyworth and Houslay, 1983), in the presence or absence of glucagon, under the conditions described in the Materials and Methods section 2.4.13. The methylxanthine, theophylline, was included in the reactions to block membrane-associated PDE activity (Butcher and Sutherland, 1962). The level of cAMP production, was then determined by the cAMP binding assay as described in the Materials and Methods section 2.4.14.

It was found that membranes from both control and transfected COS cells displayed an adenylate cyclase activity which was not significantly increased upon the addition of glucagon (table 4.1.). However, in both control and transfected COS cells, adenylate cyclase activity was significantly stimulated by the addition of GTP alone (table 4.1.).

This suggests that during cell disruption some modification of the adenylate cyclase signalling system occurs which can allow GTP-bound G_s to activate adenylate cyclase maximally.

It was also apparent that in the absence of hormone or GTP, the level of basal adenylate cyclase activity is greater in COS cells which have been transfected with the glucagon receptor than in control cells (table 4.1.). This may be a reflection of activation of the G protein by unoccupied receptors, a phenomenon which has been observed for other transfected G protein-coupled receptors (Adie and Milligan, 1994; Mullaney *et al.*, 1996; Smit *et al.*, 1996).

4.2.1.5.1. Membrane adenylate cyclase activity in different homogenisation buffers in glucagon receptor-transfected COS-7 and HEK-293 cells

It is known that exposure of cells to environmental stress such as ultraviolet radiation or osmotic or heat shock can lead to activation of a cascade of protein kinases culminating in the stimulation of stress-activated protein kinase (SAPK) (Davis, 1994; Sanchez *et al.*, 1994; Yan *et al.*, 1994). In addition, osmotic shock strongly activates the kinase cascade which leads to activation of the mammalian p38 kinase, which is similar to the yeast osmo-sensing mitogen-activated protein kinase (MAPK), high-osmolarity glycerol response 1 (HOG1) (Han *et al.*, 1994; Cano and Mahadevan, 1995). Thus it was considered possible that the oxidative stress induced by the loss of ATP during homogenisation might lead to the activation of one or more of these kinase cascades, the action of which could lead to the observed maximal stimulatory effect of GTP. To explore this possibility, membranes were prepared from glucagon receptor-transfected COS cells by homogenisation in either 1 mM $KHCO_3$ or the buffer used for the adenylate cyclase assay. The latter contains an ATP regenerating system comprising creatine phosphate and creatine kinase. Additionally, in view of the possibility that maximal stimulation of membrane adenylate cyclase by GTP might be an effect unique to COS cells, another immortalised cell type, HEK-293 cells was chosen. These cells,

which are derived from human embryonal kidney cells, were also transfected with the glucagon receptor and homogenised in the same way. Although these cells, unlike COS cells (Gluzman, 1981), do not constitutively express SV40 large T, transfection of DNA encoding the rat glucagon receptor was found to confer glucagon-induced stimulation of intracellular cAMP accumulation (figure 4.7.). On measuring adenylate cyclase activity in membranes isolated from both of these cell types, however, it was again found that glucagon did not cause any additional increase in cAMP production over that elicited by GTP alone (table 4.2.). This result was not changed by homogenising the cells in the presence of an ATP regenerating system, although this procedure was noted to cause a general increase in the cAMP levels ascertained under each of the stimulation conditions (table 4.2.).

In view of the inability to determine receptor-stimulated adenylate cyclase activity in isolated membranes, in subsequent experiments glucagon-stimulated adenylate cyclase activity was gauged exclusively by measuring intracellular cAMP accumulation under conditions where degradation of cAMP was blocked using the non-selective PDE inhibitor IBMX (Beavo and Reifsnyder, 1990; Hoey and Houslay, 1990). In homogenates of COS cells such a concentration of IBMX (1 mM) as used in these experiments was demonstrated to inhibit greater than 96% of the detectable PDE activity (see section 4.2.1.1.).

4.2.1.6. The effect of PMA on glucagon-stimulated cAMP accumulation in glucagon receptor-transfected COS cells

To determine whether treatment with the phorbol ester, 12-O-tetradecanoylphorbol 13-acetate (TPA, PMA) would lead to an inhibition of glucagon-stimulated cAMP accumulation in COS cells transfected with the glucagon receptor, such cells were stimulated with 10 nM glucagon for 10 minutes in the presence or absence of PMA at a concentration of 1 μM, after a pre-incubation period of 15 min with PMA in addition to IBMX. Control incubations were performed with DMSO at a final concentration equal to that present in the wells containing PMA.

It was observed that co-incubation with PMA failed to cause any observable reduction in the glucagon-stimulated cAMP accumulation (<2%, n=6 experiments) in GR-transfected COS cells (fig. 4.3.). This finding contrasted markedly with the previously reported finding that in hepatocytes, the phorbol ester caused a pronounced inhibition of glucagon-stimulated cyclic AMP accumulation (Heyworth *et al.*, 1984).

4.2.1.6.1. The effect of PMA on glucagon-stimulated cAMP accumulation in glucagon receptor-transfected 293 cells

It was considered possible that the PMA-mediated inhibition of this system, previously observed in hepatocytes, might require a particular environment, or set of cellular signalling components, which may be absent in COS cells. To address this issue, a different transfectable cell type was chosen in which to perform similar experiments. Thus HEK 293 cells were cultured and transfected with the GR-encoding plasmid. The degree of glucagon-stimulated cAMP accumulation in these cells was found to be 11.6 fold. Upon treatment of these cells with PMA, however, again no inhibition of glucagon-stimulated cAMP accumulation was observed (Fig. 4.7.).

4.2.1.6.2. Phosphatase inhibition by okadaic acid

The existence of multiple cellular phosphatases has been described (Hunter, 1995). In addition, a G protein coupled receptor phosphatase has been reported and has been identified as a member of the protein phosphatase type 2A (PP-2A) class (Pitcher *et al.*, 1995a). To determine whether the inability to observe PMA-mediated inhibition of the glucagon-stimulated cAMP accumulation in GR-transfected COS cells might be a consequence of potent phosphatase activity, glucagon-stimulated cAMP accumulation in GR-transfected COS cells was measured in the presence of PMA with the additional presence of okadaic acid. This substance is known to inhibit the phosphatases of both the PP-1 and the PP-2A classes (Hunter, 1995) and itself has been shown to mimic the PMA-mediated inhibition of glucagon-stimulated adenylate cyclase activity in hepatocytes (Savage *et al.*, 1995). Despite the additional presence of this inhibitor, however, no

PMA-induced attenuation of the glucagon-induced cAMP response was observed (Fig. 4.8.).

4.2.1.6.3. Varying the quantity of DNA transfected

Transient transfection of COS cells with a plasmid containing an SV40 promoter and origin of replication leads to a high level of expression of the protein encoded by the cDNA inserted into the vector 3' to the promoter. It is conceivable therefore that, if PMA-mediated inhibition of glucagon-stimulated cAMP accumulation is dependent upon phosphorylation of the glucagon receptor, that the over-expressed receptor may be so abundant that even a small proportion of unphosphorylated ligand-activated receptors might be sufficient to couple to and activate Gs maximally. This could therefore result in an inability to observe PMA-induced reduction of glucagon-stimulated cAMP accumulation in transiently transfected COS cells.

To address this possibility, dishes of COS cells were transfected with different quantities of the GR-encoding plasmid and subsequently stimulated with glucagon in the presence of PMA and okadaic acid. Such a strategy, that of transiently transfecting a range of quantities of plasmid DNA to achieve a range of expression levels, has been used successfully in other laboratories, for example that of M. Karin (Claret *et al.*, 1996).

It was observed here that transfecting with only 1 μg DNA per dish (rather than the previously used 5 μg) did indeed result in a reduction (by 44%) of the glucagon-induced cAMP response, suggesting that the expression of the receptor was related to the quantity of DNA utilised to transfect the cells. In the presence of PMA, or PMA in combination with okadaic acid, however, no inhibition of the glucagon-stimulated cAMP accumulation was observed with any of the various transfected cDNA quantities (Fig. 4.8.).

4.2.2. Western blotting for PKC isoforms in COS cells

It was regarded as likely that the failure to observe a PMA-induced inhibition of glucagon-stimulated cAMP accumulation in GR-transfected COS cells was due to inadequate expression in these cells of a signalling component required for this effect and

which is expressed in hepatocytes. Several lines of evidence suggest that in the mechanism of the PMA-mediated inhibition of glucagon-stimulated cAMP accumulation in hepatocytes, one or more PKC isoforms is a key component (Savage *et al.*, 1995). Thus it was of interest to determine whether any of the PKC isoforms expressed in hepatocytes are absent or poorly expressed in COS cells. It has been demonstrated that in rat hepatocytes the isoforms that can be detected by immunoblotting are PKC-α, βII and ε and ζ, but not PKC-βI, γ, δ or η (Tang *et al.*, 1993). Using antisera specific for PKC-α, βII and ε and ζ, therefore, Western blots were performed on COS cell and rat hepatocyte extracts (Fig. 4.9.), as described in the Materials and Methods chapter. The immunoblots indicated that although PKC-α, βII and ε and ζ were all detectable in native COS cells, the levels of PKC-βII appeared to be lower in COS cells than in hepatocytes.

4.2.3. Co-transfections of COS cells with GR and PKC isoform cDNAs

Given the high expression level of the transfected GR, it was thought possible that inadequate PKC isoform expression in COS cells may be the cause of the inability to observe PMA-mediated inhibition in COS cells. Thus it was decided to co-transfect COS cells with the GR-encoding cDNA and with plasmids encoding each of the three PMA-responsive PKC isoforms present in hepatocytes (PKC-α, βII and ε). As the activity of atypical PKC-ζ is known to be phorbol ester-independent (Ono *et al.*, 1989) this isoform was not co-transfected.

4.2.3.1. Co-transfections of COS cells with GR and PKC-α cDNAs

Exponentially growing COS-7 cells, at between 40 and 60% confluence, were transfected with expression vectors encoding the rat hepatic glucagon receptor (in pCDM8) and PKC-α (in pMT2). The cells were incubated and treated as for the single transfectants.

4.2.3.1.1. Confirmation of expression of PKC-α cDNA

In order to verify that this isoform was expressed in the co-transfected cells, Western blotting was performed using a primary antiserum specific for PKC-α. Thus, confluent flasks of native, untransfected and GR/PKC-α co-transfected COS cells were each harvested directly into Laemmli sample buffer (Laemmli, 1970) and immunoblotting undertaken as described in the Materials and Methods chapter (see section 2.4.3). In the lane loaded with the co-transfected sample, two high intensity bands were visible indicating immunoreactive species migrating with molecular weights of 84 and 88 kD (Fig. 4.11.A). In the native COS sample, however, although immunoreactive species were visible with identical molecular weights to those observed in the transfected cells, only relatively very weak signals were detected. These results suggested that transfection of COS cells with the PKC-α cDNA led to the expression of the PKC isoform, while the isoform was much less strongly expressed in native COS cells. The appearance of a doublet for PKC-α is consistent with immunoblotting results previously reported for this isoform, and has been suggested to be related to the existence of phosphorylated and non-phosphorylated species of the kinase in COS cells, with differing mobilities (Pears *et al.*, 1992).

4.2.3.1.2. Glucagon-stimulated cAMP responses in GR/PKC-α co-transfected COS cells

To investigate the effects of PKC-α co-transfection on glucagon-stimulated cAMP accumulation in GR-transfected COS cells, transfections were performed with no cDNA (mock), PKC-α cDNA alone, GR cDNA alone or PKC-α and GR cDNA together. It was observed that glucagon, as expected, did not lead to an elevation of intracellular cAMP accumulation in COS cells transfected with no cDNA or PKC-α cDNA alone. In the cells transfected with the GR-encoding plasmid alone, the cAMP accumulation after 10 minutes, in the presence of glucagon, was 9.7 fold greater than in its absence. The same incubation, however, in COS cells in which the expression vectors encoding both the glucagon receptor and PKC-α were co-transfected, gave rise to a 6.1 fold stimulation

(fig. 4.10.). This reduction in glucagon-stimulated cAMP accumulation associated with the transfection of an additional cDNA, may reflect the increased demands placed upon the finite transcriptional and translational capacity of these cells. Thus, it is likely that the expression of the cDNA encoding the glucagon receptor is reduced upon the transfection of an additional cDNA.

It was observed that, as before, co-incubation with the phorbol ester, PMA, did not lead to a reduction in glucagon-stimulated cAMP accumulation in GR-transfected COS cells. It was also found, however, that even in the additional presence of PKC-α, as confirmed by immunoblotting, PMA treatment again did not exert any inhibitory effect on the cellular cAMP response to glucagon. In fact, upon PMA treatment of the GR/PKC-α co-transfected cells, a small increase in the glucagon-stimulated cAMP accumulation was observed, although this was not statistically significant.

4.2.3.2. Co-transfections of COS cells with GR and PKC-βII and PKC-ε cDNAs

Co-transfections of COS cells were carried out with pairs of cDNAs encoding GR and PKC-βII or GR and PKC-ε. In each case immunoblotting confirmed that the designated PKC isoform was over-expressed in the cells (Fig. 4.11.B and 4.11.C), and glucagon incubations in the presence and absence of PMA were performed. In these experiments the effect of glucagon stimulation was measured at both 5 and 10 minutes. At neither of the time points, however, was a statistically significant inhibitory effect on glucagon-stimulated cAMP accumulation observed in the GR/PKC-βII or in the GR/PKC-ε co-transfected cells (table 4.3.).

4.3. Discussion

4.3.1. Glucagon-stimulated cAMP accumulation in transfected COS cells

Isobutylmethylxanthine (IBMX) elicited a profound inhibition (>96%) of the total phosphodiesterase activity in homogenates of glucagon receptor-transfected COS-7 cells. That complete inhibition was not observed may reflect the fact that IBMX is a reversible, competitive inhibitor of PDE action (Beavo and Reifsnyder, 1990; Hoey and Houslay, 1990) and perhaps the presence of the PDE type VII isoform, which has been shown to be IBMX-insensitive (Lavan *et al.*, 1989).

The initial objective was to create a model system in COS cells for glucagon-stimulated cAMP accumulation. In native COS-7 cells, challenge with glucagon in the presence of the cAMP phosphodiesterase inhibitor, IBMX, caused a small, dose-dependent increase in the intracellular accumulation of cAMP. A marked response was, however, elicited by the hormone in COS cells which had been transfected with the glucagon receptor cDNA, confirming that functional expression of the transfected receptor had been conferred upon these cells. This had been predicted as the glucagon receptor cDNA is contained within the pCDM8 plasmid which contains an SV40 origin of replication and would therefore be expected to replicate to a high copy number in COS cells (Sambrook *et al.*, 1989). The relatively small response to glucagon that was observed in native COS-7 cells suggests that although COS cells are kidney-derived (Gluzman, 1981) the level of glucagon receptor expression in these cells is low.

The observed EC_{50} value of 1.8 ± 0.4 nM for the glucagon response in the glucagon receptor-transfected COS cells is comparable to the values reported for the native response seen in intact hepatocytes in the presence of PDE inhibitors: 2 nM (Christoffersen and Berg, 1974), 0.5-3.0 nM (Sonne *et al.*, 1978) and 1.2 nM (Heyworth *et al.*, 1983). Jelinek et al. (Jelinek *et al.*, 1993) in their studies using the cloned rat glucagon receptor observed a value of 0.7 nM. In contrast, the level of glucagon detected in the serum of fasting patients has been reported to be only 30 ± 4.3

pM (Baron *et al.*, 1987). However, since glucagon is secreted by the pancreas and carried in the blood in the hepatic portal vein to the liver where it undergoes degradation (Jaspan *et al.*, 1981), only a fraction of the glucagon secreted by the pancreas can actually be measured in serum samples obtained from the peripheral circulation. Hepatocytes, therefore, are likely to be exposed to much higher concentrations of glucagon than were measured peripherally by Baron et al.

The timecourse for glucagon-stimulated cAMP accumulation in glucagon receptor-transfected COS cells showed that the response was only apparent if the non-selective PDE inhibitor, IBMX (Beavo and Reifsnyder, 1990; Hoey and Houslay, 1990), was added to assays. This is likely to reflect the potent activities of endogenous cAMP phosphodiesterases which have been demonstrated in these cells (Shakur *et al.*, 1993). As IBMX is a reversible, competitive inhibitor of PDE action then the plateau in accumulation reached after about 15 min presumably reflects a steady state where the glucagon-stimulated production of cAMP matches the degradation by the residual PDE activity.

4.3.2. Investigation of glucagon-induced lipid signalling

The inability to detect a significant stimulation of IP_3 production in mock or transfected COS cells stimulated with 10 nM glucagon is a finding that is consistent with the current literature regarding the effects of glucagon. Thus, while there have been reports of glucagon-stimulated inositol phospholipid hydrolysis, the magnitude of the reported stimulation has varied between only 18 and 25% (Wakelam *et al.*, 1986; Whipps *et al.*, 1987; Unson *et al.*, 1989). Furthermore, in rat hepatocytes, Pittner and Fain were unable to detect any glucagon-induced rise in inositol phosphate production (Pittner and Fain, 1991). While it is certainly acknowledged that glucagon causes a rapid transient increase in the level of intracellular calcium (Charest *et al.*, 1983; Mauger *et al.*, 1985; Sistare *et al.*, 1985; Blackmore and Exton, 1986; Mine *et al.*, 1988), it is likely, in view of the relatively small glucagon-induced release of inositol phosphates that this is

mediated by an influx of the ion from the extracellular medium rather than by a release from intracellular stores.

The finding that glucagon stimulation of GR-transfected COS cells led to an increase in the level of [^{3}H]-PtdOH, in the presence of 0.3% (v/v) butan-1-ol, suggests that glucagon in these cells stimulates the activity of PtdCho-PLC. However, no significant stimulation of the production of [^{3}H]-PtdBut was observed, indicating that PtdCho-PLD activity was not increased by this hormone.

Glucagon treatment of rat hepatocytes results in PKC activation, presumably by elevating DAG levels (Pittner and Fain, 1991). PtdCho-PLD coupled to phosphatidate phosphohydrolase activity was assumed by these authors to provide the DAG signal since the glucagon-stimulated formation of choline exceeded that of phosphocholine. However, this interpretation is compromised since choline and phosphocholine are readily interconverted ie. choline could be produced by the combination of PtdCho-PLC and phosphocholine phosphatase activity.

It appears from the experiments in transfected COS cells that PtdCho-PLC rather than PtdCho-PLD activity is predominant. Thus, production of DAG is a likely consequence in these cells with the implication that glucagon stimulation of COS cells will lead to the activation of PKC and other DAG-activated kinases.

4.3.3. Measurement of adenylate cyclase activity in plasma membranes of transfected cells

The inability to determine glucagon-stimulated adenylate cyclase activity in isolated membranes together with the maximal stimulation of adenylate cyclase activity that was elicited by the addition of GTP alone, was resolved neither by transfection of an alternative cell line, HEK293 cells, nor by homogenisation in the presence of an ATP regenerating system. This suggests that during cell disruption some modification of the adenylate cyclase signalling system occurs which can allow GTP-bound G_s to activate adenylate cyclase maximally. This might be a consequence of the high level of expression of transfected receptors, leading to the adoption of an active conformation by

a significant number of empty receptors, although representing only a small proportion of the total number of transfected receptors. Perhaps, due to cell disruption, an alteration in the ionic composition of the membrane environment occurs, which effects an increased stability of any receptor-G_s interactions. A proportion of empty receptors would then be able to couple to G_s, and the addition of GTP, even in the absence of glucagon, might be sufficient to permit maximal adenylate cyclase activation by these G proteins. In support of this hypothesis, it was observed that in the absence of hormone or GTP, the level of basal adenylate cyclase activity is greater in COS cells which have been transfected with the glucagon receptor than in control cells. Such an activation of membrane adenylate cyclase by unoccupied transfected receptors has been demonstrated previously in NG108-15 cells expressing either the human β_2 adrenoceptor (Adie and Milligan, 1994) or the δ-opioid receptor (Mullaney *et al.*, 1996) and in Chinese hamster ovary cells expressing histamine H_2 receptors (Smit *et al.*, 1996). In addition, the occurrence of altered signalling properties during membrane isolation has been observed in other systems (Anderson and Jaworski, 1979).

The determination of glucagon-stimulated adenylate cyclase activity was undertaken in subsequent experiments by the measurement of intracellular cAMP accumulation under conditions where degradation of cAMP was blocked using the non-selective PDE inhibitor IBMX (Beavo and Reifsnyder, 1990; Hoey and Houslay, 1990).

4.3.4. The effect of pre-incubation of transfected COS cells with PMA

Several lines of evidence (discussed in section 4.1.) have implicated a DAG-activated kinase in the desensitization of the glucagon receptor elicited both by glucagon itself and by other hormones able to elevate cellular DAG levels. In order to gain an insight into the mechanism involved in this desensitisation the intention was to study one component of this mechanism, the PMA-stimulated inhibition of glucagon-stimulated cAMP accumulation. However, in neither COS nor HEK293 cells, transfected with the rat hepatic glucagon receptor, was any PMA-stimulated inhibition of glucagon-stimulated cAMP accumulation observed. This contrasted with the published observations that in

hepatocytes, treatment with the phorbol ester PMA causes an inhibition of glucagon-stimulated cyclic AMP accumulation (Heyworth *et al.*, 1984). Furthermore, upon performing a glucagon time-course experiment with GR-transfected COS cells, the transience of the cAMP response, previously noted in hepatocytes (Christoffersen and Berg, 1974; Heyworth *et al.*, 1983; Murphy *et al.*, 1987), was not observed. Rather a continuous accumulation of cAMP ensued, over a comparable time-course to that investigated in hepatocytes.

The inability to observe any PMA-elicited inhibition of glucagon-stimulated cAMP accumulation in these transfected COS cells seemed unlikely to be due to any potent protein phosphatase activity as performing experiments in the presence of the protein phosphatase PP-1/PP-2A inhibitor, okadaic acid (Hunter, 1995), which itself has been shown to mimic the PMA-mediated inhibition of glucagon-stimulated adenylate cyclase activity in hepatocytes (Savage *et al.*, 1995), failed to allow PMA to exert an inhibitory effect on glucagon-stimulated cAMP accumulation in transfected COS cells (<2% inhibition).

It was considered that owing to the nature of transient transfection, the receptor might be over-expressed to such a degree that even full activation of the cellular kinase responsible in hepatocytes for the PMA-elicited inhibition would be incapable of phosphorylating a sufficient proportion of the receptors to prevent complete activation of the Gs population. In support of this hypothesis, it is evident from earlier published data on the rat hepatic glucagon receptor that, in fact, only a small proportion of the receptors need be occupied for activation of adenylate cyclase to occur (England *et al.*, 1983). Similarly, it has been shown that in hepatocytes, over 75% of glucagon receptors must be lost for any reduction in the glucagon-mediated stimulation of adenylate cyclase to be manifest (Houslay *et al.*, 1980). For this reason, with the objective of varying the degree to which the receptor was over-expressed, the effect of varying the quantity of transfected cDNA was studied. Transfection of varying quantities of glucagon receptor cDNA, was, indeed, found to lead to a marked variation in the glucagon-stimulated cAMP accumulation in these cells, implying that the maximum copy number reached may

depend upon the number of copies initially transfected. In fact, transient transfection using a range of plasmid DNA quantities has been used successfully by others to achieve varying levels of expression (Claret *et al.*, 1996). Reducing the quantity of cDNA transfected, in the present study, did not, however, lead to an ability of PMA to inhibit the response to glucagon.

It was subsequently considered that COS cells might lack some important cellular signalling component that is required for PMA-induced inhibition of glucagon-stimulated cAMP accumulation and which is not only present in hepatocytes but also capable of being activated by DAG or phorbol ester. The most likely candidates for such a component were considered to be the various isoforms of protein kinase C (PKC). Protein kinase C activity is supplied by a large family of isoforms (Hug and Sarre, 1993). These fall into a number of distinct classes which are: conventional (cPKCs), PKC-α, βI, βII and γ; novel (nPKCs), PKC-δ, ε, η, θ; and atypical (aPKCs), PKC-ζ, ι/λ, μ (Johannes *et al.*, 1994). The precise reasons for the existence of such a complex gene family have yet to be fully elucidated. However, there is now evidence to suggest that particular PKC isoforms can mediate specific biological effects and, that expression patterns of isoforms are cell specific (Dekker and Parker, 1994; Spence *et al.*, 1995).

It is possible that the inability to observe PMA-mediated inhibition of glucagon-stimulated cAMP accumulation in COS cells might have been due to inadequate PKC expression. Hepatocytes have been shown to express PKC isoforms PKC-α, βII and ε and ζ, but not PKC-βI, γ, δ or η (Tang *et al.*, 1993). Immunoblotting indicated that although PKC-α, βII and ε and ζ were all detectable in native COS cells, the levels of PKC-βII appeared to be lower in COS cells than in hepatocytes. In order to increase levels of PKC isoforms it was decided to co-transfect COS cells with the glucagon receptor-encoding cDNA and with plasmids encoding each of the three PMA-responsive PKC isoforms present in hepatocytes (PKC-α, βII and ε). As the activity of atypical PKC-ζ is known not to be stimulated by phorbol ester (Ono *et al.*, 1989) transfection of this isoform was not attempted. Immunoblotting analyses showed that transfection of COS cells with these various PKC cDNAs led to the over-expression of the appropriate

isoform. However, in no instance was PMA-induced inhibition of glucagon-stimulated cyclic AMP accumulation reconstituted by co-transfection with the plasmids encoding these PKC isoforms. In conclusion, the co-expression in the glucagon receptor-transfected COS cells of any individual PKC isoform present in hepatocytes, was not, in itself, sufficient to confer PMA-induced inhibition of the glucagon-stimulated cAMP accumulation.

	Untransfected		GR-transfected	
	control	PMA	control	PMA
control	94 ± 51	245 ± 47	378 ± 42	569 ± 15
glucagon	97 ± 30 ns	285 ± 55 ns	484 ± 60 ns	759 ± 104 ns
GTP	333 ± 38 *	594 ± 111 *	683 ± 26 **	1020 ± 6 **
glucagon+GTP	389 ± 9 **	591 ± 88 *	700 ± 60 **	1063 ± 24 **

Table 4.1. Glucagon and GTP-stimulated adenylate cyclase activities in membranes prepared from control and PMA-treated untransfected and GR-transfected COS cells.

Confluent untransfected or GR-transfected COS cells were incubated for 15 min with either control (0.1% DMSO) medium or 1 μM PMA at 37 °C. Membranes were then prepared as described in the Materials and Methods chapter, and incubated at 30 °C for 10 min with 10 nM glucagon, 100 μM GTP, or both together, prior to cAMP determination. The values shown, expressed in pmol/mg, represent means ± SE of three experiments. * Significant at $P<0.05$, ** Significant at $P<0.01$, ns not significant (Student's t test, relative to control, unstimulated with glucagon or GTP).

	Transfected COS-7 cells		Transfected HEK293 cells	
	1mM $KHCO_3$	ATP regen. system	1mM $KHCO_3$	ATP regen. system
control	280 ± 19	1080 ± 45	129 ± 9	642 ± 33
GTP	927 ± 39	1720 ± 63	920 ± 110	1300 ± 35
glucagon+GTP	901 ± 85	1720 ± 98	958 ± 24	1370 ± 47

Table 4.2. Glucagon and GTP-stimulated adenylate cyclase activities in membranes prepared from GR-transfected COS and HEK-293 cells.

GR-transfected COS cells and HEK-293 cells were homogenised in the presence of either 1 mM $KHCO_3$ or an ATP regenerating system containing creatine kinase and creatine phosphate. Subsequently, membranes were prepared as described in the Materials and Methods chapter and incubated at 30 °C for 10 min with 10 nM glucagon, 100 μM GTP, or both together, prior to cAMP determination. The values shown, expressed in pmol/mg, represent means ± SE of three experiments.

Transfection	Glucagon-stimulated cAMP production in presence of PMA (% of control ie. glucagon alone)	
	5 min	10 min
GR	124±7	101±6
GR+PKC α	ND	113±6
GR+PKC βII	104±8	94.5±1.1
GR+PKC ε	98.0±3.2	87.1±11.5

Table 4.3. The effect of PMA on the glucagon-stimulated cAMP response in COS cells co-transfected with cDNA encoding the GR and PKC-α, PKC-βII or PKC-ε

Transfected COS cells, grown to confluence in 6-well plates as described in the Materials and methods chapter, were pre-incubated for 15 min at 37 °C with serum-free DMEM containing 1 mM IBMX, and either 0.1% DMSO (control) or 1 μM PMA. Glucagon was then added to a final concentration of 10 nM and the incubation was continued for another 5 or 10 minutes. 2% PCA was then added, the cells were harvested and their cAMP content determined. The results shown represent means of triplicate determinations of cAMP ±SE. The glucagon stimulated cAMP production in the presence of PMA is expressed as a percentage of the control (no PMA) value. ND, not determined.

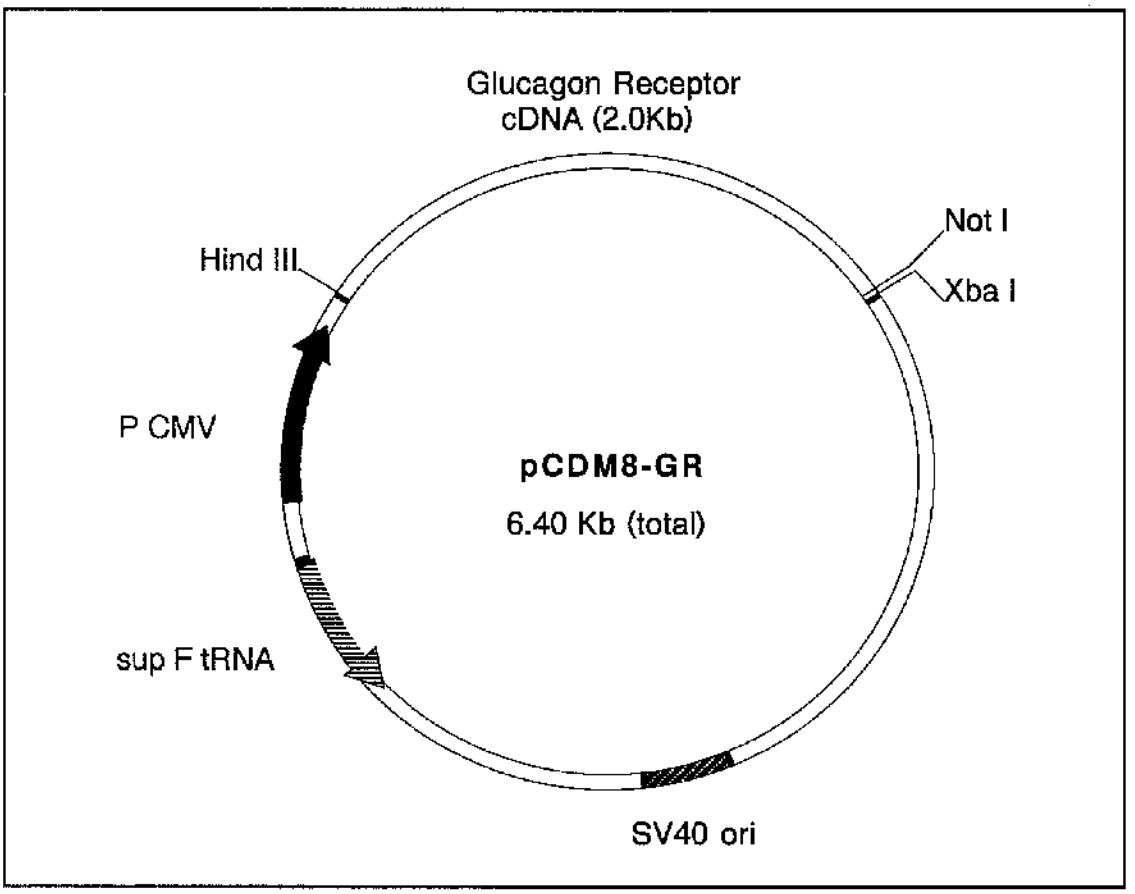

Glucagon Receptor
cDNA (2.0Kb)
Not I
Xba I
Hind III
P CMV
pCDM8-GR
6.40 Kb (total)
sup F tRNA
SV40 ori

Figure 4.1. The pCDM8-GR construct

This plasmid was constructed by the insertion of the entire intron-free rat hepatic glucagon receptor coding sequence (Genbank accession no. L04796) into the HinD III/Not I digested mammalian expression vector pCDM8 (Svoboda *et al.*, 1993b). High level expression is driven by the CMV promoter and replication to high copy number is conferred by the SV40 origin of replication. Bacterial selection is made possible by the expression of the sup F tRNA gene. The product of this gene suppresses the amber mutations in the ampicillin and tetracycline resistance genes on the P3 episome, which is contained within bacteria such as the MC1061/P3 strain of E. coli. This leads to the translation of these antibiotic resistance genes and, consequently, permits the selection of the transformed bacteria.

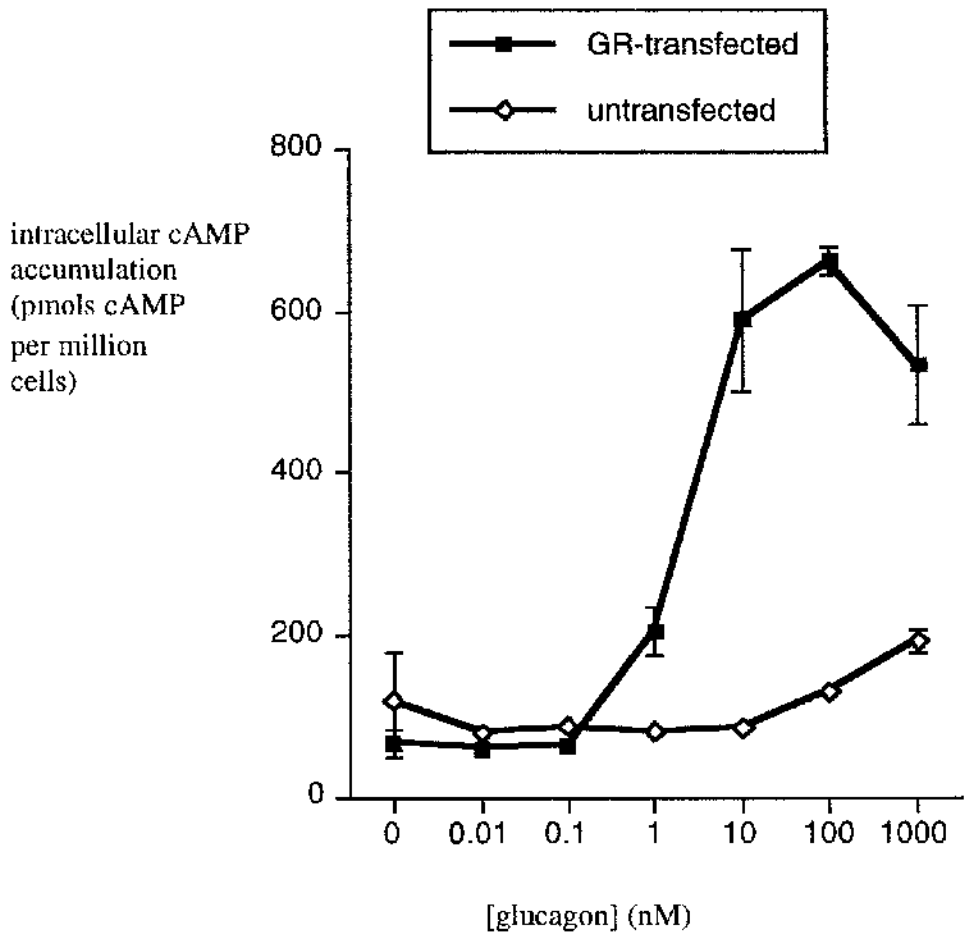

GR-transfected
untransfected
intracellular cAMP accumulation (pmols cAMP per million cells)
800
600
400
200
0
0
0.01
0.1
1
10
100
1000
[glucagon] (nM)

Figure 4.2. Dose response curve for glucagon-stimulated cAMP production in untransfected and GR-transfected COS cells.

This figure shows dose response curves for the glucagon-stimulated cAMP production in intact COS cells, either control (open diamonds) or transfected with pCDM8-GR DNA (filled squares). Cells were stimulated for 6 minutes with the indicated concentrations of glucagon, in the presence of the cAMP phosphodiesterase inhibitor, IBMX. Incubations were terminated by the addition of 2% PCA and cAMP production was determined by the cAMP binding assay. Means (±SE) of three experiments are shown.

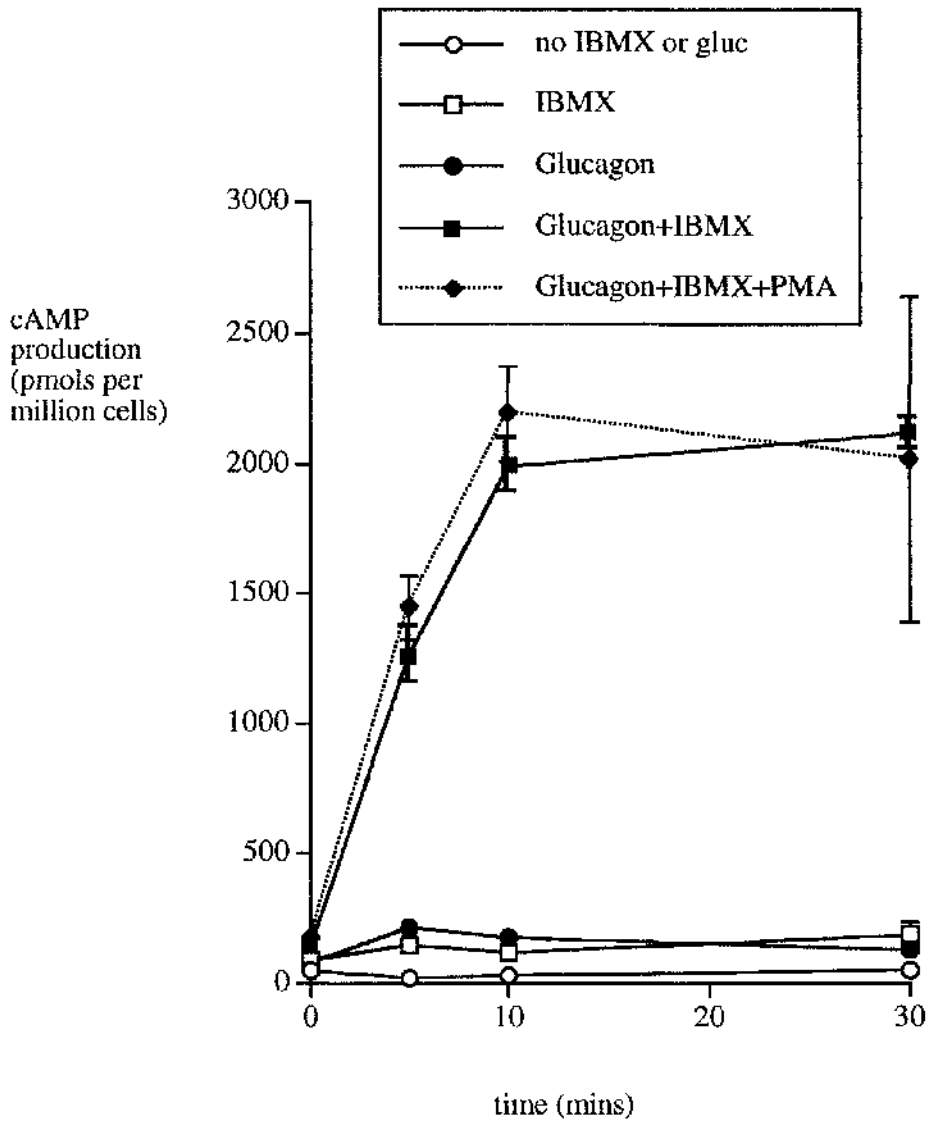
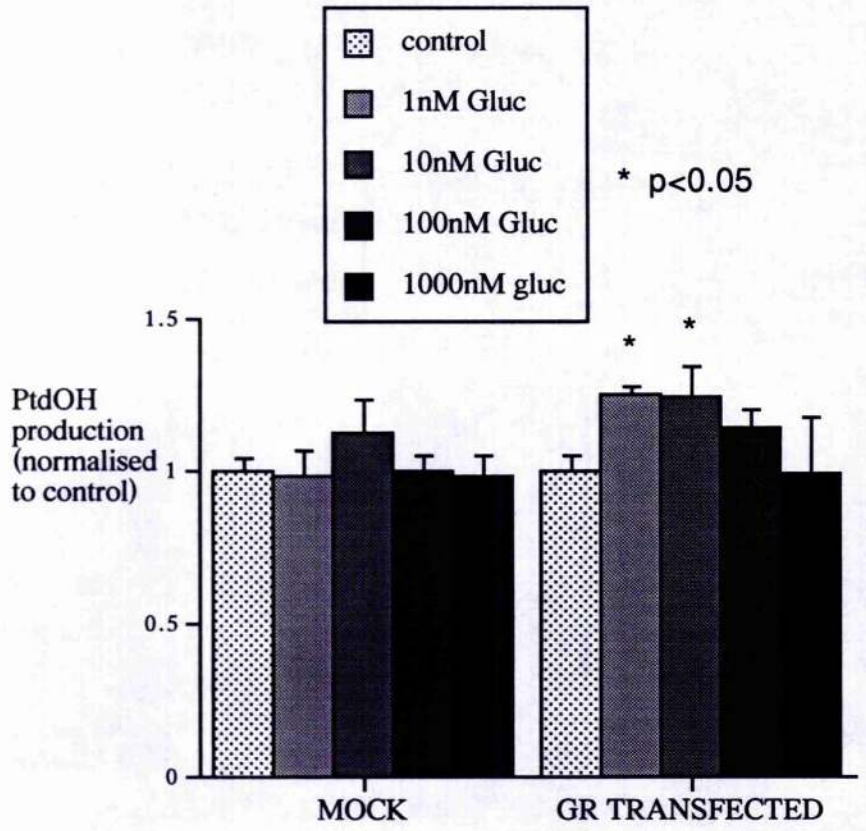

no IBMX or gluc
IBMX
Glucagon
Glucagon+IBMX
Glucagon+IBMX+PMA
cAMP production (pmols per million cells)
3000
2500
2000
1500
1000
500
0
0
10
20
30
time (mins)

Figure 4.3. Timecourses for glucagon-stimulated cAMP production in GR-transfected COS cells.

This figure shows time courses for glucagon-stimulated cAMP accumulation in GR-transfected COS cells in the presence and absence of IBMX. GR-transfected COS cells were stimulated with 10 nM glucagon (filled symbols) or control medium (open symbols), in the presence (squares) or absence (circles) of the phosphodiesterase inhibitor, IBMX, at a concentration of 1 mM. The response to glucagon, in the presence of IBMX and 1 μM PMA, is also indicated (filled diamonds). Incubations were terminated after the times indicated by the addition of 2% PCA. The values shown represent means (± SE) from three experiments.

A.


control
1nM Gluc
10nM Gluc
100nM Gluc
1000nM gluc
* p<0.05
PtdOH production (normalised to control)
1.5
1
0.5
0
*
*
MOCK
GR TRANSFECTED

B.

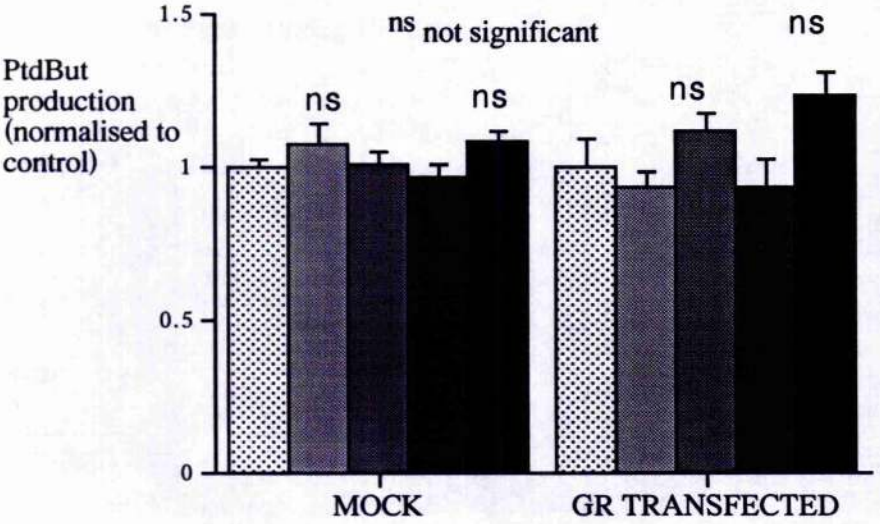

ns not significant
PtdBut production (normalised to control)
1.5
1
0.5
0
ns
ns
ns
ns
MOCK
GR TRANSFECTED

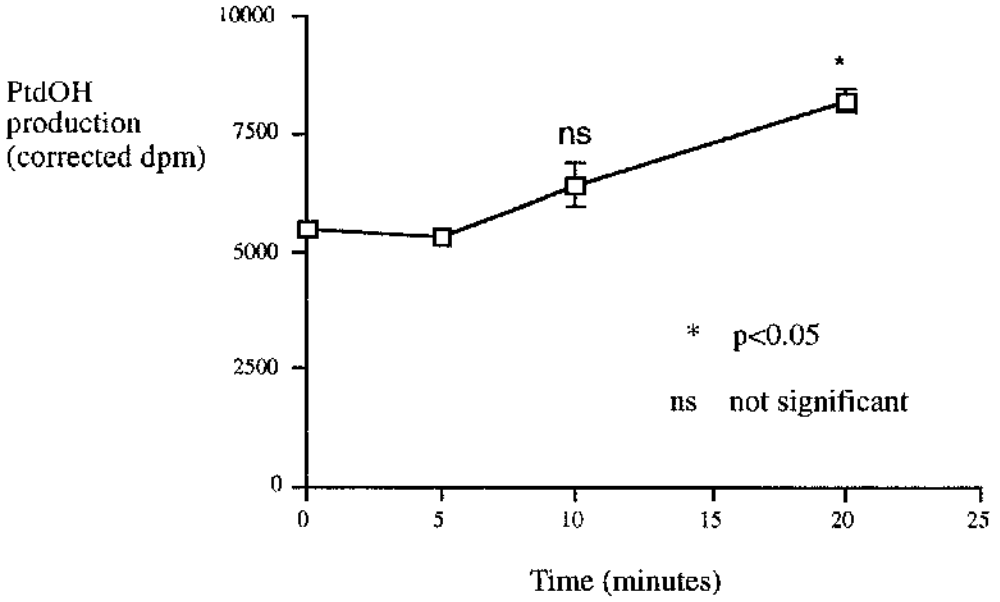
Figure 4.4. PtdOH and PtdBut production in mock and glucagon receptor-transfected COS cells upon stimulation with varying concentrations of glucagon.

COS-7 cells were either mock-transfected or transfected with the GR-encoding plasmid, and subsequently grown in 24-well plates. When 80% confluent, the culture medium was replaced with DMEM containing 1% (v/v) foetal calf serum (FCS) and 2 μCi [^{3}H]-palmitic acid/ml. After an incubation period of 24 hours the cells were then washed in 0.25 ml serum-free DMEM at 37 °C for 30 minutes prior to incubation for a further 5 min in 0.25 ml of serum-free DMEM containing 0.3% (v/v) butan-1-ol. Incubations were commenced by the addition of 28 μl glucagon in DMEM/0.3% (v/v) butan-1-ol giving the desired final concentration and were continued at 37 °C for a further 10 minutes. Incubations were terminated by aspiration of the medium and the addition of 0.2 ml of ice-cold methanol to each well.

Samples were harvested on ice and transferred to glass vials. Each well was then rinsed with a further 0.2 ml methanol which was combined with the first. Lipids were extracted for at least 15 min at room temperature with 0.4 ml of chloroform. The solvent mixture was evaporated *in vacuo* and the lipids re-dissolved in 100 μl of chloroform/methanol (19:1, v/v) by vortex-mixing. Subsequently, 10 μl of the sample was transferred to a scintillation vial for counting in order to measure total lipid labelling and allow normalisation of sample size. The remainder of the sample was applied to a Whatman LK5D 150 Å TLC plate which was developed in the organic phase of 2,2,4-trimethylpentane/ethyl acetate/acetic acid/water (5:11:2:10, by vol.) using an unlined chromatography tank, until the solvent reached 1 cm from the top of the plate.

[^{3}H]-PtdOH (A) and [^{3}H]-PtdBut (B), identified, upon iodine-staining, by their co-migration with authentic PtdOH and PtdBut standards, were excised from the plates and their radioactivity determined by scintillation counting. The counts from individual wells were corrected for sample size and the means calculated from triplicate determinations. The results were then normalised to the control value (in the absence of glucagon) for each cell type, and the means of the normalised values from two similar experiments were then calculated. Statistical significance was determined by Student's *t* test.

A


PtdOH
production
(corrected dpm)
10000
7500
5000
2500
0
ns
*
* p<0.05
ns not significant
0
5
10
15
20
25
Time (minutes)

B

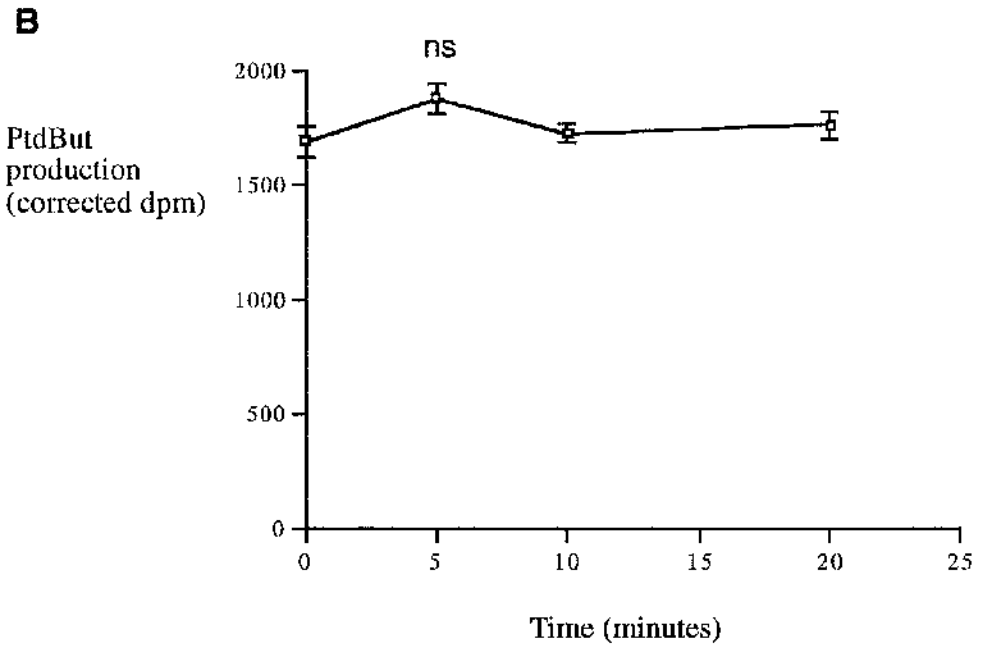

PtdBut
production
(corrected dpm)
2000
1500
1000
500
0
ns
0
5
10
15
20
25
Time (minutes)

Figure 4.5. Timecourse of PtdOH and PtdBut production in glucagon receptor-transfected COS cells in response to glucagon.

COS-7 cells were transfected with the GR-encoding plasmid, and subsequently grown in 24-well plates. When 80% confluent, the culture medium was replaced with DMEM containing 1% (v/v) foetal calf serum (FCS) and 2 μCi [^{3}H]-palmitic acid/ml. After an incubation period of 24 hours the cells were then washed in 0.25 ml serum-free DMEM at 37 °C for 30 minutes prior to incubation for a further 5 min in 0.25 ml of serum-free DMEM containing 0.3% (v/v) butan-1-ol. Incubations were commenced by the addition of 28 μl glucagon in DMEM/0.3% (v/v) butan-1-ol giving the desired final concentration and were continued at 37 °C for the times indicated. Incubations were terminated by aspiration of the medium and the addition of 0.2 ml of ice-cold methanol to each well.

Samples were harvested on ice and transferred to glass vials. Each well was then rinsed with a further 0.2 ml methanol which was combined with the first. Lipids were extracted for at least 15 min at room temperature with 0.4 ml of chloroform. The solvent mixture was evaporated *in vacuo* and the lipids re-dissolved in 100 μl of chloroform/methanol (19:1, v/v) by vortex-mixing. Subsequently, 10 μl of the sample was transferred to a scintillation vial for counting in order to measure total lipid labelling and allow normalisation of sample size. The remainder of the sample was applied to a Whatman LK5D 150 Å TLC plate which was developed in the organic phase of 2,2,4-trimethylpentane/ethyl acetate/acetic acid/water (5:11:2:10, by vol.) using an unlined chromatography tank, until the solvent reached 1 cm from the top of the plate.

[^{3}H]-PtdOH (A) and [^{3}H]-PtdBut (B), identified, upon iodine-staining, by their co-migration with authentic PtdOH and PtdBut standards, were excised from the plates and their radioactivity determined by scintillation counting. The counts from individual wells were corrected for sample size and the means calculated from triplicate determinations. Statistical significance was determined by Student's *t* test.

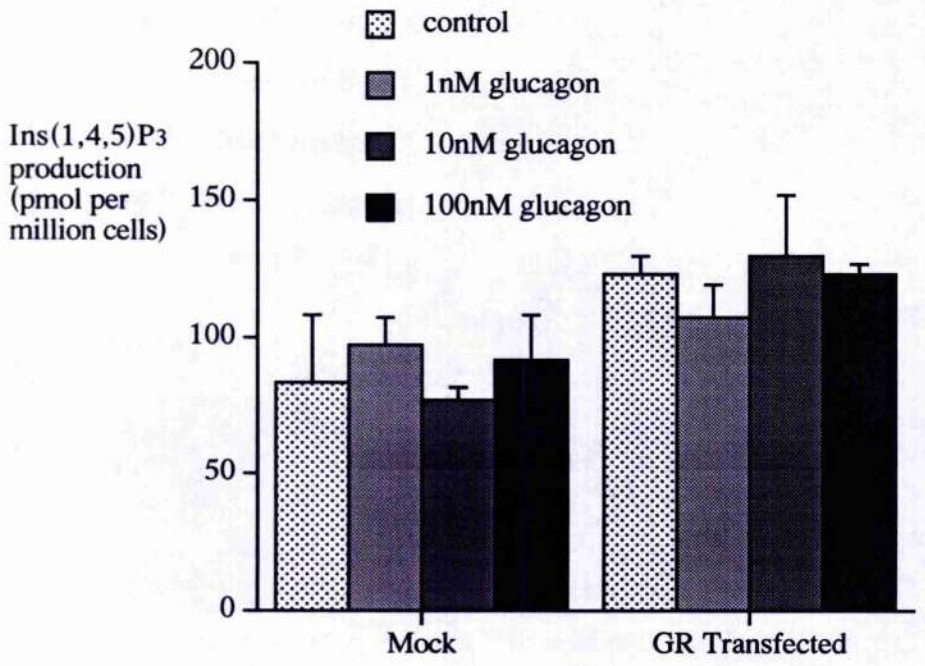

control
1nM glucagon
10nM glucagon
100nM glucagon
Ins(1,4,5)P3 production (pmol per million cells)
200
150
100
50
0
Mock
GR Transfected

Figure 4.6. Dose response of Ins(1,4,5)P_3 production upon glucagon stimulation of mock and GR-transfected COS cells

Mock and GR-transfected COS cells, grown for 48 hours in DMEM containing 10% (v/v) FCS were incubated in 0.5 ml DMEM containing 1% (v/v) FCS for a further 24 hours. The cells were then washed in 0.25 ml serum-free DMEM at 37 °C for 30 minutes. Stimulations were performed by the addition of 11 µl glucagon diluted appropriately in DMEM to give the desired final concentrations. Reactions were terminated after 10 seconds by the addition of 25 µl ice-cold 10% (v/v) perchloric acid. Samples were harvested on ice and neutralised in the presence of a trace of Universal Indicator by the addition of approximately 25-30 µl 1.5 M KOH/60 mM HEPES. A sample of 110 µl was taken for Ins(1,4,5)P_3 mass measurement. Ins(1,4,5)P_3 mass was quantified using a stereospecific radioligand binding assay (Palmer and Wakelam, 1990), as described in the Materials and Methods chapter. The values indicated represent the means ± SE calculated from triplicate determinations.

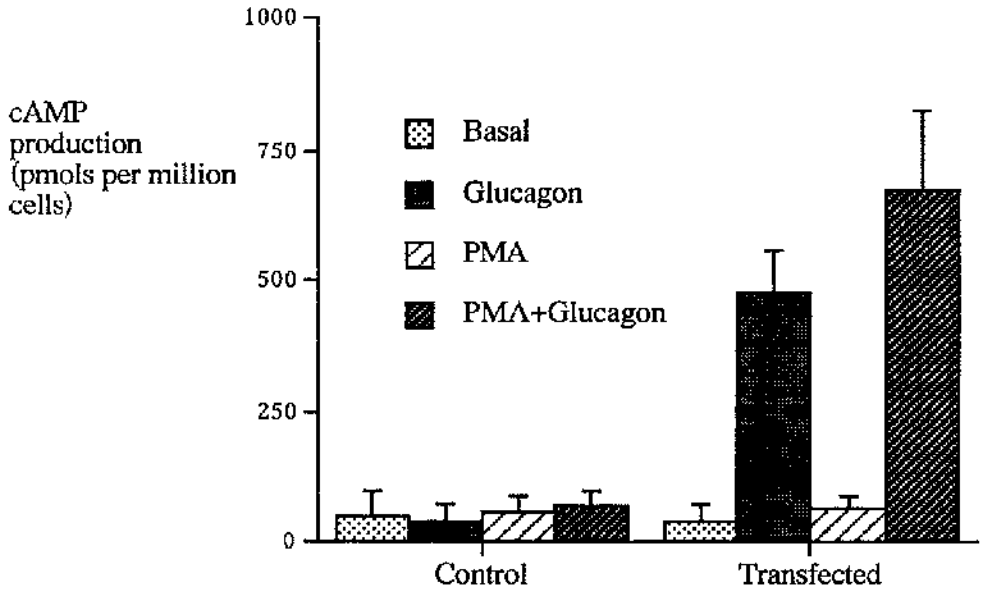

cAMP
production
(pmols per million
cells)
1000
750
500
250
0
Basal
Glucagon
PMA
PMA+Glucagon
Control
Transfected

Figure 4.7. Glucagon-stimulated cAMP production in control and PMA-treated untransfected and GR-transfected HEK-293 cells.

Confluent untransfected or GR-transfected HEK-293 cells were pre-incubated for 15 min with either control (0.1% DMSO) medium or 1 μM PMA at 37 °C, prior to stimulation for 10 minutes with 10 nM glucagon, in the presence of the cAMP phosphodiesterase inhibitor, IBMX. Incubations were terminated by the addition of 2% PCA and cAMP production was determined by the cAMP binding assay. Means (±SE) of three experiments are shown. The level of cAMP produced in response to glucagon in the presence of PMA was not significantly different from that obtained in the presence of glucagon alone (Student's *t* test).

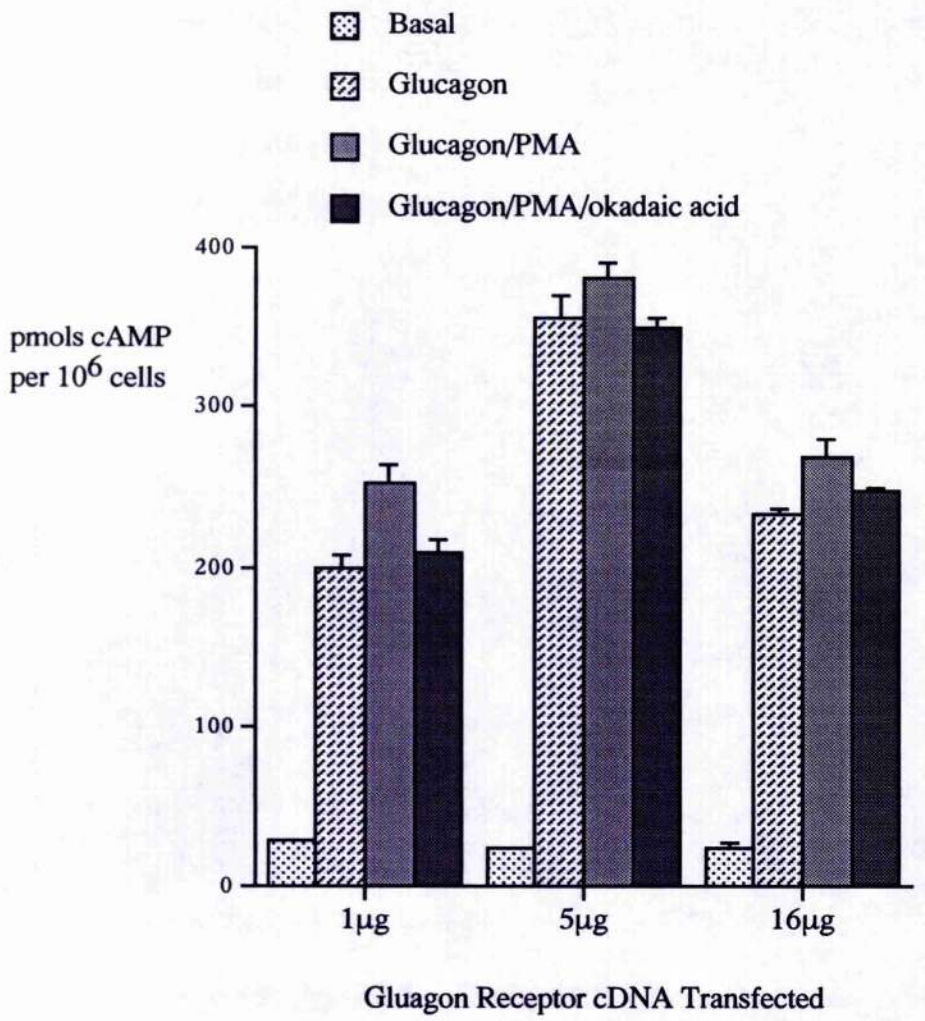

Basal
Glucagon
Glucagon/PMA
Glucagon/PMA/okadaic acid
pmols cAMP
per 10^6 cells
400
300
200
100
0
1μg
5μg
16μg
Gluagon Receptor cDNA Transfected

Figure 4.8. The effect of okadaic acid, PMA and varying the quantity of transfected GR DNA on the glucagon-stimulated cAMP response in COS cells

Intact COS-7 cells, transfected with 1, 5 or 16 μg of pCDM8-GR DNA were pre-incubated for 15 minutes with control medium, or medium containing 1 μM PMA ± 100 nM okadaic acid, and subsequently stimulated for 10 minutes with 10 nM glucagon. 1 mM IBMX was present during the pre-incubations and the glucagon stimulations. The reactions were terminated by the addition of 2% PCA and cAMP production was measured by the cAMP binding assay. Means (± SE) of triplicate determinations are shown.

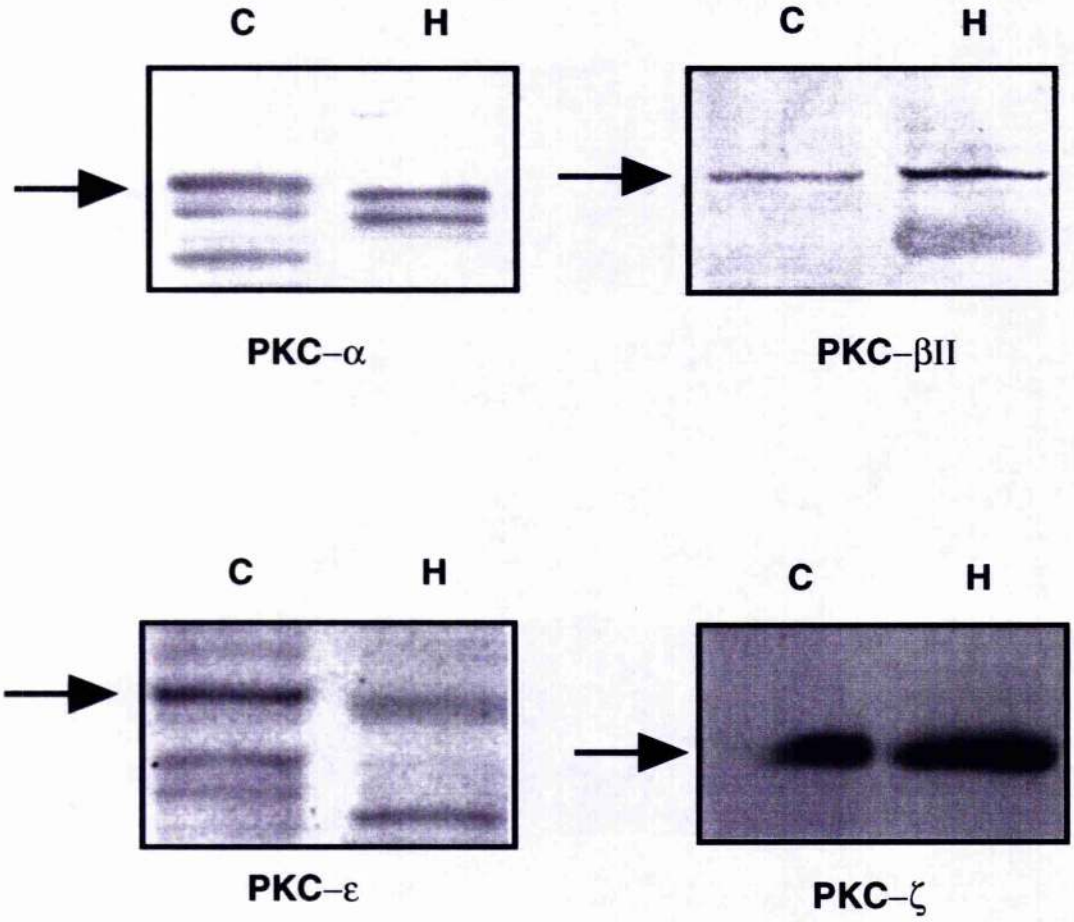

C
H
C
H
PKC–α
PKC–βII
C
H
C
H
PKC–ε
PKC–ζ

Figure 4.9. Immunoblots for PKC α, PKC βII, PKC ε and PKC ζ isoforms in untransfected COS-7 and hepatocyte extracts

Homogenates of untransfected COS-7 cells or rat hepatocytes, prepared in the presence of protease inhibitors, were boiled for 3 min following the addition of Laemmli buffer. 50μl of each sample, containing 100 μg protein, was subsequently loaded onto an 8% SDS poly-acrylamide gel. Blotting was carried out as described in the Materials and Methods chapter with primary antibody dilutions of 1 in 100.

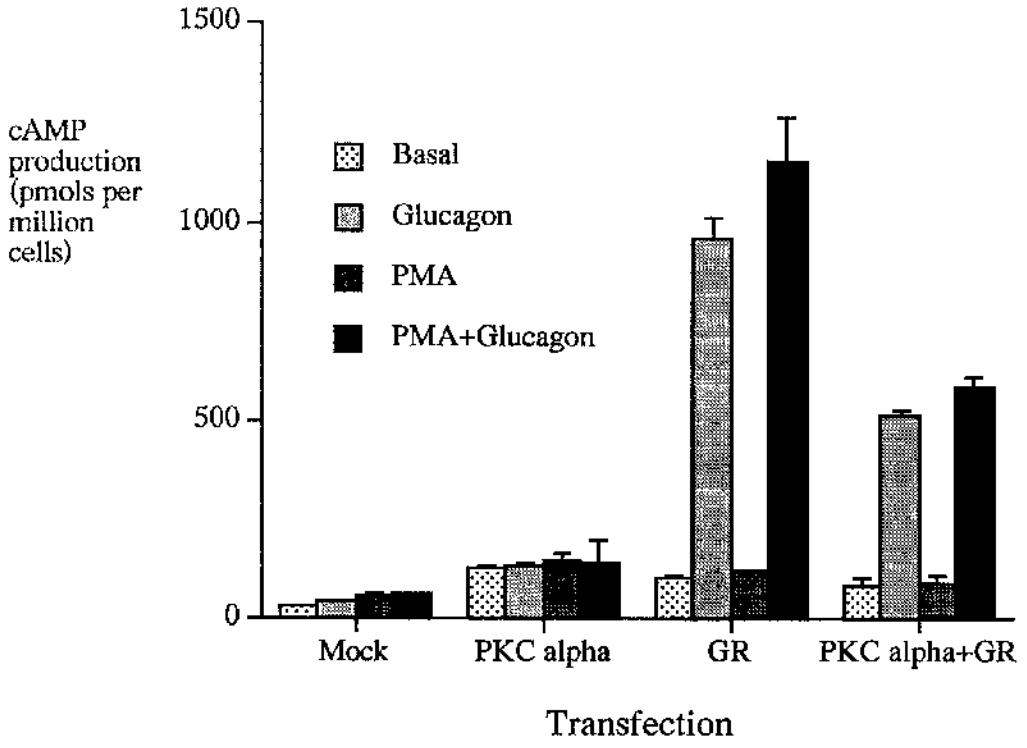

1500
1000
500
0
cAMP production (pmols per million cells)
Basal
Glucagon
PMA
PMA+Glucagon
Mock
PKC alpha
GR
PKC alpha+GR
Transfection

Figure 4.10. The effect of PMA on the glucagon-stimulated cAMP response in PKC alpha and glucagon receptor co-transfected COS cells.

Untransfected COS cells, or COS cells transfected with cDNA encoding PKC alpha, the glucagon receptor, or both plasmids together, were grown to confluence in 6-well plates as described in the Materials and Methods chapter. The cells were then pre-incubated for 15 min at 37°C with serum-free DMEM containing 1 mM IBMX, and either 0.1% DMSO (control) or 1 μM PMA. Glucagon was then added to a final concentration of 10 nM and the incubation was continued for another 10 minutes. 2% PCA was subsequently added, the cells were harvested and their cAMP content determined. The results shown represent means ± SE of three assays.

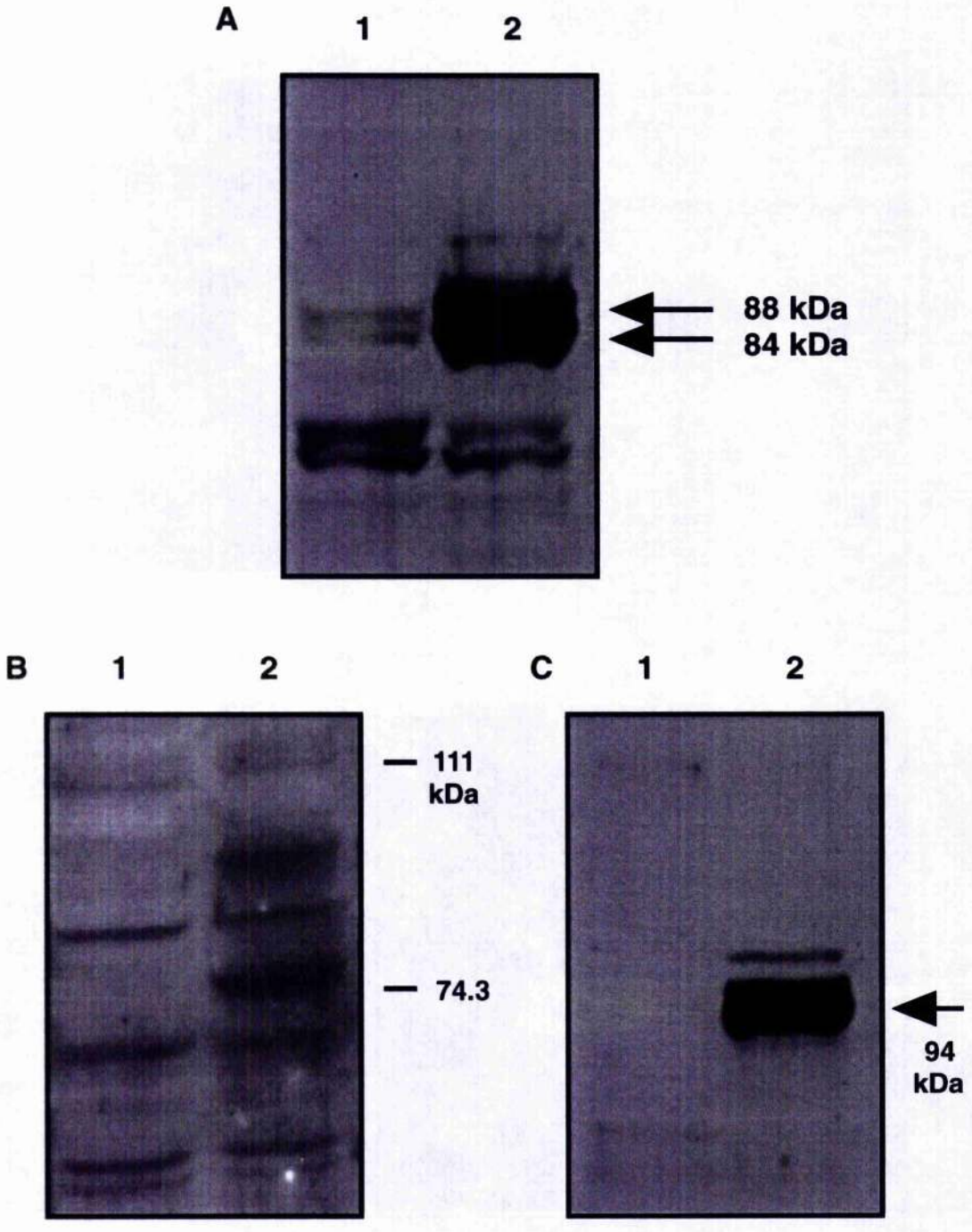

A
1
2
88 kDa
84 kDa
B
1
2
111
kDa
74.3
C
1
2
94
kDa

Figure 4.11. Confirmation of expression of transfected cDNA encoding PKC α, PKC βII and PKC ε by Western blotting.

Transfected or control COS cells were grown to confluence, washed 4 times with PBS, scraped into 0.5 ml Laemmli buffer per 75 cm^2 flask, and immediately boiled for 5 minutes. Subsequently, 30 µl of each sample was loaded onto an 8% SDS poly-acrylamide gel. Blotting was carried out as described in the Materials and methods chapter with primary antibody dilutions of 1 in 500 for PKC α (A) and PKC ε (C), and 1 in 2000 for PKC βII (B).

CHAPTER 5

Co-expression of the GR with GRK2, GRK3 and PKD in COS-7 cells, and the effects of PMA on the individual steps leading from glucagon binding to cAMP accumulation

5.1. Introduction

Following the experiments described in chapter 4 in which it was attempted to reconstitute, with co-transfected PKC isoforms, the PMA-induced attenuation of glucagon-stimulated cAMP accumulation in glucagon receptor-transfected COS cells, it was considered useful to investigate the possible alternative kinases which are known to play a role in the desensitization of G protein coupled receptor-mediated responses to extracellular signals. The kinases which are known to mediate such actions generally fall into one of two categories (Lohse, 1993), namely the receptor-specific kinases and the non-receptor-specific cellular kinases.

The receptor specific kinases constitute a family, known as the G protein coupled receptor kinases (GRKs), the identification of which was a consequence of the analysis of the regulation of adrenoceptors. There are now six cloned mammalian members of this family, known as GRK1-6 (see chapter 1), whose only known function is to specifically phosphorylate the agonist-occupied form of these receptors, thus mediating receptor-specific desensitization (Hausdorff *et al.*, 1990). The phosphorylation leads to the binding of the inhibitor proteins named arrestins, and the subsequent uncoupling of the receptor from the G protein (Lohse *et al.*, 1992).

GPCRs may also be phosphorylated and uncoupled by the action of the effector kinases of receptor systems, such as protein kinases A and C, for which such receptors are only one of a great number of potential cellular substrates. PKA has been demonstrated to mediate the desensitization of a number of GPCRs including not only the β_2-adrenergic receptor (Benovic *et al.*, 1985), but also the PGE_1 receptor (Clark *et al.*, 1988) and the peripheral DA_1-dopamine receptor (Bates *et al.*, 1991). Similarly, many receptors which lead to the activation of PKC can undergo desensitization mediated by this kinase, in addition to receptor-specific desensitization. Such receptors include the H_1-histamine receptor (Leurs *et al.*, 1991; Smit *et al.*, 1992), the thrombin receptor (Brass, 1992) the $5HT_2$ -serotonin receptor (Kagaya *et al.*, 1990), the parathyroid hormone receptor (Dunlay and Hruska, 1990), the angiotensin II receptor (Sakuta *et al.*,

1991; Oppermann *et al.*, 1996) and the glucagon-like peptide-1 (GLP-1) receptor (Widmann *et al.*, 1996).

The glucagon receptor might, as a member of the GPCR superfamily, be expected to be a substrate for one or more of the receptor-specific kinases or effector kinases. Certainly, there is now an abundance of published data supporting desensitization of the glucagon receptor system by not only glucagon, but also by other hormones and synthetic diacylglycerols (see section 4.1.3.). Moreover, exposure of hepatocytes to glucagon is known to lead not only to the elevation of cAMP, but also, to that of intracellular Ca^{2+}, IP_3 , and DAG (see section 4.1.3.) which could potentially serve to activate various kinases that might mediate desensitization of the receptor. It is unlikely that calcium itself is important in such a role, given the lack of an observable desensitization of glucagon-stimulated adenylate cyclase activity that occurred when hepatocytes were treated with the Ca^{2+} ionophore, A23187 (Savage *et al.*, 1995). Similarly the increase in cAMP levels does not appear to lead to any PKA-mediated desensitization in view of the inability of membrane-permeant cAMP analogues such as dibutyryl cAMP to induce desensitization in hepatocytes (Heyworth and Houslay, 1983).

In view of the published evidence described in section 4.1.3., it is, however, likely that a DAG/PMA-activated kinase mediates the desensitization in hepatocytes. From the data presented in the preceding chapter, this would appear not to be an isoform of protein kinase C since it was found that co-transfection of the PKC isoforms present in hepatocytes was insufficient to confer PMA-mediated inhibition of glucagon-stimulated cAMP accumulation in glucagon receptor-transfected COS cells (see section 4.2.3.). Kinases other than PKC isoforms have, however, recently been shown to be activated by PMA. These include the β-adrenergic receptor kinase-1 (βARK-1), which can effect the attenuation of functioning of G_s coupled β-adrenoceptors in a markedly PMA-enhanced fashion (Chuang *et al.*, 1995), and the recently discovered protein kinase D (PKD) (Valverde *et al.*, 1994; Van Lint *et al.*, 1995).

The mechanism of activation of βARK-1 (which corresponds to GRK2 in the GRK-terminology) certainly involves agonist stimulation of the receptor, presumably

leading to a conformational change in the receptor that is necessary for the subsequent translocation of the kinase to the membrane (Strasser *et al.*, 1986; Mayor *et al.*, 1987; Garcia-Higuera and Mayor, 1992) and its interaction with the receptor. In addition, its translocation to the membrane may be enhanced by the ability of the pleckstrin homology (PH) domain that is contained within the C-terminal, membrane targeting, region of βARK-1 and 2 (Musacchio *et al.*, 1993), to interact both with released G protein βγ-subunits (Touhara *et al.*, 1994) and with membrane lipids such as phosphatidylinositol-3,4-bisphosphate (PIP_2) (Harlan *et al.*, 1994; Pitcher *et al.*, 1995b). It now appears however, that the regulation of the βARKs is more complex as it has been reported that PKC mediates the phosphorylation and activation of βARK-1 both in vitro and in vivo (Chuang *et al.*, 1995). This action by PKC is believed to involve phosphorylation of the C-terminal region of βARK, leading to increased membrane attachment by this domain and the consequent translocation of the kinase to the plasma membrane (Lohse *et al.*, 1996).

A novel protein kinase, called protein kinase D (PKD), which shares structural identity with PKC isoforms, has recently been identified (Valverde *et al.*, 1994). Like PKCs of the conventional and novel classes, it exhibits N-terminal, cysteine-rich, zinc-finger-like domains (Nishizuka, 1988). It is not surprising therefore that a bacterially expressed fusion protein containing the regulatory domain of PKD has been found to bind phorbol ester, thus indicating that PKD can serve as a novel phorbol ester receptor (Valverde *et al.*, 1994) . Furthermore, it has been shown that transient expression of the kinase in COS cells confers phorbol ester binding on these cells and that the activity of PKD, immunoprecipitated from the transfected COS cells, can be markedly stimulated by phorbol ester in the presence of phosphatidylserine (PS) (Van Lint *et al.*, 1995). Moreover, an important finding is that PKD appears to possess a distinct substrate specificity which distinguishes it from members of the PKC family (Van Lint *et al.*, 1995).

It has been demonstrated very recently that the GRK phosphorylation sites in several receptors, including the β_2-adrenergic receptor and rhodopsin, all possess a pair

of acidic residues located on the amino-terminal side of the phosphorylation site (Fredericks *et al.*, 1996). This supports the conclusions reached originally from peptide studies that an acidic environment of a potential target residue is important for GRK2 activity (Onorato *et al.*, 1991). In this respect it is interesting that in the sequence of the rat glucagon receptor C-terminal tail, there is a pair of glutamate residues (Glu 427 and 428) on the amino-terminal side of a pair of serines (Ser 432 and 433). There are, in addition, residues in this region of the glucagon receptor which conform to the putative PKD phosphorylation site consensus sequence as defined by studies of peptide phosphorylation (Van Lint *et al.*, 1995). This will be discussed later in greater detail.

One of the studies described in this chapter involves an assay of the coupling of G_s to adenylate cyclase. This was achieved by determining the level of intracellular cAMP accumulation generated in response to cholera toxin treatment of glucagon receptor-transfected COS cells. Cholera toxin elicits activation of adenylate cyclase after a characteristic lag phase (Houslay and Elliott, 1979). During the latter it is considered (Houslay and Elliott, 1981; Orlandi *et al.*, 1993) to enter the cell by endocytosis and is subsequently processed, delivering its activated α sub-unit at the cytosol surface of the plasma membrane. This species then elicits the NAD^+-dependent ADP-ribosylation of α-G_s (Gilman, 1987; Birnbaumer *et al.*, 1990a). This action, by inactivating the GTPase of the sub-unit, leads to chronic activation of the entire G_s pool and the consequent constitutive activation of adenylate cyclase (Houslay and Elliott, 1979; Houslay and Elliott, 1981; Seamon *et al.*, 1981; Gilman, 1987; Birnbaumer *et al.*, 1990a). In addition, to gauge the effect of PMA on the activity of adenylate cyclase itself, cAMP accumulation was determined following stimulation by the diterpene, forskolin, which is known to activate the catalytic unit of adenylate cyclase directly (Gilman, 1987).

5.1.1. Aims and objectives

It was shown in the previous chapter that PMA-induced inhibition of the glucagon-stimulated cAMP accumulation was not conferred upon glucagon receptor-transfected COS cells by the co-transfection of the cDNAs encoding individual PKC isoforms that

were shown to be expressed in hepatocytes. Having identified other kinases that can be activated by phorbol ester, the subsequent objective was, therefore, to determine whether they are expressed in hepatocytes and, if so, to co-transfect cDNAs encoding them into COS cells. Subsequently, the expression of the transfected cDNAs was confirmed and assays performed in order to determine whether PMA-induced inhibition of the glucagon response had been conferred. Upon discovering that such an effect was, indeed, conferred by the co-transfection of one PMA-stimulated kinase, the objective was then to confirm that this was not due to an effect of co-transfection of this kinase upon total cellular phosphodiesterase activity, or upon the ability of IBMX to block this activity. An analysis was also undertaken to ascertain more precisely where the kinase acts in the glucagon receptor signalling system. This involved investigating its effect upon glucagon binding, receptor-G protein coupling, G_s-cyclase coupling and the catalytic unit of adenylate cyclase itself.

5.2. Results

5.2.1. Detection of PMA-sensitive kinases other than PKCs in hepatocytes

At the time of writing, the studies published have identified, other than PKC isoenzymes, only GRK2 and PKD as PMA-sensitive kinases. However, as GRK3 possesses, like GRK2, a PH domain in its membrane-targeting region (Musacchio *et al.*, 1993), and since it is this PH domain that is believed to confer PMA-enhanced activation upon GRK2 (Chuang *et al.*, 1995), it was reasoned that although it remains to be tested, the activity of GRK3, like that of GKR2, is likely to be responsive to PMA. Therefore, the expression of GRK2, GRK3 and PKD was examined in hepatocytes and COS cells.

5.2.1.1. Western blotting

A polyclonal antiserum (PA-1) raised against a 15-residue peptide contained within the C-terminal region of PKD (Valverde *et al.*, 1994) was used to perform an immunoblot on hepatocytes and COS cell extracts. While no signal was detectable in the COS cell extract using this antiserum, a weak signal was detected in the hepatocyte cytosol extract (results not shown). Unfortunately, no GRK-specific antisera were available. It was therefore decided to undertake RT-PCR analyses in order to ascertain whether PKD, GRK2 or GRK3 are expressed in rat hepatocytes and COS cells.

5.2.1.2. Design of oligonucleotide PCR primers

Using the nucleotide sequence deposited in Genbank for murine PKD (Valverde *et al.*, 1994) (accession number: Z34524), oligonucleotide PCR primers were designed as indicated below, to permit the amplification of a specific 565 bp product corresponding to nucleotides 2123-2687 of the open reading frame.

5' sense oligonucleotide ET-PKD-s1; TCGTTCACTGTGACCTCAAGC

3' antisense oligonucleotide ET-PKD-as1; CTAGCACTCAGACTGATCAGG.

Similarly, as no antisera were available specific for GRK2 or GRK3, PCR primers were designed which would specifically recognise each of these kinases. The objective was to prepare oligonucleotides which would be specific for an individual GRK yet recognise the designated kinase sequence in a species-independent fashion, detecting both rat and COS (primate) homologues in addition to the plasmid cDNA-encoded transcripts (bovine). To accomplish this, nucleotide sequences of rat, human, and bovine GRKs were obtained from Genbank, aligned using the GeneJockey II software package running on a Macintosh LC475 computer, and examined for regions of 100% identity. Where a suitable region of identity between species could not be found, a sequence with the maximum possible homology was chosen instead, and the respective oligonucleotide designed so as to recognise alternative forms of the sequence ie. with degeneracy in the appropriate nucleotide positions. By performing additional sequence alignments of differing GRKs, and by using the "modal search" facility of the GeneJockey II software package to screen a library of GRK sequences, the chosen primer sequences were also checked for inter-GRK specificity.

The sequences of the primers designed as above specific for GRK2 (βARK) were:

5' sense oligonucleotide ET-GRK2-s1; 5'-AAGCTGGAGAC(A/G)GAGGAGG

3' antisense oligonucleotide ET-GRK2-as1; 5'-TCGTCCAG(C/A)AGGATGTTGG. This allowed for the amplification of a specific 701 bp product corresponding to nucleotides 280-980 of the open reading frame of bovine GRK2 (Benovic *et al.*, 1989)(Genbank accession no. M34019).

Similarly, for GRK3 (βARK2) the primer pair designed was:

5' sense oligonucleotide ET-GRK3-s1; 5'-TTCAGAGGCATCGACTGG

3' antisense oligonucleotide ET-GRK3-as1; 5'-CATGAATGTCTCCGTCAGC

This allowed for the amplification of a specific 600 bp product corresponding to nucleotides 1357-1956 of the open reading frame of bovine GRK3 (Benovic *et al.*, 1991) (accession no. M73216).

In order to check the viability of the RNA and first strand cDNA preparations, RT-PCR was performed using primers specific for the ubiquitously expressed β-actin gene.

For this purpose the sense and antisense oligonucleotide primers designed were CATCGTCACCAACTGGGACGAC and CGTGGCCATCTCTTGCTCGAAG, respectively, allowing for the amplification of a specific 466 bp product corresponding to nucleotides 222-687 of the open reading frame of human β-actin (Ponte *et al.*, 1984) (accession no. M10278).

5.2.1.3. RT-PCR analysis of GRK2, GRK3, PKD and β-actin expression in hepatocytes and native COS cells

The GRK2, GRK3, PKD and β-actin primers, so designed, were used to perform RT-PCR on RNA prepared from rat hepatocytes and untransfected COS cells. Thus RNA was extracted as described in section 2.2.12. and 5 μg was used for first-strand cDNA synthesis as described in section 2.2.14. Thereafter, PCR was performed with the reaction conditions described in table 5.1.

These RT-PCR analyses showed that GRK2 was present in hepatocytes but not in COS cells (figure 5.1.A) and that GRK3 was not present in either cell type (figure 5.1.B). PKD transcripts were detected by RT-PCR in hepatocyte but not in COS cells (figure 5.1.C). A strong signal of the predicted size, 466 bp, was, however, detected by PCR from the first-strand cDNA prepared from both hepatocytes and COS cells using the β-actin primers (figure 5.1.D), thus providing confirmation that the RNA extraction and first strand cDNA synthesis had been correctly performed, and that nothing contained within the COS cell cDNA preparation was inhibiting the PCR reactions.

5.2.2. Co-transfection of COS-7 cells with plasmids encoding both the glucagon receptor and GRK2

On the basis of the rationale outlined in section 5.1.1., it was considered worthwhile to investigate the effect of co-expressing GRK2 with the GR in COS cells. Thus an expression plasmid, pBCβARK1, constructed by the insertion of a 3083 bp HinD III fragment containing the cDNA encoding bovine GRK2 into the multiple cloning site of pBC12BI (Benovic *et al.*, 1989), was co-transfected with that encoding the

glucagon receptor into COS-7 cells. Verification of transfection and transcription of the GRK2 cDNA was performed using the GRK2-specific primers in an RT-PCR reaction (figure 5.2.).

Co-transfection with this cDNA did not however confer upon these cells an ability of PMA to inhibit the glucagon-stimulated cAMP accumulation (figure 5.3.). In the presence of PMA the response to glucagon was 106 ± 5 % (at 5 min) and 108 ± 8 % (at 10 min) of that observed in the absence of the phorbol ester (mean ± SD, n=3). The changes were not statistically significant.

5.2.3. Co-transfection of COS-7 cells with plasmids encoding both the glucagon receptor and GRK3

To investigate whether GRK3 or a similar kinase might mediate an inhibitory effect of PMA, the construct pBCβARK2, made by blunt-end ligation of a 2725 bp EcoR1 fragment containing the βARK2 coding sequence into the blunted HinD III site of pBC12BI (Benovic *et al.*, 1991), was co-transfected with the vector encoding the glucagon receptor into COS-7 cells. Again, owing to the lack of an appropriate antiserum, RT-PCR was used to verify that the GRK was indeed expressed, at least at the level of transcription (figure 5.2.). The GRK3-specific 600 bp product was of relatively low intensity. A similar low efficiency PCR amplification was however also observed when the GRK3-specific primer pair was used to amplify from the GRK3 expression plasmid itself (results not shown), suggesting that the primer pair sequences or the PCR conditions were sub-optimal.

Importantly, co-transfecting the GRK3 cDNA failed to confer any significant PMA-induced inhibition of glucagon-stimulated cAMP accumulation (figure 5.4.).

5.2.4. Co-transfection of COS-7 cells with plasmids encoding both the glucagon receptor and PKD

The pcDNA3-PKD construct, formed by the insertion of a 3304 bp cDNA fragment containing the entire coding sequence of murine PKD into the mammalian expression

vector pcDNA3 (Van Lint *et al.*, 1995), was co-transfected with the cDNA encoding the rat hepatic glucagon receptor into COS-7 cells. A Western blot performed with the PKD-specific polyclonal antiserum described in section 5.2. strongly detected a protein of the appropriate molecular weight in PKD-transfected COS cells but not in the control COS cells, showing that the transfection led to the successful expression of PKD (figure 5.5.).

In such co-transfected cells, the level of glucagon-stimulated cAMP accumulation was found to be 10-20% (n=3) lower than that observed in cells which had been transfected with the glucagon receptor alone. However, when such co-transfected cells were challenged with PMA, then a profound further inhibition of glucagon-stimulated cAMP production was observed (table 5.2., fig. 5.6.). This effect appeared to be directed at the level of synthesis of cAMP as studies of homogenate cAMP PDE activity showed no change in total PDE activity (<5% change; n=3) and IBMX was able to inhibit a similar fraction of the total activity, namely >94% (n=3).

Co-transfection of COS cells with cDNAs encoding both a kinase-inactive form of PKD, PKDK618M (Zugaza *et al.*, 1996), and the glucagon receptor gave rise to no observable PMA-induced inhibition of the glucagon-stimulated cAMP accumulation (table 5.2).

5.2.5. Co-transfection of COS-7 cells with plasmids encoding PKD and either the β_2AR or the β_3AR

To investigate whether PMA-induced activation of transfected PKD could confer an inhibition of hormone-stimulated cAMP accumulation in COS cells transfected with GPCRs other than the glucagon receptor, cDNA encoding either the β_2-adrenoceptor (β_2AR) or the β_3-adrenoceptor (β_3AR) was transfected with that encoding PKD. Following stimulation for 10 min with isoprenaline, at a concentration of 1 μM or 100 μM, respectively, intracellular cAMP accumulation was determined as described in section 2.4.14. These incubations were carried out both in the presence and absence of 1 μM PMA. It was observed that in COS cells co-transfected with PKD and either the β_2AR or β_3AR, the isoprenaline-stimulated cAMP accumulation was reduced, in the

presence of PMA, by 31±7% and 18±4%, respectively. However, these inhibitions were found not to be statistically significant, as determined by Student's *t* tests. Similarly, no statistically significant inhibition was effected by PMA in COS cells which had been transfected with either the β_2AR or the β_3AR alone (<14% reduction from control values).

5.2.6. Investigation of the effects of PMA on the individual steps leading from glucagon binding to cAMP accumulation

5.2.6.1 The effect of PMA on glucagon binding

In order to determine whether, in COS cells co-transfected with the glucagon receptor and PKD, PMA might have an effect on the binding of glucagon to the transfected glucagon receptor, the binding of [^{125}I]-labelled glucagon to COS cell membranes was determined. This was assessed as described in the Materials and Methods chapter (see section 2.4.7.).

Levels of specific binding of [^{125}I]-glucagon to COS cell membranes were <1 fmol/mg in non-transfected COS cells and 105 ± 17 fmol/mg in glucagon receptor transfected cells (mean ± SE; n=3 separate determinations). In cells which had been co-transfected with PKD, specific glucagon binding was 114 ± 8 fmol/mg. Challenge of cells with PMA did not affect this with specific binding of 131 ± 10 fmol/mg seen for GR and PKD co-transfected cells (means ± SE; n=3 separate experiments). These results demonstrate that PMA treatment of neither the GR-transfected nor the GR/PKD co-transfected COS-7 cells results in a reduction of specific glucagon binding. This suggests that PMA treatment did not elicit its inhibitory effect in the co-transfected cells by simply attenuating the specific binding of glucagon to its receptor.

5.2.6.2. The effect of PMA on G_s-cyclase coupling and on adenylate cyclase function

In order to evaluate the possibility that PMA exerts effects on individual coupling steps of the pathway that may not be detected by measuring solely the glucagon-

stimulated cAMP accumulation, the effect of PMA on the cAMP response of COS cells to agents which activate G_s or adenylate cyclase directly, was determined. G_s-adenylate cyclase coupling was assessed by the use of cholera toxin, which elicits activation of adenylate cyclase by activating G_s, as described in section 5.1. To investigate whether PMA might effect an alteration in the functioning of adenylate cyclase, the effect of PMA was determined on intracellular cAMP accumulation in unstimulated COS cells and in COS cells treated with the diterpene, forskolin, which is known to directly activate the adenylate cyclase catalytic unit (Gilman, 1987). It was found that transfection of COS cells with PKD did not cause PMA to inhibit the accumulation of cAMP that was elicited by either cholera toxin or forskolin (table 5.3.).

5.2.6.3. The effect of PMA on the GTP-induced reduction in receptor affinity for glucagon

It has been shown (Rodbell *et al.*, 1971c; Limbird *et al.*, 1980; Rodbell, 1980) that the binding of GTP by G_s leads the associated receptor to adopt a low affinity state. Thus addition of GTP to binding assays will cause a reduced specific binding of receptor ligand. This has been shown for the glucagon receptor (Rodbell *et al.*, 1971c; Rodbell *et al.*, 1974; Rojas and Birnbaumer, 1985). As such it can be used as an index of receptor-G_s coupling.

Intriguingly, however, it was found that the ability of GTP to induce glucagon dissociation from membranes isolated from GR/PKD co-transfected COS cells was not reduced by PMA treatment (table 5.4.). This implies that PMA does not inhibit glucagon-stimulated cAMP accumulation by simply attenuating receptor-G_s coupling. Indeed it was also found that PMA does not affect GR-G_s coupling in native hepatocytes, demonstrating the similarity of these systems (table 5.4.).

5.2.7. RT-PCR analysis of PKD expression in CHO cells

A study on the phosphorylation of the human glucagon receptor stably expressed in Chinese hamster ovary (CHO) cells has been published very recently (Heurich *et al.*,

1996). The authors report that phosphorylation of the receptor was observed in response to glucagon, but, interestingly, not in response to phorbol ester. To investigate whether PKD is expressed in CHO cells, RT-PCR using the PKD-specific primers described in section 5.2.1.2. was performed on RNA extracted from these cells and from hepatocytes (figure 5.7.). While a band of strong intensity, migrating with a mobility corresponding to that predicted for the PKD-specific product, was detected in the PCR reaction using hepatocyte-derived cDNA, only a very weak signal resulted from the PCR in which the template consisted of cDNA derived from CHO cell RNA (figure 5.7.).

5.3. Discussion

The RT-PCR analyses conducted in this study showed that GRK2 was present in hepatocytes but not in COS cells and that GRK3 was not present in either cell type. The detection of the expression of GRK2, but not that of GRK3, in hepatocytes is interesting as Northern blotting has been reported to detect neither GRK2 (Benovic *et al.*, 1989) nor GRK3 (Benovic *et al.*, 1991) in bovine liver. It was also noted by these authors that the level of abundance of GRK3 mRNA was only about 10-20% of that of GRK2 in the tissues in which both transcripts were detectable by this technique (Benovic *et al.*, 1991). The detection of GRK2 in hepatocytes in the present study, therefore, may reflect both the greater sensitivity of RT-PCR and the use of isolated hepatocytes rather than liver tissue which contains a heterogeneous population of cells.

That expression of the novel protein kinase, PKD, was detected in hepatocytes (see section 5.2.1.) only by the use of the sensitive technique of RT-PCR, and not by Western blotting, suggests that the expression level of this kinase in hepatocytes is low. This conclusion is consistent with the findings of Valverde et al. (1994) who reported the detection by Northern blotting of PKD expression in liver, but at a much lower level than that detected in brain, lung, testis, heart muscle and kidney (Valverde *et al.*, 1994). In human liver samples, however, Northern blotting detected levels of mRNA of PKC-μ, the homologue of PKD, at levels which were similar to those obtained with several other tissues (Johannes *et al.*, 1994). These authors also reported that expression of this PKD homologue in control COS cells was not detected by Northern blotting or by immunoprecipitation followed by Western blotting (Johannes *et al.*, 1994). These results, therefore concur with the inability in the present study to detect PKD transcripts by RT-PCR in COS cell RNA.

Co-transfection of the glucagon receptor cDNA with cDNAs encoding each of the two G protein-coupled receptor kinases, GRK2 and GRK3, did not confer any PMA-induced inhibitory action on glucagon-stimulated cAMP accumulation in COS cells. This was unlikely to have been simply due to a failure of transfection as the appropriate

transcripts for these kinases were detected in transfected COS cells by RT-PCR, although the GRK2 signal was somewhat more intense than that of GRK3. It is conceivable, however, that another untested GRK mediates the inhibitory effect of PMA on glucagon-stimulated cAMP accumulation in hepatocytes. In this respect, it is interesting to note that GRK6, which has a similar tissue distribution to that of GRK2, appears to have a distinct substrate specificity from that of GRK1, GRK2 and GRK5 (Benovic and Gomez, 1993; Loudon and Benovic, 1994). There is, however, no evidence yet that the activity of GRK6 is increased by phorbol esters.

Co-transfection of COS cells with cDNAs encoding both the glucagon receptor and PKD was shown by immunoblotting analyses to have led successfully to the expression of PKD. In such co-transfected cells, the reduction in the level of glucagon-stimulated cAMP accumulation by 10-20% from that observed in cells which had been transfected with the glucagon receptor alone, was not specific to PKD co-transfection, as a similar reduction in glucagon-stimulated cAMP accumulation was observed following co-transfection with a control expression plasmid encoding chloramphenicol acetyl transferase. Such an effect, reflecting the increased demands placed upon the cell's finite transcriptional and translational capacity by the introduction of additional cDNA into these cells, is consistent with successful co-transfection.

When such GR and PKD cDNA co-transfected cells were challenged with PMA, a profound inhibition of glucagon-stimulated cAMP production was observed. This result contrasted markedly with the previous experiments performed on COS cells transfected either with the GR alone or with the GR together with PKC isoforms or GRK family members. Moreover, the effect appeared to be directed at the level of synthesis of cAMP rather than at its degradation as studies of homogenate cAMP PDE activity showed that total PDE activity was unchanged and that IBMX was able to inhibit a similar fraction of the total activity.

COS cells which were transfected with cDNA encoding a kinase-inactive mutant form of PKD, PKDK618M (Zugaza *et al.*, 1996), together with a plasmid encoding the GR, failed to exhibit an observable PMA-induced inhibition of glucagon-stimulated

cAMP accumulation (table 5.2). This suggests that the profound inhibitory effect of PMA observed in COS cells co-transfected with cDNAs encoding the GR and wild-type PKD is dependent upon the catalytic activity of the kinase.

In order to elucidate the precise mechanism of PMA-induced PKD-mediated inhibition of glucagon-stimulated intracellular cAMP accumulation, a variety of experimental approaches were employed. Measurement of levels of specific binding of [^{125}I]-glucagon to COS cell membranes indicated that in neither GR-transfected COS cells nor cells co-transfected with PKD did PMA treatment elicit its inhibitory effect by attenuating the specific binding of glucagon to its receptor. Such a situation is consistent with that seen for the glucagon receptor in hepatocytes (Houslay, 1994). Cholera toxin-stimulated cAMP accumulation was not inhibited in PKD-transfected COS cells by PMA treatment, indicating that phorbol ester-activated PKD does not prevent activated $G_s\alpha$ itself from stimulating adenylate cyclase. Such a conclusion is supported by the observation from studies on hepatocyte membranes that PMA treatment does not attenuate the NaAlF-stimulated adenylate cyclase response (Houslay, 1994). Furthermore, in PKD-transfected COS cells, forskolin-stimulated cAMP accumulation was not attenuated upon phorbol ester treatment, suggesting that PKD does not affect the functioning of the catalytic unit of adenylate cyclase. Intriguingly, while it might have been predicted from these observations that PMA-induced activation of PKD would interfere with the coupling between the glucagon receptor and G_s, the experiments which tested the ability of GTP to reduce the specific binding of glucagon by the receptor, did not support such a conclusion. In fact it would appear that the activation of PKD, rather than simply attenuating receptor-G_s coupling, may reduce glucagon-stimulated cAMP accumulation by an action which reduces the ability of the receptor-G_s complex to activate the catalytic unit of adenylate cyclase. Nevertheless, the modification appears to permit the activation of adenylate cyclase by G_s activated by either cholera toxin or NaAlF. Such an effect could, conceivably, be mediated by phosphorylation of the receptor, $G_s\alpha$ or adenylate cyclase by PKD. The identity of the precise target of PKD remains to be elucidated. Certainly, from these studies, the receptor itself would appear to be a possible target.

Van Lint et al. demonstrated, by peptide phosphorylation studies, that an arginine at position -3 relative to that of serine is an important requirement for phosphorylation by PKD (Van Lint *et al.*, 1995). Such a motif, RxxS, is indeed found in the cytosolic, C-terminal tail of the glucagon receptor ($R^{429}MAS^{432}$). Moreover, the PKD target is unlikely to be G_s, as the G protein can be immunoprecipitated from desensitised hepatocytes in a non-phosphorylated form (Pyne *et al.*, 1989; Tang and Houslay, 1992).

In the current studies no statistically significant PMA-induced reduction in the isoprenaline-stimulated cAMP accumulation was detectable in COS cells transfected with PKD and either the β_2AR or the β_3AR (table 5.2). This is consistent with the possibility that, in the co-transfected COS cells in which the inhibitory effect is observed, the target of PKD is the glucagon receptor itself. Interestingly, the RxxS motif, which is believed to constitute a requirement for phosphorylation by PKD (Van Lint *et al.*, 1995), is absent from the primary sequence of the β_3AR, but is found in the C-terminal tail of the GR ($R^{429}MAS^{432}$) and in both the third intracellular loop ($R^{259}RSS^{262}$) and C-terminal tail ($R^{343}RSS^{346}$) of the β_2AR. The apparent inability of PMA to effect a statistically significant inhibition of isoprenaline-stimulated cAMP accumulation in COS cells transfected with PKD and the β_2AR thus suggests that the presence of the RxxS motif in a prospective substrate may be necessary but insufficient to permit its PKD-mediated phosphorylation.

Very recently, Heurich et al., studying the phosphorylation of the human glucagon receptor expressed stably in CHO cells, detected phosphorylation of the receptor in response to treatment of the cells with glucagon but not phorbol ester (Heurich *et al.*, 1996). The finding here that the especially sensitive technique of RT-PCR yielded only a very weak PKD signal in CHO RNA (figure 5.7.) suggests, however, that the reported absence of PMA-induced glucagon receptor phosphorylation in CHO cells may be a consequence of a very low PKD expression level. Alternatively, it is possible that even if PKD is expressed at a sufficiently high level, the PKC isoforms required for PKD activation may not be. Sequence variation between the rat and human homologues of the glucagon receptor does not, in itself, appear to be sufficient to explain the lack of PMA-

induced glucagon receptor phosphorylation observed by Heurich et al. since the human receptor contains two distinct serine residues, at positions 431 and 437, respectively, which conform to the RxxS consensus.

Finally, it may be that the substrate of PKD is adenylate cyclase rather than the receptor itself. As there are at least nine isoforms of adenylate cyclase with differing tissue distribution and mechanisms of regulation, it is quite conceivable that an adenylate cyclase isoform is modified by PKD in COS cells but is not expressed in CHO cells. In fact, as can be seen from table 5.5, primary sequence inspection reveals that each of the nine isoforms of adenylate cyclase possesses at least one intracellularly-located RxxS motif which might serve as a target for phosphorylation by PKD. It is possible, however, that the presence of RxxS in a potential substrate is necessary but not sufficient to permit efficient phosphorylation by PKD, although the specific additional sequence requirements remain to be determined.

5.3.1. The possible role of PKD in hepatocytes

It is possible that, by virtue of its property of DAG-induced activation, PKD may play an important role in hepatocytes, acting to desensitize the glucagon receptor in response to hormonal activation of receptors which lead to the production of DAG. Thus it may mediate part or all of the observed desensitization of the glucagon-induced cAMP accumulation that follows treatment of hepatocytes with vasopressin, angiotensin II and glucagon itself. In this respect, it is interesting to note that Savage et al. (Savage *et al.*, 1995) demonstrated that the vasopressin-induced desensitization was completely abolished by the relatively selective PKC inhibitor chelerythrine (Herbert *et al.*, 1990) at a concentration of 1 μM whereas that induced by glucagon was only half blocked, even at a concentration of 5 μM (Savage *et al.*, 1995). Similar differential effects were observed when other PKC inhibitors were used. This might reflect the possible involvement of different PKC isoforms in the two desensitization processes, conceivably activated by different DAG species.

An alternative explanation, however, would be that the desensitization induced by glucagon itself is not mediated by PKC, but by a distinct kinase, such as PKD or even a receptor-specific kinase. In fact, PKD might also mediate the vasopressin-induced desensitization. In this scenario, the sensitivity of this process to PKC inhibitors could be accounted for by the finding that activation of PKD can be mediated not only by direct activation by phorbol ester or DAG, but also through a PKC-mediated pathway (Zugaza *et al.*, 1996).

Glucagon-induced glucagon desensitization is an example of a common feature of hormone responsive signalling, in which desensitization allows cells to attenuate their response to the continuing presence of an agonist or to reduce the effect of a subsequent agonist challenge. Desensitization of the glucagon response as a consequence of lipid signalling stimulated by other hormones, however, may also play an important physiological role. Vasopressin, for example, is already known to potentiate insulin secretion (Widmann *et al.*, 1996). Its ability to induce the desensitization of glucagon-stimulated cAMP accumulation in hepatocytes (Savage *et al.*, 1995) may therefore serve as an additional glucose-lowering mechanism by this hormone.

PKD activation in response to glucagon exposure, could, in theory, occur through a number of different mechanisms. For example, activation of PKD may be elicited by DAG generated following activation of either PC-PLC or PI-PLC (see section 1.4.1.4.1.). Alternatively DAG, produced in this fashion, may activate PKC isoforms which could then, subsequently, activate PKD (Zugaza *et al.*, 1996). A further possibility which has not been tested, is that G protein $\beta\gamma$ sub-units, released from the α sub-unit following activation of G_s by the ligand-occupied receptor, might then effect the translocation of PKD to the plasma membrane through an interaction with the pleckstrin homology domain of the kinase. In support of the latter hypothesis is the observation that G protein $\beta\gamma$ sub-units cause the membrane translocation of GRK2 by a mechanism that is believed to involve an interaction of $\beta\gamma$ with the C-terminal region of the kinase's PH domain (Pitcher *et al.*, 1995b).

	GRK2	GRK3	PKD	β-actin
Denaturation	95° 1 min	95° 1 min	95° 1 min	95° 1 min
Annealing	54.7° 2 min	52.6° 2 min	52.5° 2 min	52.5° 2 min
Extension	72° 3 min	72° 3 min	72° 3 min	72° 3 min
No. of cycles	35 cycles	35 cycles	35 cycles	35 cycles
Predicted product	701 bp	600 bp	565 bp	466 bp

Table 5.1. PCR conditions for amplification of GRK2, GRK3, PKD and β-actin

The annealing temperatures were chosen so as to be approximately 5 degrees below the true melting temperatures of the primers, which was determined by the software package Gene Jockey II.

Transfection	Glucagon-stimulated cAMP production in presence of PMA (expressed as % of control response, observed without PMA, in each transfection)
GR	101 ±6 ns (no PMA=100)
GR+GRK2	108 ±8 ns
GR+GRK3	115 ±2 ns
GR+PKD	49 ±1 **
GR+PKDK618M	99 ±4 ns
β_2AR+PKD	69 ±7 ns
β_3AR+PKD	82 ±4 ns

Table 5.2. The effect of PMA on the hormone-stimulated cAMP response in COS cells co-transfected with cDNA encoding either the GR or a βAR in addition to GRK2, GRK3 or PKD

Transfected COS cells, grown to confluence in 6-well plates as described in the Materials and Methods chapter, were pre-incubated for 15 min at 37 °C with serum-free DMEM containing 1 mM IBMX, and either 0.1% DMSO (control) or 1 μM PMA. Where the GR was transfected, glucagon was then added to a final concentration of 10 nM and the incubation was continued for another 10 minutes. Where the β_2AR or β_3AR was transfected, however, isoprenaline was added instead of glucagon to a final concentration of 1 μM or 100 μM, respectively. 2% PCA was subsequently added, the cells were harvested and their cAMP content determined. The results shown represent means ± SE of three experiments. The glucagon or isoprenaline-stimulated cAMP production in the presence of PMA is expressed as a percentage of the control (without PMA). The absolute value, in the absence of PMA, for the cAMP accumulation in the control, GR-transfected, cells following glucagon stimulation for 10 minutes was 921 ± 24 (mean ± SE) pmol cAMP per million cells. ** Significant at $P<0.01$, ^{ns} not significant (Student's *t* test).

	Control (fold change over basal)	PMA-treated (fold change over basal)
control COS		
basal	(1)	(1)
cholera toxin	48 ±6	53 ±5
forskolin	15 ± 3	17 ± 1
PKD-transfected COS		
basal	(1)	(1)
cholera toxin	48 ± 5	56 ± 4
forskolin	14 ± 3	14 ± 3

Table 5.3. The effect of PMA treatment on the forskolin and cholera toxin-induced cAMP response in control and PKD-transfected COS cells

Control or PKD-transfected COS cells, grown to confluence in 6-well plates as described in the Materials and Methods chapter, were pre-incubated for 15 min at 37 °C with serum-free DMEM containing 1 mM IBMX, and either 0.1% DMSO (control) or 1 μM PMA. Cholera toxin or forskolin, where indicated, were then added to final concentrations of 1 μg/ml and 10 μM, and the incubations were continued for 1 hour or 20 min, respectively. 2% PCA was then added, the cells were harvested, and their cAMP content determined. The intracellular cAMP levels under basal conditions for PKD-transfected cells were 70 ± 12 pmol/10^6 cells with control medium and 69 ±18 pmol/10^6 cells in the presence of PMA. For untransfected cells the cAMP levels were 85 ±16 pmol/10^6 cells and 81 ±12 pmol/10^6 cells, respectively. The results shown in the table represent means ± SD of three experiments, expressed as fold stimulation over basal values.

	Control		PMA-treated	
GTP (μM):	0	10	0	10
GR/PKD COS	114 ± 8	26 ± 12	131 ± 10	17 ± 12
hepatocytes	41 ± 6	6 ± 2	45 ± 12	5 ± 2

Table 5.4. The effect of PMA treatment on the GTP-mediated inhibition of specific ^{125}-I glucagon binding (fmol/mg) to membranes isolated from GR/PKD co-transfected COS cells and from hepatocytes.

Hepatocytes, or COS cells co-transfected with cDNA encoding the GR and PKD, were treated for 15 min with 1 μM PMA or control (0.1% DMSO) at 37 °C. Hepatocytes were then pelleted by centrifugation for 2 min at 1000g at 4°C. Homogenisation, membrane preparation and [^{125}I]-glucagon binding were then performed as described in the Materials and Methods chapter. 10 μg hepatocyte or COS cell membrane proteins were added per binding. Results, expressed in fmol/mg, representing specific binding following the subtraction of non-specific binding, are given as means ± SE of three experiments.

AC	Species	Acc. No.	Sequence	Position	Intracellular ?
I	bovine	M25579	RTAS	542	yes
			RNRS	549	yes
			RVSS	748	yes
			RMDS	1011	yes
			RRGS	1032	yes
II	rat	M80550	RVFS	106	no
			RLAS	300	yes
			RTKS	540	yes
			RMDS	1029	yes
			RSLS	1081	yes
III	rat	M55075	RMES	1073	yes
IV	rat	M80633	RLFS	3	yes
			RPLS	90	no
			RPES	163	no
			RLAS	284	yes
			RSCS	940	yes
			RMES	1005	yes
			RTGS	1057	yes
V	rat	M96159	RSAS	80	yes
			RLHS	175	no
			RTNS	420	yes
			RLRS	488	yes
			RMDS	965	yes
VI	rat	L01115	RSGS	7	yes
			RYMS	55	yes
			RQDS	216	no
			RSPS	245	no
			RANS	585	yes
			RAFS	600	yes
			RVHS	751	no
			RMDS	1128	yes
VII	murine	U12919	RLTS	26	yes
			RLAS	292	yes
			RSAS	534	yes
			RMES	1040	yes
VIII	rat	L26986	RKAS	63	yes
			RSGS	109	yes
			RRKS	175	yes
			RRNS	608	yes
			RLNS	849	yes
			RMDS	1117	yes
IX	murine	Z50190	RASS	85	yes
			RVDS	201	no
			RSRS	305	yes
			RESS	598	yes
			RPAS	973	no
			RVLS	1214	yes

Table 5.5. RxxS consensus searches in primary sequences of adenylate cyclase isoforms

The primary sequence of each adenylate cyclase isoform was obtained, in Genbank format, from the server of the National Centre for Biotechnology Information, using the accession number shown. After converting each file to a format which could be recognised by the Apple Macintosh GeneJockey II software package, the occurrences in the sequence of the amino acid motif RxxS were identified using the Consensus Search facility of the program. The presence of the RxxS sequence in a potential substrate protein appears to be a pre-requisite for its phosphorylation by PKD (Van Lint *et al.*, 1995).

The sequences of all occurrences of RxxS in each isoform are shown, together with their locations in the protein sequence. Whether or not these residues are located intracellularly was predicted by ascertaining their position with respect to the predicted transmembrane regions of the isoform concerned.

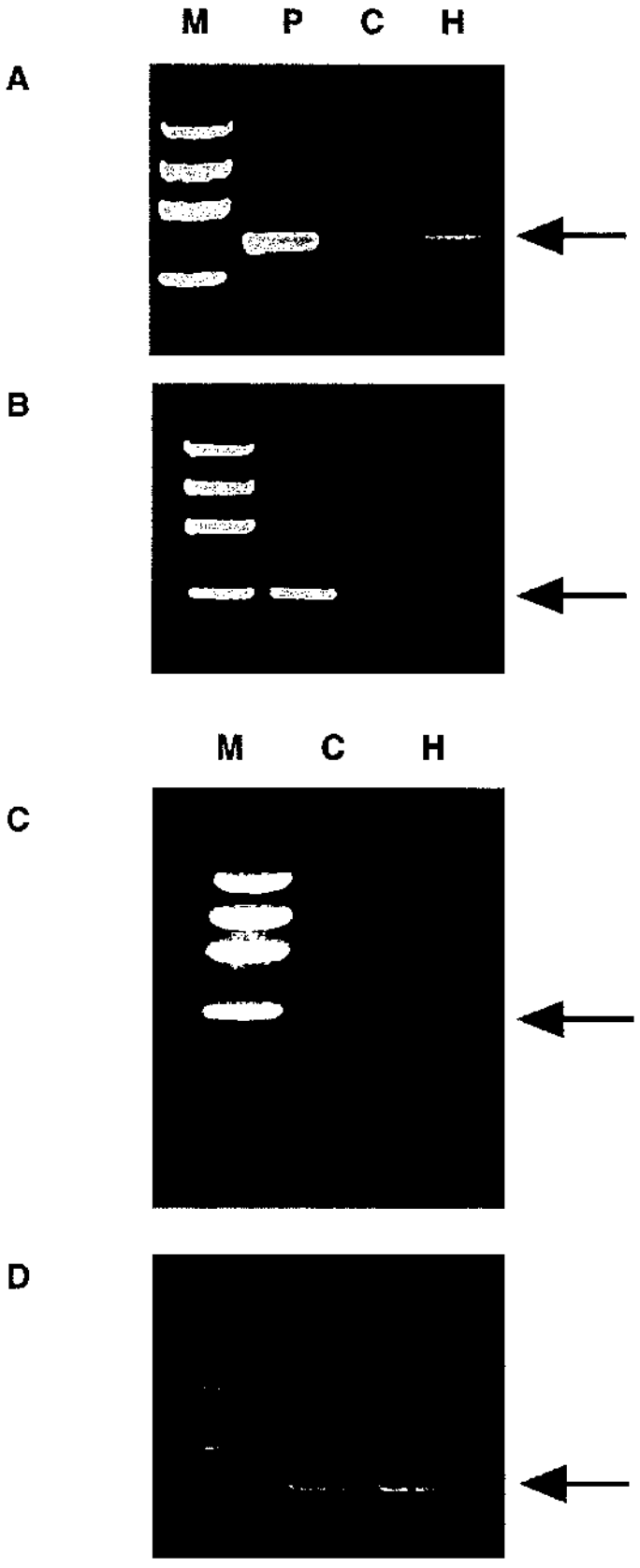

M
P
C
H
A
B
M
C
H
C
D

Figure 5.1. RT-PCR detection of GRK2, GRK3, PKD and β-actin in hepatocyte and untransfected COS cell RNA

RNA was prepared from washed hepatocytes or COS cells using Tri Reagent™ as described in the Materials and Methods chapter. First strand cDNA synthesis was carried out using identical quantities of RNA, and was checked by PCR using oligonucleotide primers specific for β-actin. The GRK 2 (panel A), GRK 3 (panel B), PKD (panel C) and β-actin (panel D) primers were designed to amplify 701 bp, 600 bp, 565 bp and 466 bp DNA sequences, respectively. PCR reactions were carried out as described in the Materials and Methods chapter using a template consisting of the cDNA derived from COS cells (lanes C), hepatocytes (lanes H) or plasmid control DNA containing the appropriate cDNA (lanes P). Promega ϕx174/HaeIII DNA markers were run in lanes (M) alongside the PCR products on a 2% agarose gel.

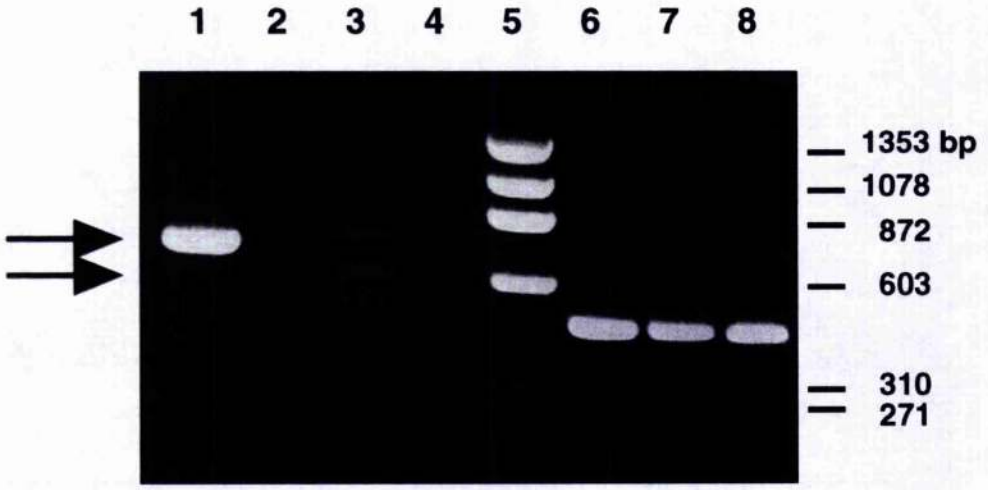

1
2
3
4
5
6
7
8
1353 bp
1078
872
603
310
271

Figure 5.2. RT-PCR detection of GRK2, GRK3 and β-actin in transfected and untransfected COS cell RNA

RNA was prepared from washed transfected or native COS cells using Tri Reagent™ as described in the Materials and Methods chapter. First strand cDNA synthesis was carried out using identical quantities of RNA, and was checked by PCR using oligonucleotide primers specific for β-actin. The GRK 2 (lanes 1 and 2), GRK 3 (lanes 3 and 4) and β-actin (lanes 6, 7 and 8) primers were designed to amplify 701 bp, 600 bp and 466 bp DNA sequences, respectively. PCR reactions were carried out as described in the Materials and Methods chapter using a template consisting of the cDNA derived from GRK2-transfected COS cells (lanes 1 and 6), GRK3-transfected COS cells (lanes 3 and 7) or untransfected control COS cells (lanes 2, 4, and 8). Promega ϕx174/HaeIII DNA markers were run in lane 5 alongside the PCR products on a 2% agarose gel.

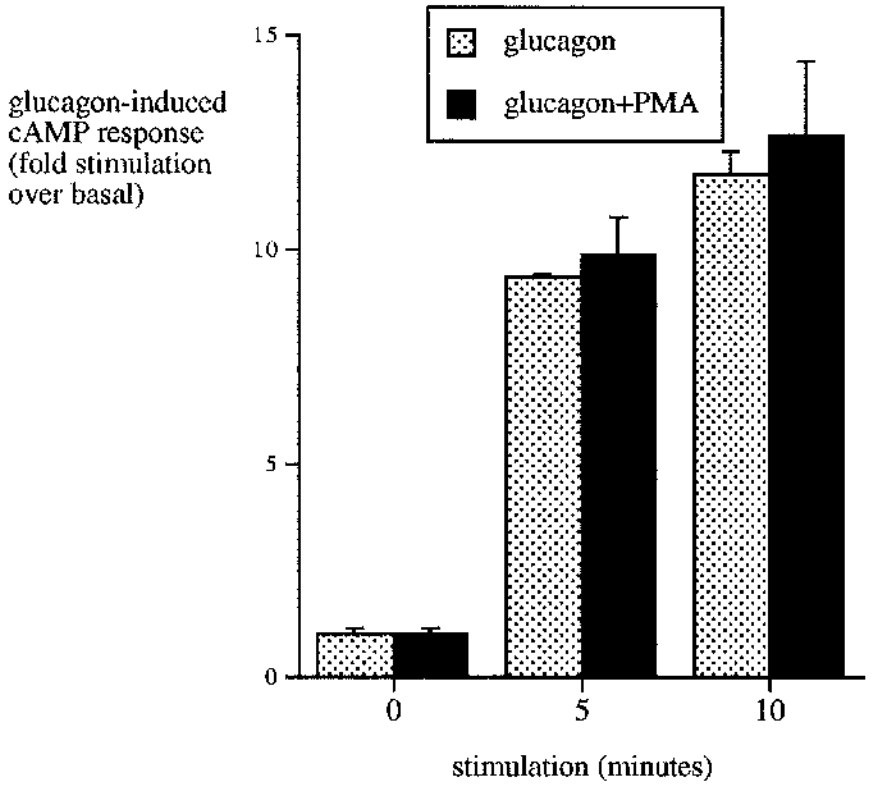

glucagon-induced cAMP response (fold stimulation over basal)
15
10
5
0
glucagon
glucagon+PMA
0
5
10
stimulation (minutes)

Figure 5.3. The effect of PMA on the glucagon-stimulated cAMP response in GRK2 and glucagon receptor co-transfected COS cells.

COS cells co-transfected with cDNA encoding both the glucagon receptor and GRK2 were grown to confluence in 6-well plates as described in the Materials and Methods chapter. The cells were then pre-incubated for 15 min at 37°C with serum-free DMEM containing 1 mM IBMX, and either 0.1% DMSO (control) or 1 μM PMA. Glucagon was then added to a final concentration of 10 nM and the incubation was continued for another 10 minutes. 2% PCA was subsequently added, the cells were harvested and their cAMP content determined. The results shown represent means ± SD of three incubations.

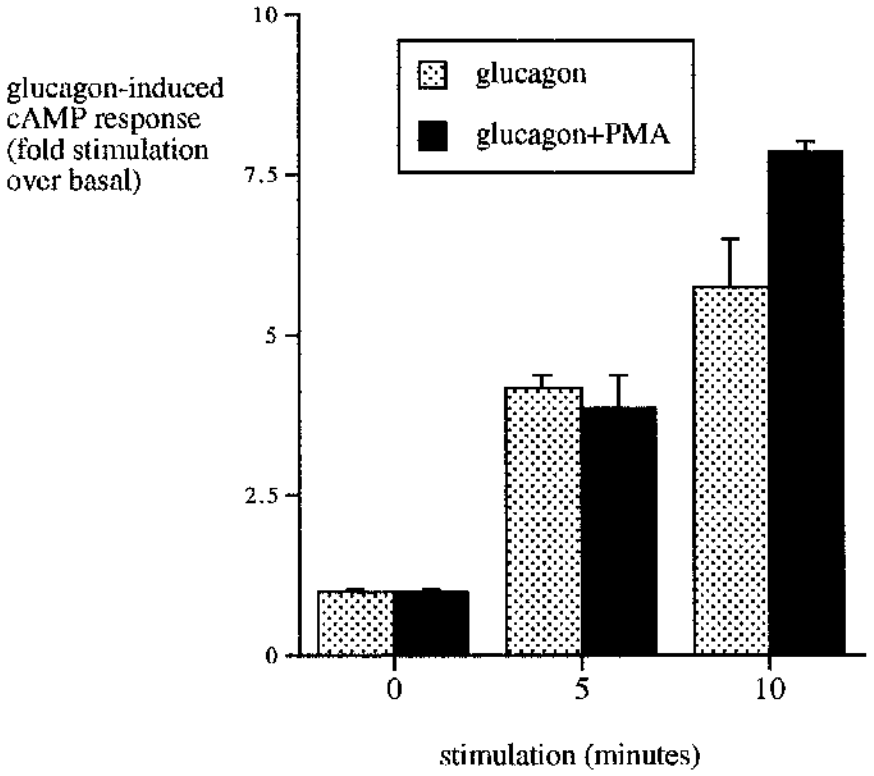

glucagon-induced cAMP response (fold stimulation over basal)
10
7.5
5
2.5
0
glucagon
glucagon+PMA
0
5
10
stimulation (minutes)

Figure 5.4. The effect of PMA on the glucagon-stimulated cAMP response in GRK3 and glucagon receptor co-transfected COS cells.

COS cells co-transfected with cDNAs encoding both the glucagon receptor and GRK3 were grown to confluence in 6-well plates as described in the Materials and Methods chapter. The cells were then pre-incubated for 15 min at 37°C with serum-free DMEM containing 1 mM IBMX, and either 0.1% DMSO (control) or 1 μM PMA. Glucagon was then added to a final concentration of 10 nM and the incubation was continued for another 10 minutes. 2% PCA was subsequently added, the cells were harvested and their cAMP content determined. The results shown represent means ± SD of three incubations.

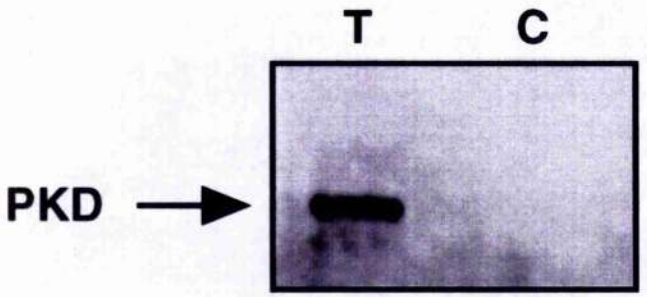

T
C
PKD

Figure 5.5. Immunoblot confirming the expression of transfected cDNA encoding PKD in transfected COS cells.

Transfected or control COS cells were washed 4 times with PBS, scraped into 0.5 ml Laemmli buffer per confluent 75 cm^2 flask, and immediately boiled for 3 min. 30 µl of each sample was subsequently loaded onto an 8% SDS poly-acrylamide gel. Blotting was carried out as described in the Materials and Methods chapter with a primary antibody dilution of 1 in 500.

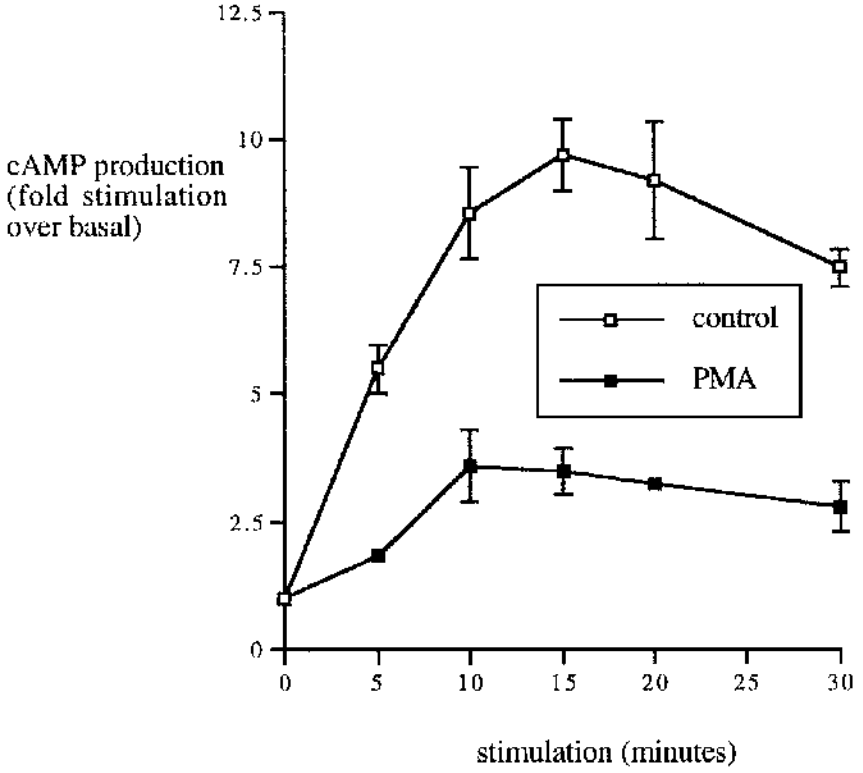

cAMP production
(fold stimulation
over basal)
12.5
10
7.5
5
2.5
0
0
5
10
15
20
25
30
control
PMA
stimulation (minutes)

Figure 5.6. Timecourses for glucagon-stimulated cAMP production in the presence and absence of PMA in COS cells transfected with cDNAs encoding both the GR and PKD.

Following a pre-incubation for 15 minutes in the presence (filled symbols) or absence (open symbols) of the phorbol ester, PMA, at a concentration of 1 μM, glucagon was added to a final concentration of 10 nM. Incubations were terminated after the times indicated by the addition of 2% PCA. IBMX was present throughout at a concentration of 1 mM. The values shown are expressed as fold stimulation over basal values and represent means (± SD) from three incubations.

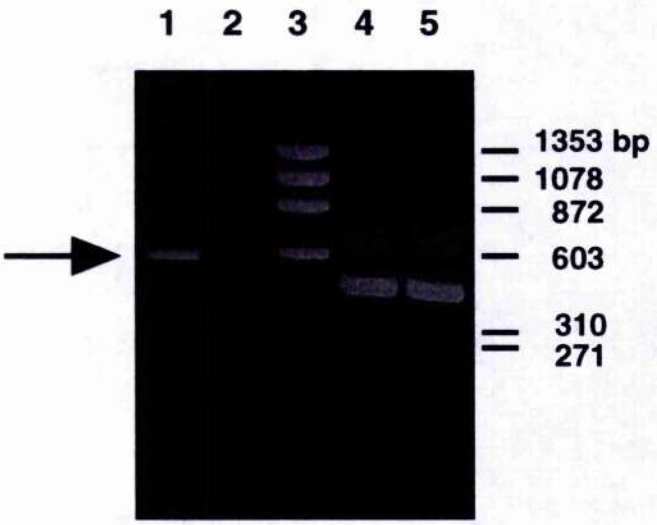

1 2 3 4 5
1353 bp
1078
872
603
310
271

Figure 5.7. RT-PCR detection of PKD and β-actin transcripts in hepatocyte and CHO cell RNA

RNA was prepared from washed hepatocytes or CHO cells using Tri Reagent™ as described in the Materials and Methods chapter. First strand cDNA synthesis was carried out using identical quantities of RNA, and was checked by PCR using oligonucleotide primers specific for β-actin. The PKD (lanes 1 and 2) and β-actin (lanes 4 and 5) primers were designed to amplify 565 bp and 466 bp DNA sequences, respectively. PCR reactions were carried out as described in the Materials and Methods chapter using a template consisting of the cDNA derived from hepatocytes (lanes 1 and 4) or from CHO cells (lanes 2 and 5). Promega ϕx174/HaeIII DNA markers were resolved in lane 3 alongside the PCR products on a 2% agarose gel.

CHAPTER 6

Relationship between the induction of diabetes and the expression levels of PMA-activated kinases in rats

6.1. Introduction

6.1.1. The use of streptozotocin to induce diabetes

The diabetic state can be induced in animals by the injection of streptozotocin (Gawler *et al.*, 1987). Streptozotocin is a toxin which causes necrosis of the pancreatic β cells with consequent loss of insulin secretion, resulting in hypoinsulinaemia, hyperglycaemia and insulin resistance in liver and adipose tissue (Houslay, 1990). This diabetic state, therefore, resembles that of human type I, insulin-dependent diabetes mellitus (IDDM). Insulin resistance is a recognised feature of this condition, although it is more typically associated with type II, non-insulin-dependent diabetes mellitus (NIDDM), obesity and hypertension (Donnelly and Connell, 1992).

The regulation of adenylate cyclase is believed to be defective in a number of animal models of diabetes and obesity, and in humans. Such reported defects include reduced levels of expression of G_i-2 in the liver of streptozotocin or alloxan-treated rats, and the loss of the "tonic" inhibitory input that is normally supplied by this G protein (Gawler *et al.*, 1987; Murphy *et al.*, 1989). These defects have also been found in the adipocytes of genetically diabetic (db/db) mice (Begin-Heick, 1992), obese (fa/fa) Zucker rats (Strassheim *et al.*, 1991) and in human platelets (Livingstone *et al.*, 1991). The reduced expression is likely to result from a decrease in the levels of the mRNA which encodes such G proteins, in particular α-G_i-2 and α-G_i-3, whose mRNA has been shown to be less abundant in hepatocytes derived from streptozotocin-treated than from control rats (Bushfield *et al.*, 1990a; Griffiths *et al.*, 1990). In addition, loss of functional G_i and impaired Gs function has been observed in liver plasma membranes of obese (fa/fa) but not lean (Fa/Fa) Zucker rats (Houslay *et al.*, 1989).

Thus, in the diabetic state it is conceivable that, due to G protein abnormalities, the ability of glucagon to stimulate the activity of adenylate cyclase may be altered, perhaps contributing to the hyperglycaemia associated with the condition. In fact, it has been observed that the degree of glucagon-induced stimulation of adenylate cyclase activity is

increased following the induction of diabetes by streptozotocin (Gawler *et al.*, 1987). Moreover, the regulation of glucagon-induced adenylate cyclase stimulation is of particular importance given that in diabetes, the level of circulating glucagon is known to be elevated (Baron *et al.*, 1987), and the hepatic cAMP content is increased (Pilkis *et al.*, 1974).

6.1.2. Changes in expression and activity of PKC isoforms in diabetes

In diabetes, a number of important changes have been demonstrated in the activity, detectable levels and intracellular distribution of PKC isoforms. Induction of streptozotocin diabetes in rats is associated with the phosphorylation of G_i-2, with the consequent inactivation of this G protein subunit (Bushfield *et al.*, 1990a). It is believed that this covalent modification, which has been shown to occur at the protein kinase C phosphorylation site (Morris *et al.*, 1996), rather than the reduced expression level of the G protein, is the principal change underlying the observed aberrant G_i functioning in these animals (Houslay, 1994). Interestingly, insulin treatment inhibits this phosphorylation (Morris *et al.*, 1995b), perhaps through an inhibition of PKC, stimulation of a phosphatase or by effecting normoglycaemia and thus reducing the production of DAG. Such a generation of DAG in response to hyperglycaemia may occur by *de novo* synthesis (Hoffman *et al.*, 1991; Wolf *et al.*, 1991; Inoguchi *et al.*, 1992), or, alternatively, may result from the increased circulating level, in the diabetic state, of the hormone vasopressin (Houslay, 1990), which is known to cause the generation of DAG by stimulating phospholipase C (Bocckino *et al.*, 1985).

In addition to changes in the activity of PKC isoforms in diabetes, alterations in the intracellular localisation and levels of these enzymes have been demonstrated. Thus, in the membrane fraction of aorta and heart of streptozotocin diabetic rats, the level of PKC-βII was found to be elevated whereas that of PKC-α was unchanged (Inoguchi *et al.*, 1992). The authors of this study, however, were unable to determine whether this was due to membrane translocation alone or PKC synthesis and translocation, since no significant change in the level of cytosolic PKC-βII was detected (Inoguchi *et al.*, 1992).

In a separate study, investigating PKC isoform levels in hepatocytes from such animals, the induction of streptozotocin diabetes was found to be associated with marked increases in the levels of both cytosolic and membrane PKC-α and PKC-βII (Tang *et al.*, 1993). Thus the total cellular level of these isoforms is increased, reflecting either an increased rate of synthesis or a reduced rate of degradation. The former could be mediated by a DAG-stimulated PKC activity leading to activation of the MAPK pathway (Kolch *et al.*, 1993), and the subsequent stimulation of the transcription of these PKC isoforms through the phosphorylation of the transcription factor complex, AP-1 (Meek and Street, 1992).

It remains to be ascertained, however, whether such changes in PKC levels occur in the principal peripheral target tissues for insulin-stimulated glucose uptake, namely adipose tissue and skeletal muscle.

6.1.3. The use of semi-quantitative RT-PCR to determine levels

The technique of RT-PCR was used to determine whether specific transcripts were present in a variety of tissues, and also to examine whether expression varied with the induction of the diabetic state. In such experiments, the viability of the first strand cDNA preparations was verified by conducting PCR with control primers. In addition, a dose response experiment was undertaken, to check that increasing quantities of RNA did indeed lead to the generation of signals of greater intensities. The use of PCR to determine whether a specific DNA sequence is present or absent is a well-established practice, for example, in the diagnosis of viral infections. Several groups have, however, used RT-PCR to evaluate in a semi-quantitative (Johannes *et al.*, 1994), and even a quantitative (Firsov *et al.*, 1995), manner the abundance of a particular transcript. RT-PCR has also been used recently in our laboratory to determine the effect of PMA treatment on PDE-1 transcript levels in Chinese hamster ovary cells (Spence *et al.*, 1995).

6.1.4. Aims and objectives

The aim of the work presented in this chapter was to investigate changes in PKC isoform levels between normal and diabetic rats. An additional objective was to examine

the expression of PKD in various rat tissues and to ascertain whether streptozotocin-induced diabetes is associated with changes in the level of expression of PKD, GRK2 or GRK3.

6.2. Results

6.2.1. Determination of changes in PKC isoform levels in diabetes

A series of polyclonal antisera were raised previously, against peptides corresponding to unique regions within the amino acid sequences of PKC-α, βI, βII, γ, δ, e, and ζ (Tang *et al.*, 1993). The isoform-specificity of the antisera thus obtained was verified previously (Tang *et al.*, 1993). Using these antisera, immunoblotting was performed to investigate the expression of these isoforms in hepatocytes and adipocytes of both streptozotocin diabetic and control rats.

6.2.1.1. Immunoblotting of hepatocytes

Hepatocytes were isolated from two streptozotocin diabetic and two control rats as described in section 2.4.9. with the kind assistance of Dr Li Zeng. Following homogenisation in buffer (20 mM Tris pH 7.5, 10 mM EGTA, 2 mM EDTA, 0.5% Triton X100 and 50 mM β-mercaptoethanol) containing protease inhibitors as described in section 2.4.11, equal quantities of protein were boiled in Laemmli buffer and applied to each lane on an 8% SDS poly-acrylamide gel. Electrophoresis and immunoblotting were subsequently performed as described in sections 2.4.1. and 2.4.3. While no differences were apparent between control and diabetic rats in the electrophoretic mobilities of the immunoreactive species, certain differences were observed in the relative intensities of the respective signal (figure 6.1.). Blots probed with the antisera specific for PKC-α, βII, γ and ε all exhibited an immunoreactive species of approximately 87 kD. Interestingly, no signal was detected with the antisera specific for PKC-βI, while that raised against the peptide corresponding to a sequence present in PKC-ζ gave rise to several immunoreactive species. Of these, the species with the highest mobility (corresponding to a molecular weight of approximately 77 kD) is likely to represent this isoform as purified PKC-ζ has been reported to migrate on SDS polyacrylamide gels with a MWt of 78 kD (Nakanishi and Exton, 1992). The blot probed with the PKC-δ-

specific antiserum revealed a prominent immunoreactive species of approximately 160 kD, possibly representing a homo-dimer, and, additionally two species of much lower intensity of approximately 83 kD. A densitometer was used to compare the signal intensities observed in blots of hepatocytes isolated from streptozotocin diabetic rats with those of control rats. It was found that the signal obtained from blots probed with antisera specific for PKC-α, βII, ε and ζ were more intense (2.0, 1.5, 1.4 and 1.6 x fold increases, respectively) in hepatocytes isolated from the diabetic rats than in those prepared from the control animals (fig. 6.1.).

6.2.1.2. Immunoblotting of adipocytes

As no data had been published pertaining to the expression levels of PKCs in adipocytes, one of the principal peripheral targets for insulin action, it was decided to isolate such cells from two sources, namely epididymal and peri-nephric fat. These tissues were excised from the abdomen of a normal male rat and the adipocytes isolated by the use of collagenase as described in section 2.4.10. Subsequently, the adipocytes were washed five times in fresh buffer containing protease inhibitors. Upon immunoblotting the adipocyte proteins, it was found, however, that no immunoreactive species of the predicted molecular weight was detectable. Instead, a species of very high intensity was detected in both adipocyte preparations, which co-migrated with albumin contained within the molecular weight markers. Such a signal is likely to represent the albumin which is included in the buffer used in order to stabilise the cells during the collagenase incubation. It is probable, therefore, that the albumin remained bound to the adipocytes, despite repeated washing of the cells. Interestingly, it has been found recently that the albumin can be replaced by 200 nM adenosine, which maintains stabilisation of the adipocytes while permitting collagenase digestion (I. Fleming, personal communication, 1996).

In view of the difficulty described above (and experienced also by others in our department) in utilising polyclonal antisera in immunoblots of adipocytes, it was decided to homogenise and fractionate epididymal fat tissue, per se, instead of performing a

collagenase incubation. Thus the epididymal fat pads were excised from two control and two streptozotocin diabetic rats, homogenised, and subjected to a low speed spin to permit removal of fat and cell debris. This yielded a crude homogenate which was then fractionated by ultracentrifugation to sediment the plasma membrane fraction, leaving a supernatant containing the cytosol.

Immunoblots were subsequently performed (with equivalent quantities of protein) on the crudely extracted homogenates, and the cytosol and membrane fractions (figure 6.2.), using two antisera, specific for PKC-βII and PKC-ε, which had been found consistently to yield good immunoblots of hepatocyte proteins. In this instance, it was found that the PKC-βII antiserum strongly detected an immunoreactive species in all of the samples, particularly in the homogenates and in the cytosol fractions. Notably, no difference was observed between the intensity of the signal that was elicited from the control and that from the diabetic samples. With the PKC-ε antiserum, however, an immunoreactive species was identified in the crude homogenate and the cytosol but not in the membrane fraction. Interestingly, an increased intensity of this species was noted in the samples of homogenate prepared from diabetic rats, but not in the cytosol fraction. It is possible that the proteins in both of the membrane fractions had undergone degradation during preparation, and the lack of increase in intensity in the cytosolic signal may reflect translocation of PKC to the membrane.

A similar method was used to prepare crude homogenates from human adipose tissue. Despite immunoblotting with PKC-α, PKC-βII, PKC-ε and PKC-ζ, however, no immunoreactive species was detected. This may reflect degradation of the PKCs or extremely low expression levels in this tissue. Alternatively, it may be a result of sequence variations, between the human and rat PKC homologues, in the sequence corresponding to that of the peptide used to raise the antisera.

6.2.2. PKD expression studies

6.2.2.1. Optimisation of PKD-specific PCR

The initial PCR reactions performed with the primers designed so as to amplify the cDNA between nucleotides 2123 and 2687 of the PKD open reading frame, as described in section 5.2.1.2, yielded signals of low intensity. In order to improve the PKD-specific PCR signal and to permit the maximum subsequent amplification of PKD-specific products by RT-PCR, a number of preliminary experiments were carried out. In these experiments, the PKD-encoding expression plasmid was used as a template.

To prevent extension from any primer dimer pairs that might have been formed, a "hot-start" technique was used, in which the Taq polymerase was added to the reaction mixture only once the denaturation temperature had been reached. This technique, however, was not found to improve the signal (figure 6.3.).

Additionally, in order to test the possibility that the primer solutions may be defective, perhaps containing residual salts or ethanol, both oligonucleotides were re-purified from the original stocks. Furthermore, a fresh dilution of the dNTPs was prepared. PCR reactions were subsequently performed using the newly purified primers at the previously used concentration of 75 pmol per 50 µl reaction volume and also at a 10-fold higher concentration. The freshly prepared dNTPs were used in a separate reaction, and, for comparison, the original constituents were used in a further PCR. It was observed that the use of newly purified primers, even at the higher concentration, did not lead to an improvement in the PCR yield. Similarly, an increased template dilution, undertaken prior to PCR, did not permit efficient amplification. Surprisingly, however, the utilisation of a freshly prepared dilution of dNTPs, did lead to a signal of a considerably greater intensity (figure 6.4.), implying that the dNTPs in the previous mixture had undergone degradation. Fresh dNTPs were used in all subsequent PCR reactions.

As slight variations in the $MgCl_2$ concentrations have been shown previously to compromise the efficiency of the PCR (Saiki, 1989) a further experiment was undertaken

to optimise the PKD-specific PCR with respect to this salt. Using a range of $MgCl_2$ concentrations, however, it was found that the original concentration used, that of 75 nmols in 50 µl (i.e. 1.5 mM) yielded the optimal result (figure 6.5.).

6.2.2.2. PKD tissue distribution

In order to examine the tissue distribution of PKD, RT-PCR was performed on RNA (extracted as described in section 2.2.12.) using oligonucleotide primers designed as described in section 5.2.1.2. to amplify a 565 bp specific PCR product from the C-terminal region of the PKD cDNA sequence. Thus RNA was extracted from heart, striated muscle, lung, and fat tissues freshly excised from male Sprague-Dawley rats, and an equal quantity, 5 µg, was used in each case for the immediate synthesis of first strand cDNA, as described in section 2.2.14. In addition, a sample of human fat tissue was excised from a healthy female patient, snap-frozen in liquid nitrogen, and, following thawing, subsequently subjected to the same processes of first strand cDNA synthesis and PCR as described above. A sample of excised rat striated muscle was also snap-frozen in order to allow the subsequent comparison of fresh isolation of RNA with extraction from tissues after freeze-thawing.

No PKD-specific DNA product of the predicted size (565 bp) was detected in the RT-PCR reactions undertaken with RNA isolated from snap-frozen rat striated muscle or human fat. Such species were, however, detected in the reactions performed with RNA isolated fresh from rat epididymal fat, heart, and lung (figure 6.6.). Only a weak signal of the predicted size was visualised in RT-PCR from freshly-isolated rat striated muscle RNA, and interestingly this reaction also gave rise to an additional band of lower mobility (corresponding to a size of approximately 700 bp) which was also detected in the reactions performed with RNA from heart and fat. While such signals may represent non-specific amplification, it is possible that they result from the presence of an alternative splice form of the PKD transcript.

6.2.3. Detection of expression of PMA-activated kinases and the glucagon receptor in control and diabetic animals

In view of the potential role for PKD in mediating glucose homeostasis implied by the above studies, additional experiments were undertaken to compare the levels of expression of PKD between diabetic rats and control rats. As no immunoreactive species could be detected in rat hepatocyte homogenates by Western blotting with an antiserum raised against a peptide derived from PKD (figure 6.7.), the more sensitive technique of RT-PCR was employed for this purpose. The primers utilised were those specific for the C-terminal region of the PKD coding sequence, allowing the amplification of a 565 bp specific product (corresponding to the PKD open reading frame between nucleotides 2123 and 2687).

6.2.3.1. Transcript detection in hepatocytes

From normal or diabetic rats, RNA was extracted from freshly isolated hepatocytes, as described in the Materials and Methods chapter. Confirmation of the diabetic state was achieved by the observation of signs of diabetes such as thirst and polyuria, and by testing for glycosuria. In addition, following euthanasia, the presence of hyperglycaemia was confirmed.

Equal quantities of the extracted RNA were reverse-transcribed from the poly-adenylated RNA species using a First-Strand cDNA Synthesis Kit as described in the Materials and Methods chapter, and equal volumes subsequently used as a substrate for PCR with the conditions that were optimised as described in section 6.2.2.1.

It was found that while a signal of the predicted mobility could be amplified from RNA extracted from control rat hepatocytes, no such product was detectable following PCR performed on RNA extracted from diabetic rat hepatocytes (figure 6.8.).

RT-PCR was also performed, in a similar manner, on these first-strand cDNA preparations in order to examine the expression in such animals of the G protein-coupled receptor kinases, GRK2 and GRK3. The primers used were those designed as described in section 5.2.1.2, so as to amplify specific products of 701 and 600 bp, corresponding

to nucleotides 280-980 and 1357-1956 of the bovine GRK2 and GRK3 open reading frames, respectively. While GRK2 transcripts were detected with signals of equal intensity in both control and diabetic hepatocytes, no GRK3-specific signal was detected utilising either source of RNA (figure 6.9.).

In addition, RT-PCR was undertaken to ascertain whether the level of glucagon receptor mRNA itself is altered in the diabetic state. Thus PCR was performed using primers designed as described in section 3.2.1. so as to amplify a specific 941 bp product (encompassing nucleotides 1 to 932 of the GR-encoding open reading frame in addition to a 9 bp extension). A glucagon receptor-specific product was detected, however, in the RNA prepared from both sources (figure 6.10.) with no apparent difference in intensity. This finding is consistent with a previously reported study of the relative levels of mRNA in diabetic (db/db) and control (db/+) mice, in which no difference was detected (Yoo-Warren *et al.*, 1994).

6.2.3.2. PKD-specific transcript detection in epididymal fat

In order to explore further the apparent loss of PKD transcripts accompanying the induction of diabetes, PKD transcripts were analysed in adipose tissue. This tissue was chosen on account of its known important role in extra-hepatic glucose homeostasis, and as it had been found, as discussed above, to express PKD.

Epididymal fat was excised from three healthy rats and a total of 13 rats in which diabetes had been induced by the injection of streptozotocin. Five of these diabetic animals were then treated for 7 days with insulin (10-12 units administered twice daily by sub-cutaneous injection) in order to eliminate the hyperglycaemia, which was confirmed by checking for the absence of the signs described above. RT-PCR was carried out on cDNA prepared as described above on RNA freshly extracted from adipose tissue immediately after the euthanasia of these animals. The PCR primers utilised to amplify PKD-specific cDNA were those described above in section 5.2.1.2. so as to amplify a 565 bp specific PCR product from the C-terminal region of the PKD cDNA sequence. In addition, in order to verify the condition of the first-strand cDNA preparation, and thus

the quality of the RNA preparation and cDNA synthesis reaction, additional RT-PCR reactions were undertaken utilising β-actin-specific primers in combination with the cDNA preparations obtained from the adipose tissue excised from these animals. The primers used were those designed as described in section 5.2.1.2, allowing for the amplification of a specific 466 bp product corresponding to nucleotides 222-687 of the open reading frame of human β-actin.

In order to verify that the intensity of the resulting PKD-specific RT-PCR product would reflect, to an appreciable extent, the level of PKD-specific mRNA available for first strand cDNA synthesis, a range of quantities of RNA were used in a series of parallel cDNA reactions. From each, an equal volume was subsequently added to a PCR reaction mixture, containing PKD-specific primers. The products from this series of PCR reactions were compared following electrophoresis in an agarose gel (figure 6.11.). While no signal was detected when a quantity of less than 37 ng RNA was utilised for the first strand cDNA reaction, with the use of higher quantities, up to 9.4 μg the signal was observed to increase in intensity. This, therefore, provided support for the supposition that the magnitude of the PCR amplification would increase with greater quantities of template RNA. The quantity of RNA routinely used in the first-strand cDNA syntheses was 5 μg, which thus lies within this range.

It was found that while strong signals resulted in all cases from β-actin-specific RT-PCR on the cDNAs thus isolated from the healthy controls, the insulin-treated diabetic animals, and the untreated diabetic animals, the intensity of the resulting PKD-specific signal varied greatly between these groups, despite the use of identical RT-PCR conditions. PKD was detected in the cDNAs prepared from all 3 untreated healthy animals, but of the eight cDNA samples prepared from untreated diabetic rats, three failed to yield a detectable product and the other five gave rise to an amplification product of only a very weak intensity (fig. 6.12. and table 6.1.). Furthermore, the treatment of diabetic animals with sufficient insulin to reverse those clinical features of the condition that were described above, led to an apparent restoration of the PKD transcript level, as cDNAs prepared from 4 out of the 5 animals thus treated exhibited a detectable PKD-

specific RT-PCR signal which was of a similar intensity to that observed following the RT-PCR from healthy animals' RNA (fig. 6.12. table 6.1.).

6.3. Discussion

6.3.1. Immunoblotting for PKC isoforms

The inability to detect an immunoreactive species when immunoblotting hepatocyte proteins with the PKC-βI-specific antiserum is a finding that is consistent with the recognised limited tissue distribution of this isoform (Wetsel *et al.*, 1992). It has been reported to be expressed principally in brain and spleen and to be undetectable in liver tissue (Wetsel *et al.*, 1992). Similarly, the very weak signal elicited by the use of the PKC-γ-specific antiserum is consistent with the observations of others that this PKC isoform is expressed only in cells of the nervous system (Wetsel *et al.*, 1992; Hug and Sarre, 1993).

Immunoblotting with PKC isoform-specific antibodies suggested differences in the level of abundance of individual isoforms between hepatocytes isolated from streptozotocin diabetic and those obtained from normal rats. Specifically, PKC-α, βII, ε and ζ appeared to be present at higher levels (2.0, 1.5, 1.4 and 1.6 x fold increases, respectively) in hepatocytes isolated from the diabetic than in those prepared from normal rats. Increased levels of PKC-α, PKC-βII and PKC-ε have been reported previously in hepatocytes (Tang *et al.*, 1993) and an elevated level of PKC-βII has been observed in macrovascular tissues (aorta and heart) (Inoguchi *et al.*, 1992) of streptozotocin diabetic rats. Such changes were shown to be reversible upon insulin treatment (Tang *et al.*, 1993; Inoguchi *et al.*, 1994).

The results of the present study, indicating that changes may occur in the levels of members of all three classes of PKC isoforms in diabetes, constituted, however, a novel finding at the time when this work was undertaken. More recently, all four PKC isoforms mentioned above, PKC-α, βII, ε and ζ, have been shown to be expressed in the livers of Zucker diabetic rats at higher levels than in the control animals (Considine *et al.*, 1995). Furthermore, three of the four isoforms, PKC-α, ε and ζ, were shown by immunoblotting of human liver samples, to be expressed in patients with NIDDM at

levels which are approximately two-fold higher than in control patients (Considine *et al.*, *al.*, 1995).

It is believed that changes in PKC expression levels such as those observed in the present study, may be one of the factors which underlie insulin resistance, a phenomenon associated with human diabetes of type I (IDDM) and especially with that of type II (NIDDM). Several lines of evidence support this contention. Firstly, it has been shown that the insulin receptor's tyrosine kinase activity is crucial to the mediation of many of insulin's effects (Kilgour, 1993). It has also been demonstrated that TPA-induced activation of PKC causes not only the phosphorylation of solubilised insulin receptors but also a reduction of the insulin receptor tyrosine kinase activity (Bollag *et al.*, 1986). This was subsequently confirmed in Fao hepatoma cells, in freshly isolated hepatocytes (Takayama *et al.*, 1988; Caro *et al.*, 1992) and, more recently, in CHO cells transfected with the human insulin receptor and cDNAs encoding specific PKC isoenzymes (Chin *et al.*, 1993). The role of PKC in this process has been further supported by the ability of staurosporine to block the TPA-induced insulin receptor phosphorylation (Duronio and Jacobs, 1990), and by the observed potentiation of insulin receptor auto-phosphorylation which follows the PMA-induced down-regulation of PKC (Pillay *et al.*, 1990). Phosphorylation of the insulin receptor by PKC occurs on the β subunit at Thr-1336 (Lewis *et al.*, 1990), Thr-1348 and Ser-1305/1306 (Chin *et al.*, 1993).

In addition to the implication from the above experimental systems that PKC can phosphorylate the insulin receptor and thus inhibit its tyrosine kinase activity, several studies have shown that in patients with NIDDM, the activity of the insulin receptor tyrosine kinase is indeed reduced. This has been demonstrated for liver (Caro *et al.*, 1986), muscle (Arner *et al.*, 1987; Caro *et al.*, 1987; Obermaier-Kusser *et al.*, 1989) and fat (Freidenberg *et al.*, 1987; Sinha *et al.*, 1987).

It remains to be determined, however, whether PKC isoenzymes are involved in this process in vivo, and, if so, then which specific isoforms are important. The immunoblotting studies discussed above (Tang *et al.*, 1993; Considine *et al.*, 1995), and the results of the present study, certainly indicate an association of altered PKC

expression with diabetes in rat models of diabetes, and in man. Such changes, however, are possibly secondary consequences of hyperglycaemia, and may occur independently of changes in insulin receptor functioning. Nevertheless, PKC isoforms have been shown to phosphorylate and regulate not only the insulin receptor, but also the type 1A angiotensin II receptor (Oppermann *et al.*, 1996) and the glucagon-like peptide-1 (GLP-1) receptor (Widmann *et al.*, 1996). Thus it is likely that aberrant expression of specific PKC isoforms does cause alterations in cellular signalling pathways, leading to hormone resistant states.

6.3.2. PKD expression studies

The detection of PKD-specific transcripts in rat lung and heart, and, albeit weakly, in muscle tissue is consistent with previous observations from Northern blotting of RNA extracted from mouse tissues (Valverde *et al.*, 1994). As PKD expression in adipose tissue was not examined by Valverde et al., the detection of a PKD-specific signal resulting from RT-PCR performed on RNA isolated from fat, is a novel finding.

Freeze-thawing of tissue prior to RNA extraction was found to lead to an inability to detect any RT-PCR product. Thus, while 5 µg of RNA was obtained for first strand cDNA synthesis, it is likely that, owing to degradation of RNA occurring during freeze-thawing, only small cDNA fragments were available after cDNA synthesis for the subsequent PCR amplification.

The additional product observed following RT-PCR reactions performed on RNA isolated from freshly excised rat striated muscle, heart and adipose tissue, indicated that alternatively-spliced PKD transcripts might exist. This possibility could be further investigated using Northern and Western blotting of RNA and protein extracts, respectively. Such alternative transcripts have not, however, been reported previously in the studies on PKD and PKC-µ expression (Johannes *et al.*, 1994; Valverde *et al.*, 1994; Van Lint *et al.*, 1995) and it is more likely that the 700 bp band resulted from non-specific annealing of one or both of the oligonucleotide primers.

The greatly reduced PKD-specific PCR signal obtained from cDNA prepared from hepatocytes and adipose tissue from diabetic rats is likely to indicate a reduction in the level of PKD transcript associated with the induction of this condition in these animals. The efficient amplification of β-actin-specific PCR products from the cDNAs prepared from these animals indicates that the failure of amplification of PKD products is not merely a consequence of poor quality cDNA synthesis or RNA isolation. Nor, in view of the results obtained with this β-actin control, is it likely to be due to the presence in the diabetic animal cDNA preparations of a substance which non-specifically inhibits the PCR reaction. The increase in intensity of the PKD-specific signal which was observed to result from increasing the quantity of RNA available for the first strand cDNA synthesis reaction lends further support to these conclusions and, additionally, demonstrates the absence, in the cDNA prepared from diabetic rats, of any inhibitor of PKD amplification by PCR. It would appear that the treatment with insulin is associated, in a number of animals, with the restoration of PKD transcript levels to that which supports the amplification by RT-PCR of a detectable signal.

The insulin treatment serves to reduce the elevated blood sugar levels in these animals, with the observable consequence that polyuria is prevented and thirst is diminished. While the mechanism of the diabetes-induced apparent PKD transcript reduction is not clear, it may be postulated that it is a consequence of the hyperglycaemia which is present in the diabetic rats but not in those that have been treated with insulin. In fact, such a glucose-mediated regulation of the levels of a specific transcript has been reported, for instance, for the glucagon receptor mRNA, for which a small but significant increase in levels was detected following the incubation of rat hepatocytes in hyperglycaemic medium overnight (Abrahamsen *et al.*, 1995). Of great interest, in view of the changes in PKD mRNA levels implied by the present study, is the demonstration that immunoreactive PKC-β levels are reduced by a factor of 10 following overnight treatment of A-10 vascular smooth muscle cells with 25 mM glucose (Cooper *et al.*, 1993). It is thus possible that a similar hyperglycaemia-mediated reduction of PKD level occurs in the adipocytes of diabetic rats.

It is possible that the de novo DAG synthesis and PKC activation that has been shown in a number of cell types, including rat adipocytes, to be a consequence of hyperglycaemia (Hoffman *et al.*, 1991; Wolf *et al.*, 1991; Inoguchi *et al.*, 1992; Berti *et al.*, 1994; Williams, 1995) could, by activating Raf (Kolch *et al.*, 1993; Marquardt *et al.*, 1994) lead to the subsequent covalent modification of DNA-binding proteins (Meek and Street, 1992), and thus modify the transcriptional activity of individual genes, including perhaps, that of PKD. The precise nature of the upstream DNA regulatory elements of the PKD gene remains to be elucidated, but the role of such a PKC-mediated mechanism in the apparent reduction in the level of PKD transcripts in fat cells of diabetic animals is supported by the finding that PKC is indeed activated in rat adipocytes subjected to prolonged hyperglycaemia (Hoffman *et al.*, 1991; Muller *et al.*, 1991). An alternative mechanism for the reduction in PKD transcript level is that a reduction in mRNA stability may be a consequence in these cells of the diabetic state. A less likely, but nevertheless conceivable further alternative, would be a reduction in polyadenylation of the PKD transcripts, resulting in failure of the oligo-dT primed cDNA synthesis.

While the effects discussed above are based on the assumption that the observations are a result of the effects of hyperglycaemia, it is, of course, feasible that insulin itself may play a pivotal role in the maintenance of PKD transcription. In this scenario, the diminution in insulin production resulting from streptozotocin treatment (Gawler *et al.*, 1987), would lead to an abrogation of insulin-stimulated transcription (O'Brien and Granner, 1991) of genes, perhaps including that of PKD.

6.3.3. Implications of results for the pathogenesis of diabetes

6.3.3.1. Postulated physiological role of PKD in glycaemic homeostasis

It is well established from studies in a variety of cell types, including rat adipocytes (Hoffman *et al.*, 1991), vascular smooth muscle cells (Cooper *et al.*, 1993; Williams, 1995) and endothelial cells (Wolf *et al.*, 1991; Inoguchi *et al.*, 1992) that hyperglycaemia leads to an elevation of cellular diacylglycerol levels, possibly through the de novo

synthesis of this second messenger (Inoguchi *et al.*, 1992). Thus, as PKD is a DAG-activated kinase (Valverde *et al.*, 1994; Rozengurt *et al.*, 1995; Van Lint *et al.*, 1995), and as the results discussed in the previous chapter have shown that its action attenuates the response of adenylate cyclase to glucagon, it follows that, under normal circumstances, PKD may mediate an auto-regulatory process involved in glucose homeostasis. Thus, activation of PKD might occur physiologically in response to hyperglycaemia and lead to an attenuation of glucagon-stimulated gluconeogenesis and glycogenolysis. Such a negative feedback mechanism may operate in concert with, or independently of, the mechanism of glucagon-induced homologous desensitisation of the glucagon stimulation of adenylate cyclase.

6.3.3.2. Implications of diminished expression of PKD

If PKD does indeed play such a role in glycaemic control, then the finding that the expression of PKD appears to be diminished in the diabetic state may have important implications for the understanding of the pathogenesis of the hyperglycaemia that is associated with untreated diabetes mellitus. It might be envisaged that the impairment of such a PKD-mediated regulation of excessive action of glucagon, a hormone which is counter-regulatory to insulin, would have the consequence of further elevating blood glucose levels in diabetic patients. This would potentially lead to an exacerbation of the condition, and thus contribute to an increased tendency to hyperglycaemic episodes.

Inappropriate elevation of basal hepatic glucose output (bHGO) is a well documented finding in patients with NIDDM (Baron *et al.*, 1987). This phenomenon is believed to play a significant role in the pathogenesis of the disease, as there is a strong correlation between the degree of bHGO and the level of fasting hyperglycaemia, and, in particular, between improvements in glycaemic control and reduction in bHGO subsequent to therapeutic intervention (Best *et al.*, 1982). Moreover, it is clear that in untreated NIDDM hyperglycaemia results not only from insulin resistance but also from a paradoxical hyperglucagonaemia. In fact, in diabetic subjects, the contribution to HGO

attributable to the hyperglucagonaemia was determined to be as high as 58% (Baron *et al.*, 1987).

6.3.3.3. Homologous glucagon desensitization in diabetic rats

Previous workers in our laboratory, who studied the ability of glucagon pre-exposure to induce desensitization of the subsequent stimulation of adenylate cyclase by glucagon in hepatocytes isolated from diabetic or from control rats, found that desensitization was a feature of both populations of cells (Murphy *et al.*, 1989). The degree of desensitisation observed in the control hepatocytes was however, 53% greater than in the cells isolated from diabetic animals (Murphy *et al.*, 1989). These results would, therefore, be consistent with a role for PKD in the desensitization process, and with the reduced expression of this kinase in the diabetic state. That the glucagon-induced desensitisation observed in the diabetic rat hepatocytes was not completely abolished is consistent with the observation that PKD is often detectable by RT-PCR in the tissue excised from the diabetic animals, albeit at a much reduced intensity (table 6.1.).

It is also conceivable that PKD is not the only kinase involved in this regulatory process. It is possible, in this respect, that a kinase of the GRK class may also be involved, in a manner which might be similar to that demonstrated for the β2-adrenoceptor. This receptor is believed to be phosphorylated by an effector kinase, such as PKA, at low agonist concentrations, while at higher concentrations of agonist, phosphorylation by the β-adrenergic receptor kinase takes place (Clark *et al.*, 1988; Hausdorff *et al.*, 1989; Lohse *et al.*, 1990).

Finally, it is interesting to speculate on the mechanism of the increase, by approximately 70%, in the magnitude of the response of adenylate cyclase to glucagon, that is observed in hepatocytes isolated from streptozotocin-diabetic rats relative to the results obtained from the hepatocytes of healthy rats (Gawler *et al.*, 1987). While such an increase could certainly be solely attributable to the loss of functional G_i described above, it is also conceivable that it reflects a loss of a regulatory tonic inhibition by PKD.

Rat	PKD	β-Actin
Control		
1	+++	+++
2	+++	+++
3	+++	+++
STZ-treated		
1	---	+++
2	--+	+++
3	---	+++
4	---	+++
5	--+	+++
6	--+	+++
7	--+	+++
8	--+	+++
STZ+Insulin -treated		
1	+++	+++
2	+++	+++
3	+++	+++
4	---	+++
5	+++	+++

Table 6.1. RT-PCR of PKD-specific cDNA from rat epididymal fat

Adult male Sprague-Dawley rats were injected, by the intraperitoneal route, with 80 mg/Kg streptozotocin dissolved in 0.3 ml 0.1M sodium citrate, pH 4.5. After 4 days, the urine glucose level was tested using Diabur-Test 5000 (Boehringer Mannheim) test strips, to confirm the induction of diabetes mellitus. The glucose concentration was typically at least 280 mM. At the time of dissection of killed diabetic rats, the level of blood glucose was also checked using Dextrostix and found, typically, to be greater than 450 mg/ml. Animals which were treated with insulin, received 10 units of a long-acting insulin preparation (Humulin Lente), every 12 hours, for 7 days. Urine was checked as above to confirm the absence of glucose.

First strand cDNA synthesis and PCR were carried out as described in the Materials and Methods chapter using the PKD-specific oligonucleotide primers:

5' sense oligonucleotide ET-PKD-s1; TCGTTCACTGTGACCTCAAGC

3' antisense oligonucleotide ET-PKD-as1; CTAGCACTCAGACTGATCAGG.

This allowed for the amplification of a specific 565 bp product (see figure 6.12) corresponding to nucleotides 2123-2687 of the open reading frame of murine PKD (accession no. Z34524). The relative intensity of the specific RT-PCR product was gauged under ultraviolet light, following staining with ethidium bromide, and is represented thus: +++ strong; ++- weak; --+ very weak; --- not detected. Excluded from the above table are two animals: one which was injected with streptozotocin but failed to become diabetic, and one whose RNA failed to yield a viable cDNA preparation.

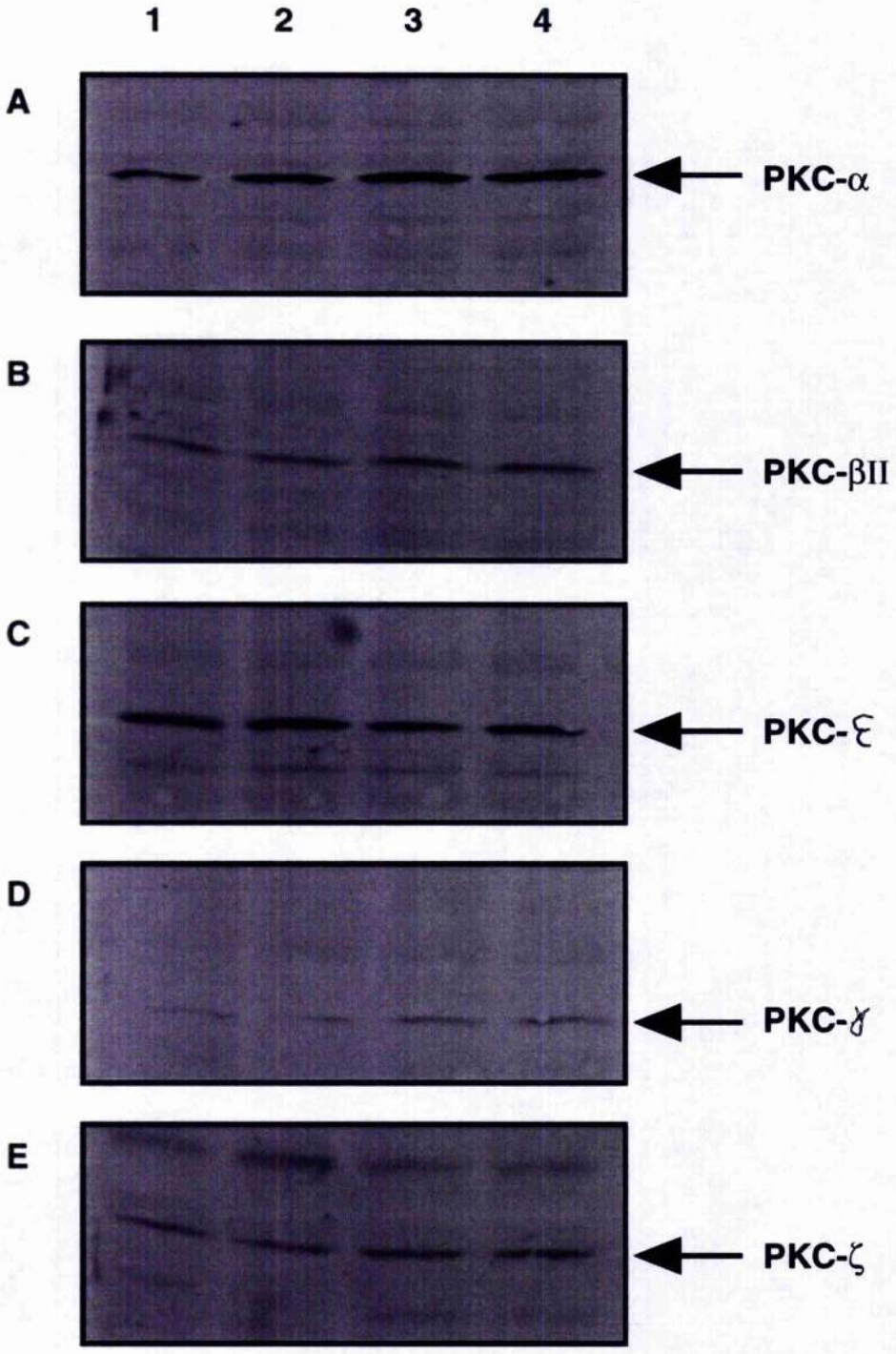
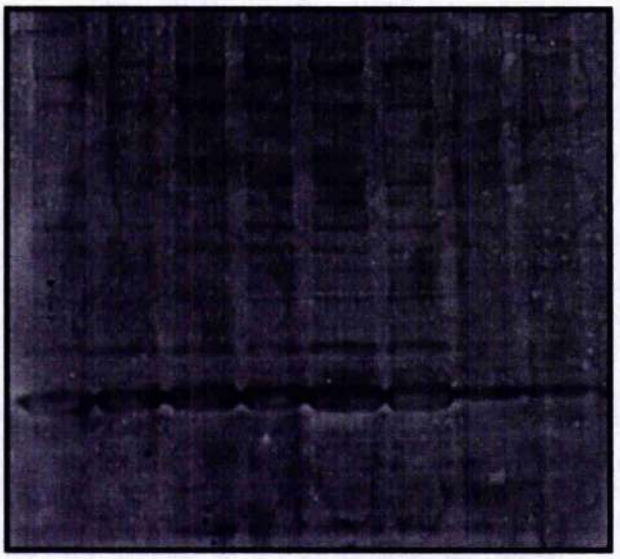

1
2
3
4
A
PKC-α
B
PKC-βII
C
PKC-ε
D
PKC-γ
E
PKC-ζ

Figure 6.1. Immunoblots for PKC alpha, beta II, epsilon and zeta in extracts of hepatocytes prepared from control and streptozotocin diabetic rats

An 8% polyacrylamide SDS gel was loaded with equal quantities of protein extracted from whole hepatocytes prepared from two control (lanes 1 and 2) and two streptozotocin diabetic rats (lanes 3 and 4). In each case an aliquot containing 100 μg of protein was boiled in Laemmli sample buffer prior to loading. Following electrophoresis, the proteins were transferred to nitrocellulose and blocking was performed by incubation in 5% (w/v) dried milk protein. The nitrocellulose blots were then incubated for 2 hours at room temperature with antisera specific for PKC alpha (A), PKC beta II (B), PKC gamma (δ), PKC epsilon (ε), PKC zeta (E) diluted 1 in 100. Following the removal of unhybridised antibodies by successive washing, the blots were incubated in HRP-conjugated anti-rabbit IgG, and detection performed by enhanced chemiluminescence.

A 1 2 3 4 5 6 7 8



B 1 2 3 4 5 6 7 8

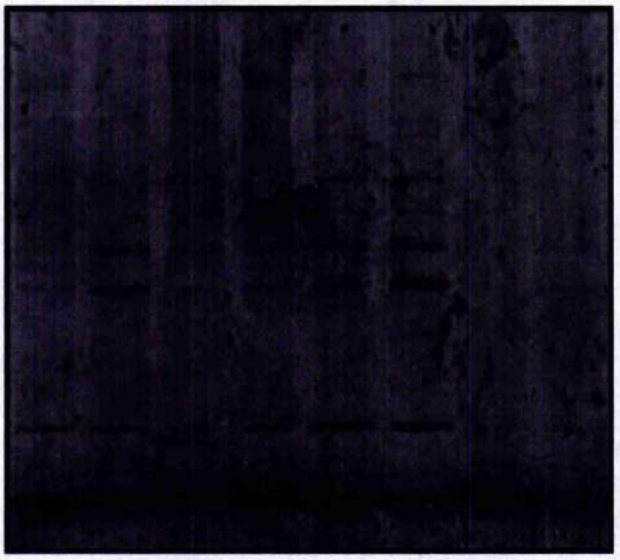


Figure 6.2. Immunoblots for PKC beta II and epsilon in extracts of epididymal fat prepared from control and streptozotocin diabetic rats

An 8% polyacrylamide SDS gel was loaded with equal quantities of protein from crude homogenate (lanes 1-4), cytosol (lanes 5 and 6) or membranes (lanes 7 and 8), which were prepared from control rats (lanes 1, 3, 5 and 7) or diabetic rats (lanes 2, 4, 6 and 8). In each case an aliquot containing 200 μg of protein was boiled in Laemmli sample buffer prior to loading. Following electrophoresis, the proteins were transferred to nitrocellulose and blocking was performed by incubation in 5% (w/v) dried milk protein. The nitrocellulose blots were then incubated for 2 hours at room temperature with antisera specific for PKC beta II (A) or PKC epsilon (B), diluted 1 in 100. Following the removal of unhybridised antibodies by successive washing, the blots were incubated in HRP-conjugated anti-rabbit IgG, and detection performed by enhanced chemiluminescence.

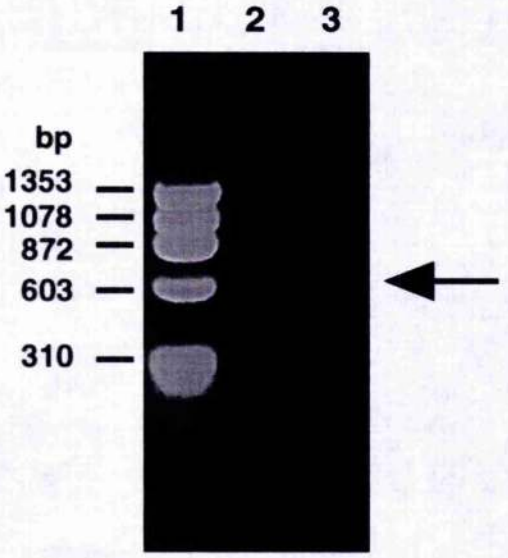

1
2
3
bp
1353
1078
872
603
310

Figure 6.3. Amplification by PCR of the cDNA encoding protein kinase D

Ethidium bromide stained 2% agarose gel electrophoresis of the PCR amplification product from the PKD-encoding plasmid with the PKD-specific primers with Taq polymerase added either prior to PCR (lane 3) or following the initial denaturation ie. "hot start" (lane 2). Lane 1 contained ϕX174 / Hae III DNA markers. The sizes are indicated in bp. The arrow indicates the predicted amplification product which was detected only weakly, under either condition.

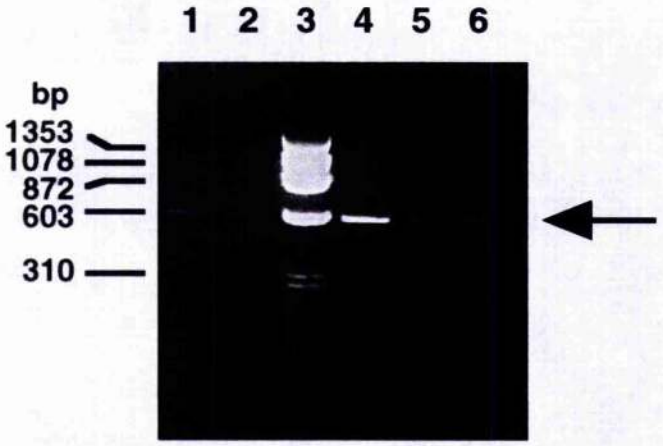

1 2 3 4 5 6
bp
1353
1078
872
603
310

Figure 6.4. Amplification by PCR of the cDNA encoding protein kinase D

Ethidium bromide stained 2% agarose gel electrophoresis of the PCR amplification product from the PKD-encoding plasmid in the presence of the original preparation of PKD-specific primers (lane 1) or a fresh preparation of primers (lanes 2,4-6). The primer concentrations were 75 pmol per 50 µl in each reaction, but were 4-fold higher in the PCR reaction loaded in lane 5. The quantity of PKD plasmid template was 100-fold less in the PCR reaction loaded in lane 6. The concentration of dNTPs was 20 µM in each reaction and a freshly prepared stock was utilised for the reaction loaded in lane 4. Lane 3 contained ϕX174 / Hae III DNA markers. The sizes are indicated in bp. The arrow indicates the predicted amplification product which was detected with greatest intensity when freshly prepared dNTPs were added.


1 2 3 4 5 6 7 8
bp
1353
1078
872
603
310

Figure 6.5. Effect of varying the $MgCl_2$ concentration upon PKD-specific RT-PCR

Ethidium bromide stained 2% agarose gel electrophoresis of the PCR amplification products from the first-strand cDNA synthesised from rat hepatocyte RNA, in the presence of varying $MgCl_2$ concentrations: 87.5 nmol/50 µl (lane 1), 62.5 nmol/50 µl (lane 2), 50 nmol/50 µl (lane 3) and 75 nmol/50 µl (lane 6). The primer concentrations were 75 pmol per 50 µl in each reaction. In order to provide a positive control, PKD plasmid template was included in the PCR reaction loaded in lane 8. The concentration of dNTPs was 20 µM in each reaction. Lane 4 contained ϕX174 / Hae III DNA markers. The sizes are indicated in bp. The arrow indicates the predicted PKD-specific amplification product, which was detected using first-strand cDNA as template with greatest intensity when the $MgCl_2$ concentration employed was 75 nmol/50 µl.


1 2 3 4 5 6 7 8

Figure 6.6. Amplification by PCR of the cDNA encoding protein kinase D from RNA isolated from various tissues

Ethidium bromide stained 2% agarose gel electrophoresis of the PKD-specific PCR amplification product from first strand cDNA prepared from equal quantities of RNA extracted from freeze-thawed human fat (lane 1) and rat striated muscle (lane 2), and from freshly excised rat epididymal fat (lane 3), rat heart (lane 4), rat lung (lane 6) and rat striated muscle (lane 7). In addition, to provide a PCR control, amplification was also performed in parallel from the PKD-encoding plasmid (lane 8). The primer concentrations were 75 pmol per 50 μl in each reaction. Lane 5 was loaded with 1 kilobase DNA markers. The arrow indicates the predicted PKD-specific PCR amplification product.

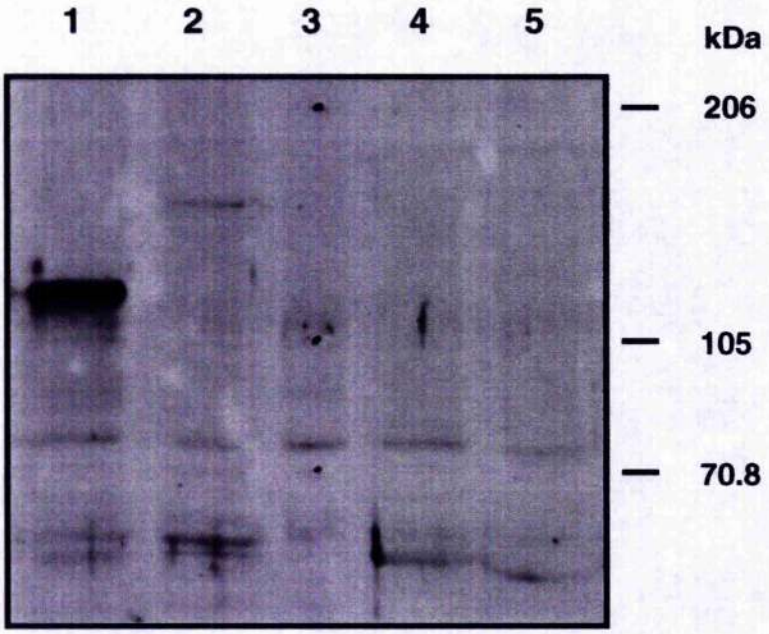

1
2
3
4
5
kDa
206
105
70.8

Figure 6.7. Immunoblots for PKD in homogenates of rat hepatocytes and control and transfected COS cells

An 8% polyacrylamide SDS gel was loaded with 50 µg of protein from PKD cDNA-transfected COS cells (lanes 1), 100 µg protein extracted from control COS cells (lane 2), 100 µg (lane 4) and 400 µg (lane 5) of protein extracted from whole hepatocytes, and protein standards in lane 3. In each case the protein extract was boiled in Laemmli sample buffer prior to loading. Following electrophoresis, the proteins were transferred to nitrocellulose and blocking was performed by incubation in 5% (w/v) dried milk protein. The nitrocellulose blots were then incubated for 2 hours at room temperature with antisera specific for PKD, diluted 1 in 200. Following the removal of unhybridised antibodies by successive washing, the blots were incubated in HRP-conjugated anti-rabbit IgG, and detection performed by enhanced chemiluminescence.

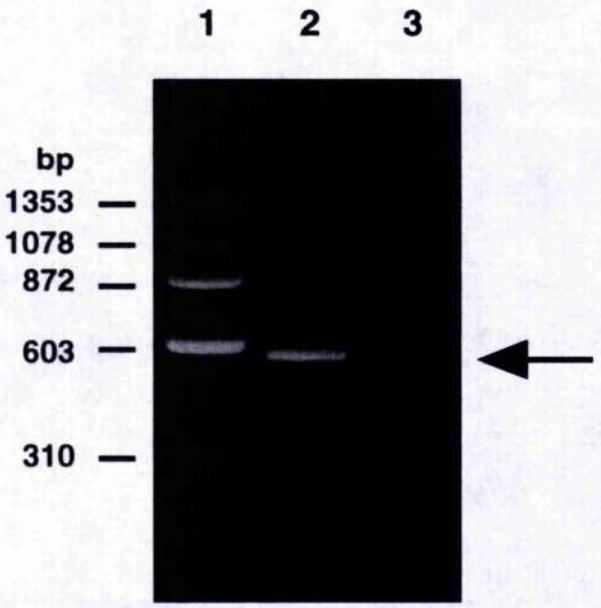

1
2
3
bp
1353
1078
872
603
310

Figure 6.8. Amplification by RT-PCR of the cDNA encoding protein kinase D from RNA isolated from control and diabetic rat hepatocytes

Ethidium bromide stained 2% agarose gel electrophoresis of the PKD-specific PCR amplification product from first strand cDNA prepared from equal quantities of RNA extracted from hepatocytes isolated from a healthy animal (lane 2) and one which had been treated with streptozotocin to induce a diabetic state (lane 3). The primer concentrations were 75 pmol per 50 µl in each reaction. Lane 1 contained ϕX174 / Hae III DNA markers. The arrow indicates the predicted PKD-specific PCR amplification product.

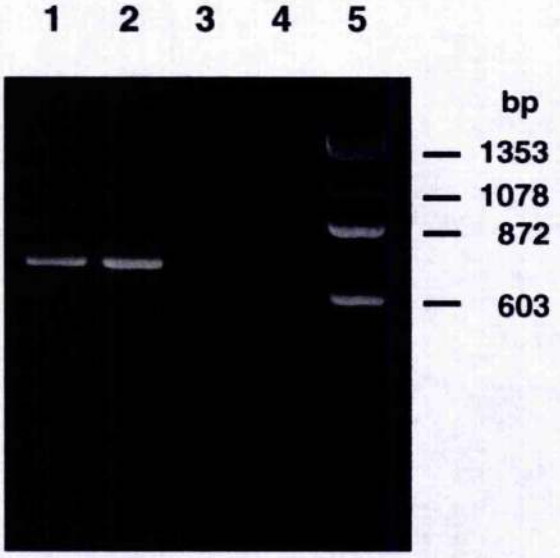

1
2
3
4
5
bp
1353
1078
872
603

Figure 6.9. Amplification by RT-PCR of the cDNAs encoding GRK2 and GRK3 from RNA isolated from control and diabetic rat hepatocytes

Ethidium bromide stained 2% agarose gel electrophoresis of the PCR amplification products following PCR reactions using first strand cDNA preparations as template. The latter were synthesised from equal quantities of RNA extracted from hepatocytes isolated from a healthy animal (lanes 1 and 3) or from one which had been treated with streptozotocin to induce a diabetic state (lanes 2 and 4). GRK2-specific primers (lanes 1 and 2) or GRK3-specific primers (lanes 3 and 4) were present at concentrations of 75 pmol per 50 µl in each reaction. Lane 5 was loaded with ϕX174 / Hae III DNA markers.

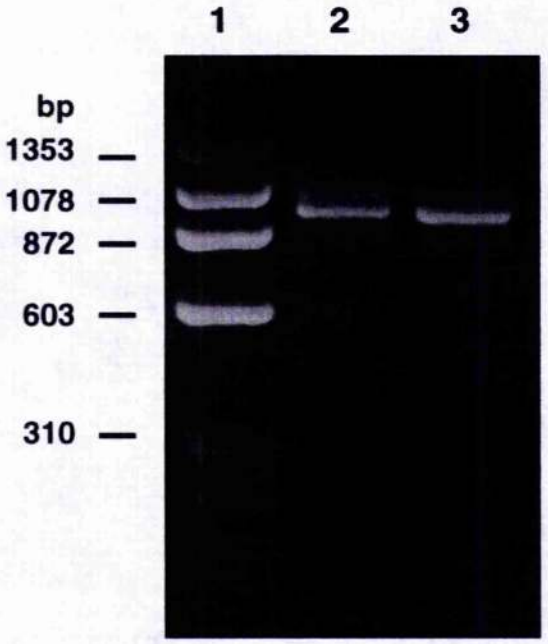

1
2
3
bp
1353
1078
872
603
310

Figure 6.10. Amplification by RT-PCR of glucagon receptor cDNA from RNA isolated from control and diabetic rat hepatocytes

Ethidium bromide stained 2% agarose gel electrophoresis of the PCR amplification products following PCR reactions, using as template, preparations of first strand cDNA synthesised from equal quantities of RNA extracted from hepatocytes which had been isolated either from a healthy animal (lane 2) or one which had been treated with streptozotocin to induce a diabetic state (lane 3). Glucagon receptor-specific primers were present (lanes 2 and 3) at concentrations of 75 pmol per 50 µl in each reaction. Lane 1 contained ϕX174 / Hae III DNA markers.

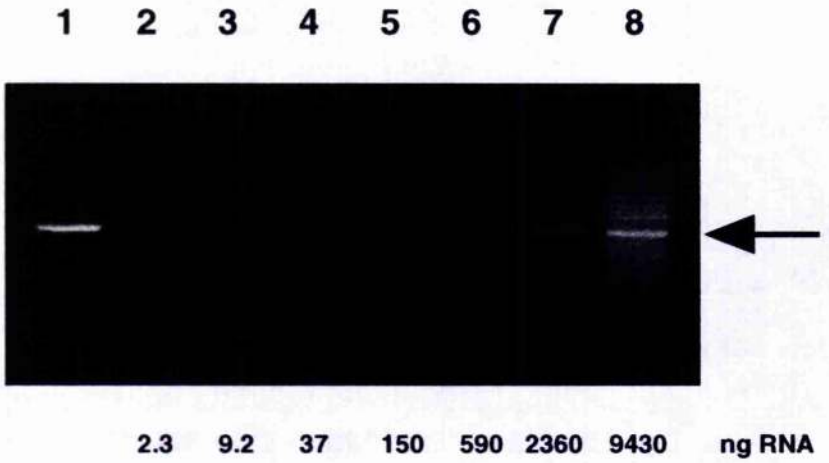

1 2 3 4 5 6 7 8
2.3 9.2 37 150 590 2360 9430 ng RNA

Figure 6.11. RT-PCR of the cDNA encoding protein kinase D from a range of quantities of RNA isolated from rat epididymal fat

Ethidium bromide stained 2% agarose gel electrophoresis of the PKD-specific PCR amplification product from first strand cDNA mixtures prepared from a range of quantities of RNA extracted from epididymal fat excised from a healthy animal. The quantities of RNA used as a template for each first-strand cDNA synthesis reaction ranged from 2.3 ng (lane 2) to 9.4 μg (lane 8) increasing in successive 4-fold steps as indicated. In addition, as a PCR control, amplification was also performed in parallel from the PKD-encoding plasmid (lane 1). The primer concentrations were 75 pmol per 50 μl in each reaction. The arrow indicates the predicted PKD-specific PCR amplification product, which was detected in lanes 6-8, and, in higher exposures (not shown), also in lanes 4 and 5.

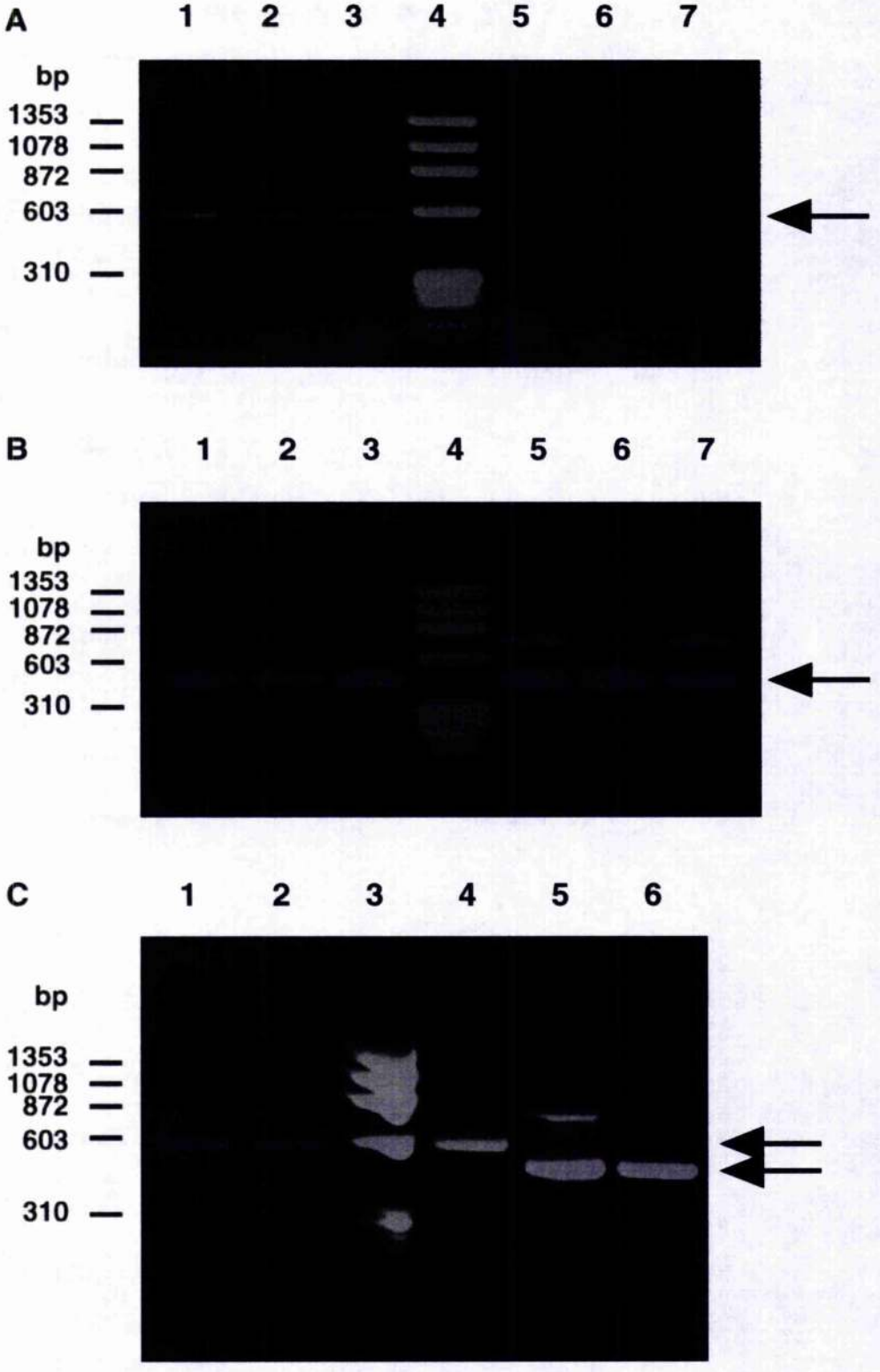

A
1 2 3 4 5 6 7
bp
1353
1078
872
603
310
B
1 2 3 4 5 6 7
bp
1353
1078
872
603
310
C
1 2 3 4 5 6
bp
1353
1078
872
603
310

Figure 6.12. Amplification by RT-PCR of the cDNAs encoding protein kinase D and β-actin from RNA isolated from control, diabetic and insulin-treated diabetic rat epididymal fat

A and B. Ethidium bromide stained 2% agarose gel electrophoresis of the products resulting from PCR with PKD-specific primers (A) from first strand cDNA preparations synthesised from equal quantities of RNA extracted from rat epididymal fat. The tissue was excised from three healthy animals (lanes 1-3) and three which had been treated with streptozotocin to induce a diabetic state (lanes 5-7). In addition, to confirm the viability of the cDNA preparations, amplifications were also performed in parallel, using primers specific for β-actin, from the same first-strand cDNA preparations (B). The primer concentrations were 75 pmol per 50 µl in each reaction. Lane 4 contained ϕX174 / Hae III DNA markers.

C. Ethidium bromide stained 2% agarose gel electrophoresis of the products obtained by PCR with PKD-specific primers (lanes 1, 2 and 4) from template DNA consisting of the PKD expression construct (lane 4) or of first strand cDNA prepared from equal quantities of RNA extracted from rat epididymal fat (lanes 1 and 2). The tissue was excised from two animals which had been treated with streptozotocin to induce a diabetic state and, subsequently, treated with 10 units of a long-acting insulin preparation (Humulin Lente), every 12 hours, for 7 days. Urine was checked in order to confirm the absence of glucose. To confirm the viability of the cDNA preparations, amplifications were also performed in parallel, using primers specific for β-actin, from the same two first-strand cDNA preparations (lanes 5 and 6). The primer concentrations were 75 pmol per 50 µl in each reaction. Lane 3 contained ϕX174 / Hae III DNA markers.

CHAPTER 7

Conclusions

7.1. Analysis and manipulation of the glucagon receptor cDNA

At the outset of this study I was provided with a cDNA clone containing the coding sequence encoding part of the rat hepatic glucagon receptor. An initial BamH I / HinD III analysis, however, suggested to me that this clone might contain a 300 bp intron, and, unfortunately, the results of a number of subsequent restriction digests on this clone confirmed my suspicion. The incorporation of this intronic DNA into the completed construct would have compromised the expression of the receptor (Svoboda *et al.*, 1993a). It is unusual, but certainly not without precedent, for a clone isolated from a cDNA library to contain intronic sequences, implying that despite the formation of the library from poly-adenylated mRNA, a number of these mRNA molecules had not been fully spliced prior to polyadenylation. This phenomenon has been described previously (Lewin, 1985; Svoboda *et al.*, 1993a), and is unlikely to be due to anomalous splicing signals, as the donor and acceptor splice sites of each of the four introns present in the GR genomic sequence do conform to the consensus dinucleotide sequences GT and AG, respectively (Ohshima and Gotoh, 1987). Fortunately a plasmid construct was subsequently obtained, obviating the necessity to use the intron-containing cDNA species.

While the incorporation of a coding sequence for a VSV epitope tag at the 5' end of the glucagon receptor sequence was successfully achieved by PCR, difficulties were encountered in the subsequent ligation of the 832 bp product into the expression vector containing the remainder of the coding sequence of the GR. The possible reasons for this include a failure of efficient restriction digestion at one or both extremities of the PCR product. Such incomplete digestion might have resulted from the degradation of the ends of the amplified products by the exonuclease activity of the proof-reading polymerase during PCR. Another possible explanation for the difficulty encountered in ligating the PCR product into the expression vector was that despite the precautions taken, such as the use of two different restriction enzymes and the inclusion of a phosphatase step, the

cut vector may have re-circularised without the inclusion of the insert. Furthermore, additional difficulties were created on account of the vector containing the GR-encoding sequence being pCDM8, a plasmid which contains few unique restriction sites at the 5' and 3' ends of the inserted cDNA sequence and which necessitates the use of the MC1061/P3 strain of E. coli (Seed, 1987). These bacteria contain an endonuclease which makes plasmid purification and subsequent manipulations difficult, and their use relies upon an antibiotic selection system involving the suppression by a plasmid-encoded suppressor tRNA of premature stop-codons in the antibiotic resistance genes of the P3 episome. This process may, itself, yield false-positive colonies. Thus, in retrospect, in order to facilitate further studies, it might well have been beneficial to sub-clone the coding region of the glucagon receptor from pCDM8 into a more easily manipulated mammalian expression vector, such as pcDNA3 or pSV-Sport1.

7.2. Transient transfection studies

A model system for the investigation of glucagon receptor function was created by transfecting COS-7 cells with the pCDM8 expression plasmid containing the glucagon receptor cDNA. In such transfected cells the GR was found to be functionally expressed, yielding a cAMP response to glucagon which greatly exceeded that observed in untransfected COS cells, with an observed EC_{50} value of 1.8 ± 0.4 nM. In fact, similar values have been reported for the native response seen in intact hepatocytes in the presence of PDE inhibitors: 2 nM (Christoffersen and Berg, 1974), 0.5-3.0 nM (Sonne *et al.*, 1978) and 1.2 nM (Heyworth *et al.*, 1983). That the response observed here was only apparent if the non-selective PDE inhibitor, IBMX (Beavo and Reifsnyder, 1990; Hoey and Houslay, 1990) was added to assays, is likely to reflect the potent activities of endogenous cAMP phosphodiesterases which have been demonstrated in these COS cells (Shakur *et al.*, 1993). Moreover, as IBMX is a reversible, competitive inhibitor of PDE action then the plateau in accumulation reached after about 15 min of exposure to glucagon presumably reflects a steady state where the glucagon-stimulated production of cAMP matches the degradation by the residual PDE activity.

Stimulation of mock or transfected COS cells with 10 nM glucagon failed to lead to a detectable significant stimulation of IP_3 production. This is not surprising, given the current literature regarding the effects of glucagon. For example, in rat hepatocytes, Pittner and Fain were unable to detect any glucagon-induced rise in inositol phosphate production (Pittner and Fain, 1991) and, in those instances where glucagon-stimulated inositol phospholipid hydrolysis has been reported, the magnitude of the reported stimulation has varied between only 18 and 25% (Wakelam *et al.*, 1986; Whipps *et al.*, 1987; Unson *et al.*, 1989).

Glucagon-stimulated hydrolysis of phosphatidylcholine was, however, observed in the present study in the COS cells which had been transfected with GR-encoding cDNA, with a predominance of PtdCho-PLC rather than PtdCho-PLD activity. Thus, glucagon-induced production of DAG would be predicted to occur in these cells, with the implication that the stimulation of these COS cells by glucagon is likely to lead to the activation of PKC and other DAG-activated kinases.

From several lines of evidence it has been inferred that the reduction in the response of adenylyl cyclase to glucagon that can be elicited in hepatocytes by the prior exposure of the cells to this hormone, is mediated by a DAG-activated kinase. In order to gain an insight into the mechanism involved in this desensitisation the intention was to study the PMA-stimulated inhibition of glucagon-stimulated cAMP accumulation in transfected cells.

The unexpected finding, however, was that in neither COS nor HEK293 cells, transfected with the rat hepatic glucagon receptor, was any PMA-stimulated inhibition of glucagon-stimulated cAMP accumulation observed. This contrasted with the published observation that treatment of hepatocytes with the phorbol ester, PMA, caused an inhibition of glucagon-stimulated cyclic AMP accumulation (Heyworth *et al.*, 1984). From subsequent experiments, discussed in chapter 4, the lack of detectable inhibition by PMA appeared to reflect neither any potent protein phosphatase activity nor the quantity of DNA with which the COS cells were transfected.

It was subsequently considered that the PMA-induced inhibition of glucagon-stimulated cAMP accumulation might depend upon a specific cellular signalling component that is absent in COS cells but which is present in hepatocytes and is capable of being activated by DAG or phorbol ester. It appeared that the various members of the protein kinase C (PKC) family were the most likely candidates for such a component, and that the inability to observe PMA-mediated inhibition of glucagon-stimulated cAMP accumulation in COS cells might have been due to the inadequate expression of a particular PKC isoform.

Immunoblotting studies had previously shown that hepatocytes express PKC-α, βII, ε and ζ isoforms, but not PKC-βI, γ, δ or η (Tang *et al.*, 1993). In the experiments discussed in chapter 4, PKC-α, βII, ε and ζ were all detected in native COS cells, although the levels of PKC-βII appeared to be lower in COS cells than in hepatocytes. In order to increase the expression levels of PKC isoforms it was decided to co-transfect COS cells with the glucagon receptor-encoding cDNA and with plasmids encoding each of the three PMA-responsive PKC isoforms known to be present in hepatocytes (PKC-α, βII and ε). Over-expression of the appropriate PKC isoform, following the transfection of COS cells with the respective cDNA, was demonstrated, but in no instance did co-transfection with the plasmids encoding these PKC isoforms lead to the reconstitution of PMA-induced inhibition of glucagon-stimulated cyclic AMP accumulation. It was therefore concluded that the co-expression in the glucagon receptor-transfected COS cells of any individual PKC isoform present in hepatocytes, was not, in itself, sufficient to confer PMA-induced inhibition of the glucagon-stimulated cAMP accumulation.

In order to identify the PMA-activated kinase responsible for the glucagon-induced inhibition of the response of hepatocytes to glucagon, the cDNA encoding the glucagon receptor was then co-transfected into COS-7 cells with plasmids encoding each of three additional PMA-responsive kinases. These kinases, GRK2, GRK3 and PKD, were known to possess substrate specificities and structural characteristics distinct from those of PKC isoforms. Although transcription of the cDNAs encoding GRK2 and GRK3

was confirmed using RT-PCR, no PMA-induced inhibition of the glucagon-stimulated adenylate cyclase response could be detected. Intriguingly, however, co-expression of protein kinase D (PKD) with the glucagon receptor led to a striking PMA-induced inhibition of glucagon-stimulated cAMP accumulation in these co-transfected COS cells. The inability of a transfected inactive mutant PKD enzyme to confer this inhibition suggests that the effect is indeed due to the catalytic activity of the kinase. Furthermore, from the determination of the cellular PDE activity, it was ascertained that the inhibition of cAMP accumulation was directed at the level of cAMP synthesis rather than at its degradation.

7.3. Investigations into the mechanism of the PKD-mediated inhibition

The mechanism by which glucagon-stimulated intracellular cAMP accumulation might be inhibited by PMA-induced PKD activation was investigated by measuring the effect of PMA treatment on both the level of specific binding of [^{125}I]-glucagon to COS cell membranes and the stimulation of cAMP accumulation elicited by cholera toxin or forskolin. From these experiments it appeared that the action of PKD may reduce glucagon-stimulated cAMP accumulation by an action which reduces the ability of the receptor-G_s complex to activate the catalytic unit of adenylate cyclase.

Although PMA treatment markedly attenuated the glucagon-stimulated cAMP accumulation in COS cells co-transfected with cDNAs encoding the GR and PKD, it did not elicit a statistically significant reduction in the isoprenaline-stimulated cAMP accumulation in COS cells co-transfected with cDNAs encoding PKD and either the β_2-AR or the β_3-AR. This is consistent with the possibility that, in the co-transfected COS cells in which the inhibitory effect is observed, the glucagon receptor, itself, is a target of PKD.

7.4. Inferences regarding the possible roles of PKD in hepatocytes

The above findings have important implications for our understanding of glucagon action in hepatocytes. Firstly, in view of the activation of PKD by DAG, it is conceivable that this kinase plays an important role in hepatocytes. In these cells it may effect the desensitization of the glucagon receptor and possibly a number of other GPCRs, in response to the generation of DAG induced by glucagon and other hormones such as vasopressin. Secondly, desensitization of the glucagon response, specifically, may play an important additional role, responding not only to hormone-stimulated lipid signalling, but also to DAG generated *de novo* in response to hyperglycaemia.

The detection of a PKD-specific signal resulting from RT-PCR performed on RNA isolated from fat was, in itself, a novel finding. Intriguingly, the PKD-specific PCR signal obtained using hepatocytes and adipose tissue of streptozotocin diabetic rats was of much lower intensity than that obtained using samples from control rats. Moreover, this difference appeared to be eliminated upon the treatment of the diabetic rats with insulin, suggesting that a reduction in the level of PKD-specific mRNA may be a consequence of hyperglycaemia itself.

A number of potential mechanisms might be envisaged by which the streptozotocin-induced diabetic state could bring about the reduction in PKD mRNA. One possibility is that the *de novo* DAG synthesis and PKC activation that has been shown in a number of cell types, including rat adipocytes, to be a consequence of hyperglycaemia (Hoffman *et al.*, 1991; Wolf *et al.*, 1991; Inoguchi *et al.*, 1992; Berti *et al.*, 1994; Williams, 1995), could effect the activation of Raf kinase (Kolch *et al.*, 1993; Marquardt *et al.*, 1994) and, consequently, MAPK, with the subsequent modulation by phosphorylation of transcription factor activity (Meek and Street, 1992). An alternative mechanism, however, might be envisaged, if the gene encoding PKD were to constitute one of those whose transcription is markedly stimulated by insulin itself. In such a situation, the diminution in insulin production resulting from streptozotocin treatment (Gawler *et al.*, 1987) could lead to an abrogation of insulin-stimulated transcription (O'Brien and

Granner, 1991) of the kinase. The mechanisms by which PKD gene transcription is regulated, however, remain to be characterised.

An inhibition, by 51%, of the glucagon-induced desensitization of the response to glucagon was observed in hepatocytes (Savage *et al.*, 1995) in the presence of the PKC inhibitor, chelerythrine (Herbert *et al.*, 1990). Interestingly, recent studies on the regulation of PKD expressed in Swiss 3T3 and Rat-1 cells have indicated that the activation of PKD *in vivo* may be dependent upon its phosphorylation by PKC isoforms (Zugaza *et al.*, 1996). The chelerythrine-induced inhibition of the desensitization might therefore be a consequence of the inhibition by this compound of the PKC isoforms whose action is required for the activation of PKD. The alternative possibility, that chelerythrine can inhibit PKD directly, remains to be investigated, although other inhibitors that exhibit specificity towards PKC isoforms, such as calphostin C and Ro 31-8220, appear to have no effect on the activity of PKD *in vitro* (Johannes *et al.*, 1995; Zugaza *et al.*, 1996).

7.5. Postulated physiological and pathological importance of PKD

As PKD is a DAG-activated kinase (Valverde *et al.*, 1994; Rozengurt *et al.*, 1995; Van Lint *et al.*, 1995), and as the results discussed above have indicated that its action can attenuate the response of adenylate cyclase to glucagon, I propose that PKD may mediate a homeostatic mechanism involved in glycaemic control. Thus, the activation of PKD upon *de novo* DAG synthesis might occur physiologically in response to hyperglycaemia and lead to an attenuation of glucagon-stimulated gluconeogenesis and glycogenolysis (fig. 7.1.). Conceivably, a glucagon-induced desensitisation of the response of adenylate cyclase to glucagon could operate either in concert with, or independently of, this postulated negative feedback mechanism.

If PKD does play such a role in glucose homeostasis, the diminished expression of the kinase, which appears to be associated with the diabetic state, may have important implications for our understanding of the pathogenesis of the hyperglycaemia that is

associated with untreated diabetes mellitus. Thus, in untreated diabetic patients, the impairment of such a PKD-mediated regulation of the glucagon-induced glucose production, could have the consequence of further elevating blood glucose levels, potentially exacerbating the condition, and thus contributing to an increased tendency to hyperglycaemic episodes. Supporting such a hypothesis is the reported finding that an inappropriate elevation of the hepatic glucose output is indeed a significant pathogenic factor in patients with NIDDM (Baron *et al.*, 1987).

The apparent reduction in the level of PKD mRNA and the inhibitory role of PKD proposed above are both consistent with previous observations regarding glucagon-stimulated cAMP accumulation in rat hepatocytes. Firstly, in the hepatocytes of rats which have been treated with streptozotocin, thus rendering them diabetic, the magnitude of the response of adenylate cyclase to glucagon has been shown to be approximately 70% greater than that which is observed in hepatocytes isolated from the hepatocytes of healthy rats (Gawler *et al.*, 1987). Such an increase could reflect a loss of a regulatory tonic inhibition by PKD, although alternative mechanisms have been described involving the loss of functional G_i (Gawler *et al.*, 1987; Murphy *et al.*, 1989; Bushfield *et al.*, 1990a; Morris *et al.*, 1996). Secondly, the ability of glucagon pre-exposure to induce desensitization of the subsequent stimulation of adenylate cyclase by this hormone in hepatocytes isolated from control rats has been shown to be 53% greater than in hepatocytes from diabetic animals (Murphy *et al.*, 1989). Again these results would be consistent both with a role for PKD in the desensitization process and with the reduced expression of this kinase in the diabetic state.

7.6. Future studies

Many aspects of PKD expression and functional characteristics remain to be investigated. These include its precise mechanism of activation, substrate preference and role in cellular signalling and disease. For instance, it is conceivable that the activation of PKD is associated with a translocation in a manner analogous to the membrane translocation of GRK2. The latter occurs by a mechanism that is believed to involve an

interaction of G protein βγ sub-units, released from the α sub-unit following the activation of G_s by the ligand-occupied receptor, with the pleckstrin homology (PH) domain of the kinase (Pitcher *et al.*, 1995b). Thus, although PKD is known to be activated by DAG, and is thus likely to be stimulated following the hormone-induced stimulation of either PC-PLC or PI-PLC, it is interesting to speculate that, given the possession by PKD of a PH domain, the mechanism of activation of the kinase may also involve its translocation to the plasma membrane by an interaction with G protein βγ sub-units. In fact, as a prelude to the testing of this hypothesis, I have sub-cloned the cDNAs which encode the $G\beta_1$ and $G\gamma_2$ sub-units into expression vectors. This should permit the future determination of whether co-expression of these sub-units with PKD can lead to membrane translocation of the kinase in COS cells.

Particularly desirable would be an experiment designed to test the hypothesis proposed above that PKD mediates a hyperglycaemia-induced inhibition of glucagon-stimulated cAMP accumulation in hepatocytes. If viable hepatocytes could be sustained for a sufficient period, then it should be possible to test whether prolonged exposure of these cells to raised extracellular glucose (20-25 mM) would cause an attenuation of the subsequent response of adenylate cyclase to glucagon. Incubation with a PKD-specific antisense oligonucleotide could then be used to determine whether the expression of this kinase is a pre-requisite for the inhibitory effect.

Finally, the mechanisms by which PKD effects an inhibition of glucagon-stimulated cAMP accumulation could be investigated by studying the phorbol-ester stimulated phosphorylation of the glucagon receptor, G proteins or adenylate cyclase isoforms *in vitro* or in transfected COS cells. In fact, as mentioned above, analysis of the glucagon receptor coding sequence has identified a potential phosphorylation site for PKD in the C-terminal tail. Thus, using the PCR-based strategy outlined in figure 7.2, the cDNA encoding the receptor could be subjected to site-directed mutagenesis to change the amino acid sequence in this region from RMASS to RMAAA, thus eliminating the two potential target residues, Ser 432 and Ser 433. The mutant cDNA construct could

then be employed in subsequent transfection or phosphorylation experiments to determine the effects of these amino acid substitutions.

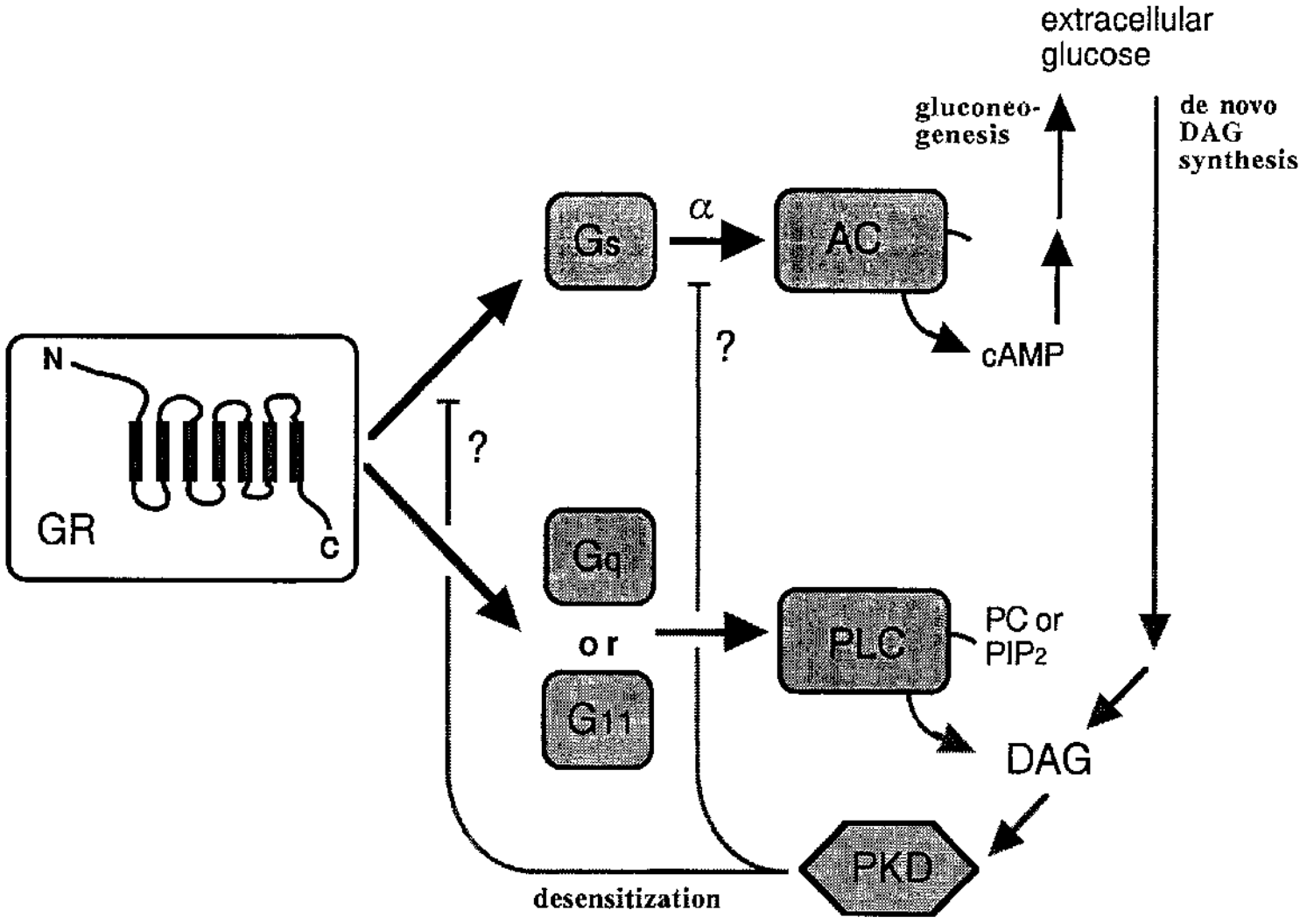

extracellular
glucose
gluconeo-
genesis
de novo
DAG
synthesis
Gs
α
AC
cAMP
?
N
GR
C
?
Gq
or
G11
PLC
PC or
PIP2
DAG
PKD
desensitization

Figure 7.1. The proposed role of PKD in a homeostatic mechanism regulating hepatocyte gluconeogenesis

It is postulated that a raised extracellular glucose level may, via the induction of *de novo* synthesis of diacylglycerol (DAG), lead to the stimulation of PKD. This, in view of the data presented in the preceding chapters, is proposed to cause the inhibition of glucagon-stimulated cAMP accumulation, thus negatively regulating gluconeogenesis in these cells. Several studies have demonstrated hyperglycaemia-induced synthesis of DAG (Hoffman *et al.*, 1991; Wolf *et al.*, 1991; Inoguchi *et al.*, 1992; Berti *et al.*, 1994; Williams, 1995), which has been shown to activate PKD (Valverde *et al.*, 1994; Rozengurt *et al.*, 1995; Van Lint *et al.*, 1995). The consequence of a loss of PKD expression in the diabetic state would therefore be a loss of this regulatory mechanism, with a resulting exacerbation of the diabetes-associated hyperglycaemia.

In this figure, the arrows depicted in black represent steps which have previously been demonstrated to occur, while those indicated in grey represent steps in a hypothetical pathway, proposed on the basis of the evidence presented in the preceding chapters.

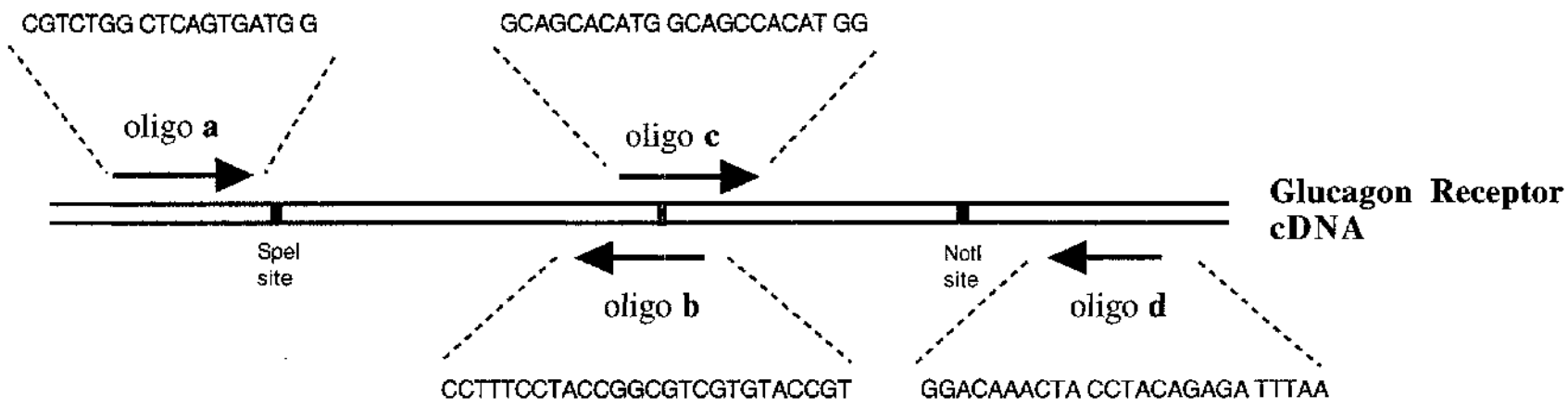

CGTCTGG CTCAGTGATG G
GCAGCACATG GCAGCCACAT GG
oligo a
oligo c
Glucagon Receptor cDNA
SpeI site
NotI site
oligo b
oligo d
CCTTTCCTACCGGCGTCGTGTACCGT
GGACAAACTA CCTACAGAGA TTTAA

Figure 7.2. Strategy for the site-directed mutagenesis of the coding sequence of the rat hepatic glucagon receptor.

This scheme is based on the technique of overlap extension PCR (Ho *et al.*, 1989). Two pairs of PCR oligonucleotide primers (a+b and c+d) are designed in such a way as to result in complementarity between oligonucleotides b and c. Thus, the PCR reactions generates two DNA products with overlapping ends. The novel DNA sequence containing the desired specific nucleotide substitutions is incorporated into the region of overlap. In a subsequent 'fusion' reaction, the overlapping ends are permitted to anneal, allowing the 3' overlap of each strand to serve as a primer for extension, with the other member of the overlapping primer pair serving as template. The product of this fusion reaction may then be amplified by successive rounds of PCR, thus incorporating the mutant sequence into all of the resulting DNA copies. By this method, using the indicated oligonucleotide primers, the putative site for phosphorylation by PKD may be mutated from RMASS to RMAAA thus eliminating the target residues, Ser^{432} and Ser^{433}.

APPENDIX

Attempts to obtain a glucagon receptor-specific antiserum

A.1. Testing antisera raised against GR-based peptides

In order to raise GR-specific antisera, a previous worker in our laboratory had obtained synthetic peptides corresponding to the regions of the GR amino acid sequence indicated in table A.1. These peptides were conjugated, as haptens, to the carrier molecule, keyhole limpet haemocyanin (KLH), prior to injection into New Zealand White rabbits. Pre-immune sera were obtained from these rabbits prior to peptide injection. Initially, the antisera from rabbits YE625 and YE627 raised against the C-terminal peptides 4 and 5, respectively, were tested in parallel with the respective pre-immune sera, in order to determine which immunoreactive species could be detected in liver membranes by the immune but not by the pre-immune antisera. The YE625 immune serum was found to detect immunoreactive species of 75 and 72 kDa which were, however, also detectable, albeit at very low intensity, by the respective pre-immune serum (figure A.1.). In addition, the YE625 immune serum detected a species of 40 kDa which was not detected by the pre-immune serum. This, however, was considered to be of too high a mobility to represent the glucagon receptor, which, in early cross-linking studies, had been observed to migrate with an apparent molecular mass of approximately 63 kDa (Iyengar and Herberg, 1984).

The YE627 immune serum, in contrast, detected two immunoreactive species of apparent molecular weights 88 kDa and 65 kDa which were not detectable by the respective pre-immune serum (figure A.2.). The 65 kDa species, whose signal was of much greater intensity, was considered to possibly represent the glucagon receptor.

In subsequent immunoblots, to test its specificity, this YE627 antiserum was again used to probe liver membrane proteins, after pre-incubation of the antiserum with either peptide 4 (non-specific) or the peptide against which this antiserum was raised, peptide 5, as described by Tang et al. (Tang *et al.*, 1993). In such immunoblots, however, it was found that the detection of the two immunoreactive species was impaired by both of

these peptides, with the non-specific peptide 4 apparently having the greatest inhibitory effect (figure A.3.).

Subsequently, the YE627 pre-immune and immune antisera were used to probe extracts of untransfected or GR cDNA-transfected COS-7 cells. Thus crude lysate, membrane and cytosol fractions were prepared from these COS cells and 100 µg protein applied to individual wells of an 8% SDS poly-acrylamide gel. The immunoblot again revealed the presence of an immunoreactive species with an apparent molecular weight of 65 kD, in the crude lysate and membrane fractions, that was detectable with the immune serum but at relatively lower intensity with the pre-immune serum. This species, however, was observed to be of the same intensity in both untransfected and GR cDNA-transfected COS cell extracts (figure A.4.).

Subsequently, a series of Western blots of GR-transfected and control COS cells undertaken with the other antisera raised against GR-peptides in rabbits YE619-628, with the exception of the antisera from rabbits YE623 and YE624 which showed negligible immunoreactivity by ELISA (performed by Dr. A. Campbell in our department). It was found that the antisera obtained from rabbits YE621, 622, 625, 628 (injected with peptides 2, 2, 4 and 5, respectively) detected immunoreactive species of approximately 60-65 kDa (figure A.5.). In none of these immunoblots, however, was the signal any less intense in the untransfected control COS cells than in the GR cDNA-transfected COS extract. This was unlikely to have been a result of failure of GR expression in the transfected cells, as such cells exhibited a marked adenylate cyclase response to glucagon (see chapter 4). Moreover, following the electrophoretic separation, transfer to nitrocellulose and protein staining of the proteins extracted from both the transfected and control cells, an additional band was visible in the transfected COS cell extract with an apparent molecular weight of 65 kDa (figure A.6.). As this was the only protein species to be detected in transfected but not in control COS, it is likely that this reflected the expression of the glucagon receptor cDNA. Interestingly, a very intensely stained protein band was detected in the hepatocyte extract, migrating with a similar MWt to that of the glucagon receptor. The abundance of this protein, which may represent albumin,

in hepatocyte preparations may account for the species detected previously by the antisera raised against glucagon receptor peptides, which may therefore have detected this protein non-specifically.

A.2. Testing antisera raised against a GST-GR fusion protein

A fusion protein was engineered by the in-frame fusion of the entire C-terminal tail of the glucagon receptor to the GST-encoding sequence of the pGEX-3X vector. The GST-GR fusion protein was subsequently expressed in E. coli, purified and injected into two rabbits. The antisera, Ig7 and Ig8, thus obtained were immuno-purified by adsorption onto a GST column, in order to remove GST-reactive antibodies. This work was done by Dr. Pascal Julien in our laboratory.

The antisera obtained pre- and post-purification were then used to perform immunoblots of hepatocyte homogenate, and of homogenate, P1 and P2 fractions of both transfected and control COS cells. In addition, purified extracts from E. coli expressing cDNA encoding either GST-GR or GST were included, serving as positive and negative controls, respectively. Both of the unpurified antisera yielded strong signals corresponding to both the predicted 34 kDa GST-GR fusion protein and the 28 kDa GST protein, implying the presence in both antisera of GST-reactive antibodies (results not shown). Following purification, while both antisera yielded a strong signal corresponding to the predicted 34 kDa GST-GR fusion protein, no signal (Ig8) or one of only relatively low intensity (1g7) was detected corresponding to the 28 kDa GST protein, thus confirming that the GST-reactive antibodies had been removed (figure A.7.). Interestingly, an immunoreactive species of 74 kDa was detected in both the GR-GST and the GST extracts by the purified Ig7 antiserum. This is likely to reflect a bacterial protein co-purified with the fusion protein by the glutathione beads, as a similar species has been detected in our laboratory with a different fusion protein which was purified by the same method (Dr. I. Wilkinson, personal communication). Unfortunately, despite the detection of the GR-GST fusion protein by both the Ig7 and

Ig8 antisera following purification, no immunoreactive species were detected by either of the two antisera in the hepatocyte or COS cell extracts (figure A.7.).

A.3. Testing the ability of fusion protein antisera to immunoprecipitate the GR

The antisera were also tested for their ability to immunoprecipitate the GR, as measured by a [^{125}I]-glucagon binding assay, from a hepatocyte membrane fraction. It was found however that the binding activity immunoprecipitated by neither of the antisera was significantly greater than that obtained with the pre-immune sera (figure A.8.).

A.4. Phosphatase treatment of membrane proteins prior to immunoblotting

The possibility was considered that phosphorylation of the C-terminal tail of the GR by a cellular kinase may have prevented recognition by the antisera Ig7 and Ig8, which were raised against a fusion protein containing the sequence corresponding to this region. To address this hypothesis, 200 μg of membrane proteins prepared from either untransfected or GR cDNA-transfected COS cells were treated, where indicated, in a total volume of 100 μl with 20 units of alkaline phosphatase for 1 hour. This was done following a 15 min pre-incubation either at 70 °C to inactivate any kinases isolated with the membranes, or on ice (control). These samples were then immunoblotted as before with Ig7 and Ig8 antisera. Despite these treatments, no immunoreactive species of a MWt corresponding to the GR became detectable with either of the antisera in the transfected COS cells (figure A.9.).

A.5. Re-coupling of peptides to the carrier molecule KLH

To address the possibility that the previous conjugation of the peptides to the carrier molecule, KLH, may have been ineffective, the work undertaken by a previous worker in our laboratory was repeated. The peptides corresponding to sequences within the C-terminal tail of the GR (peptides 4 and 5) were chosen to undergo this process. This

conjugation was done using a modification (as described in section 2.3.2.2.) of the protocol utilised previously in order to allow more time for the coupling of the hapten to the carrier.

The coupled peptides was then injected into male New Zealand White rabbits in complete Freund's adjuvant, and again, 10 and 21 days later, in incomplete Freund's adjuvant. The antisera obtained 10 days later were tested by immunoblotting. While these antisera detected a number of immunoreactive species in both GR-transfected and untransfected COS cell, no species was detected in the transfected cells with the intensity predicted for an over-expressed protein (figure A.10.A and B).

Discussion

In testing the antisera raised against peptides corresponding to regions of the GR amino acid sequence, it was found that the antiserum derived from rabbit YE627 detected an immunoreactive species in hepatocytes of an apparent MWt similar to that of the GR. It could, perhaps, be envisaged that the inability to block the detection of this species by the prior incubation of the antiserum with the peptide against which the antiserum was raised, was the result of the possible adoption of a different conformation by this peptide in solution from that which it adopts when conjugated to KLH. However, the pre-incubation of an antiserum with the peptide against which it was raised prior to immunoblotting is a method which has been used, for example, to demonstrate the specificity of PKC isoform-specific antisera in our laboratory (Tang *et al.*, 1993) and by others (Wetsel *et al.*, 1992).

Furthermore, the subsequent inability of this antiserum to detect the GR expressed in transfected COS cells more strongly than in untransfected COS cells, together with the inability of peptide competition to block the detection of the hepatocyte protein by this antiserum, suggests that the species detected by this antiserum was not, in fact, the GR but some other cross-reacting protein.

It is intriguing that none of the antisera raised against the peptides or the fusion protein was able to detect an immunoreactive species of the MWt of the GR more strongly in COS cells transfected with the cDNA encoding the GR than in control COS cells. This may be due to one or more of a number of possible reasons. For instance it is conceivable that the antisera do recognise epitopes of the GR but that these antigenic determinants are masked by one or more post-translational modifications. One such modification, glycosylation of the receptor, is indeed known to occur, as evidenced by the step-wise increase in electrophoretic mobility of the GR which follows endo-β-N-acetylglucosaminidase F treatment of the receptor (Iyengar and Herberg, 1984) and by the presence of potential sites for glycosylation in the extracellular, N-terminal region of the amino acid sequence of the rat hepatic GR. While such glycosylation of the receptor, might prevent recognition of extracellular epitopes by antisera raised against peptides 1-3 it should not have prevented those antisera raised against peptides 4, peptide 5 and the fusion protein, from interacting with the antigenic determinants located on the intracellular aspect of the receptor.

Alternatively, phosphorylation of the receptor in a manner similar to that known to occur for other G-protein coupled receptors (GPCRs), might be envisaged to block recognition of epitopes located on the intracellular loops and C-terminal tail of the receptor. However, alkaline phosphatase treatment of hepatocyte membranes did not lead to the detection of the GR by either of the antisera raised against the fusion protein, implying that the failure of these antisera to successfully immunoblot the GR was not a result of stearic hindrance of the interaction by phosphate groups. An additional manner in which antigenic determinants might be obscured is by lipid modification of the receptor. Indeed, it is believed that, in several GPCRs, palmitoylation of one or more cysteine residues in the C-terminal region of the receptor occurs, and that such lipid moieties may even insert themselves into the plasma membrane in such a manner as to create a fourth intracellular loop (Baldwin, 1994). The rat hepatic GR does, in fact, contain three cysteines in the C-terminal tail at positions 444, 449 or 465 of the open reading frame, but they do not reside in sequences that conform to the consensus for

palmitoylation. Finally, as immunoblotting was performed under denaturing conditions, in the presence of the reducing agent, β-mercaptoethanol, it is unlikely that the epitopes were masked by secondary or tertiary protein structure, nor by any possible complex formation with, for example, the G protein, Gs.

Rather than the epitopes of the GR being masked, it is possible that the peptides failed to act as effective immunogens. This is unlikely to be due to inadequate length of the sequences as although four of the peptides were 12 amino acids or less, the other was of 16 residues in length. One possibility is that the peptide synthesis was incomplete, resulting in two or more species of individual peptides, of varying lengths. However, HPLC analysis performed recently on these peptides at the author's request, indicated that this was not the case, with only a single peak recorded for each peptide. Alternatively, it is conceivable that the coupling process, itself, failed to result in effective conjugation of the peptide to the carrier molecule, KLH, resulting in the presence of only unconjugated hapten.

It was, perhaps, not surprising that none of the antisera raised against the peptides or the fusion protein was able to detect the glucagon receptor effectively, in view of the paucity of available antisera to any GPCR. In fact, in instances when the successful immunodetection of such receptors has been described this has been by virtue of the incorporation of epitope tags into the respective coding sequences (Oppermann *et al.*, 1996). This strategy was employed in the work described in chapter 3.

Table A.1. Amino acid sequences and corresponding locations of the synthetic peptides based on the primary sequence of the glucagon receptor

Peptide	Length	Sequence (residues)	Location on GR	Rabbit
1	12	YSQKIGDDLSVS(C) (203-214)	extracellular loop	619+620
2	12	WTSNDNMGFWWI(C) (296-307)	extracellular loop 2	621+622
3	9	AQGTLRSTK(C) (374-382)	extracellular loop 3	623+624
4	10	SLPRLADSPT(C) (476-485)	C terminal tail (distal)	625+626
5	16	AELLRRWRRWQEGKAL(C) (410-425)	C terminal tail (proximal)	627+628

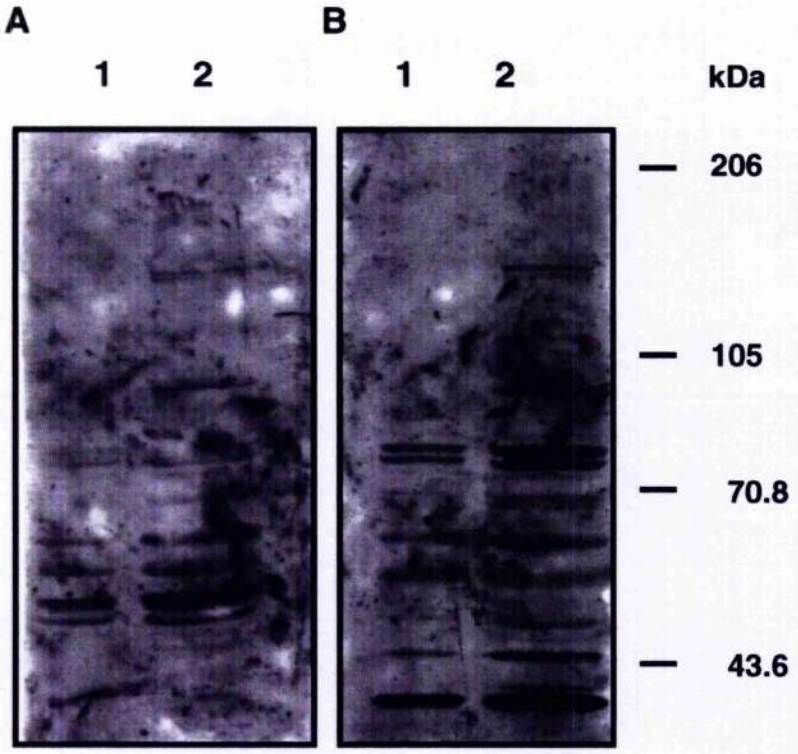

A
B
1
2
1
2
kDa
206
105
70.8
43.6

Figure A.1. Immunoblot of liver membranes using as primary antibody either pre-immune serum or an antiserum raised against peptide 4 of the glucagon receptor

100 µg (lane 1) or 400 µg (lane 2) of liver plasma membrane proteins were boiled in Laemmli sample buffer and subjected to electrophoresis on an 8% polyacrylamide SDS gel prior to transfer to nitrocellulose and blocking in 5% (w/v) dried milk protein. The nitrocellulose blots were then incubated for 2 hours at room temperature with either pre-immune (A) or immune (B) serum of rabbit YE625, diluted 1 in 100. Following the removal of unhybridised antibodies by successive washing, the blots were incubated in HRP-conjugated anti-rabbit IgG, and detection performed by enhanced chemiluminescence.

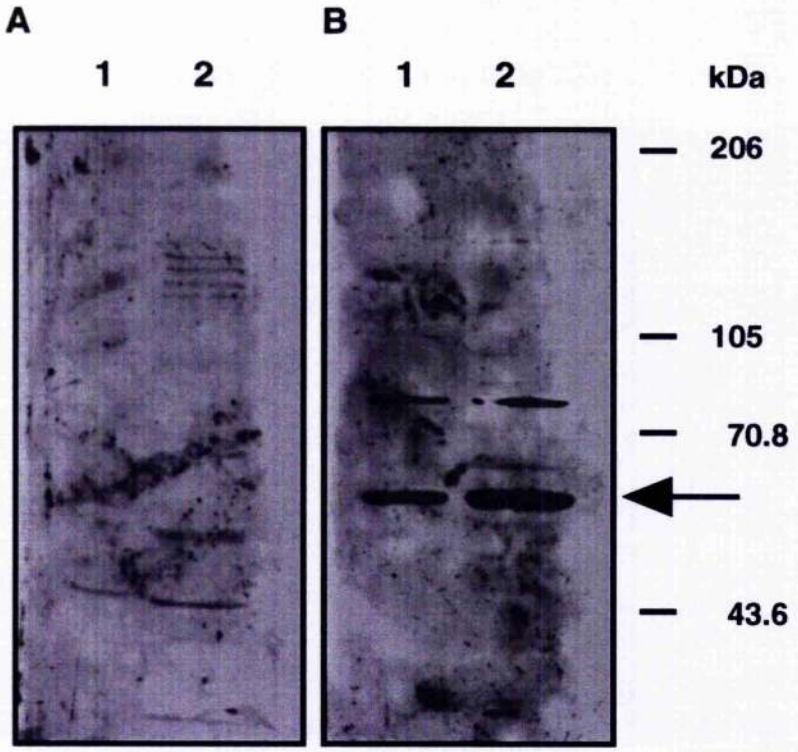

A
1
2
B
1
2
kDa
206
105
70.8
43.6

Figure A.2. Immunoblot of liver membranes using as primary antibody either pre-immune serum or an antiserum raised against peptide 5 of the glucagon receptor

100 μg (lane 1) or 400 μg (lane 2) of liver plasma membrane proteins were boiled in Laemmli sample buffer and subjected to electrophoresis on an 8% polyacrylamide SDS gel prior to transfer to nitrocellulose and blocking in 5% (w/v) dried milk protein. The nitrocellulose blots were then incubated for 2 hours at room temperature with either pre-immune (A) or immune (B) serum of rabbit YE627, diluted 1 in 100. Following the removal of unhybridised antibodies by successive washing, the blots were incubated in HRP-conjugated anti-rabbit IgG, and detection performed by enhanced chemiluminescence. The arrow indicates the immunoreactive species considered to possibly represent the glucagon receptor.

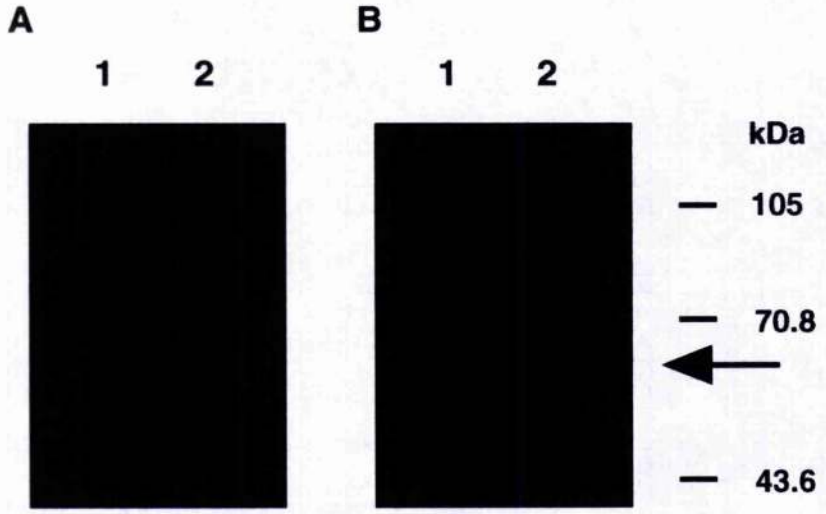
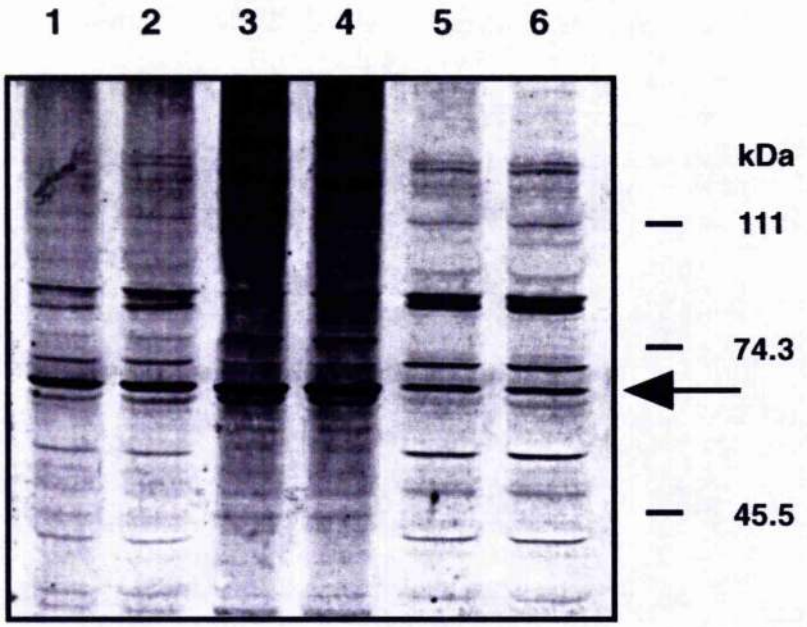

A
1
2
B
1
2
kDa
105
70.8
43.6

Figure A.3. The effect of peptide competition on immunodetection by YE627 antiserum

400 μg (lane 1) or 100 μg (lane 2) of liver plasma membrane proteins were boiled in Laemmli sample buffer and subjected to electrophoresis on an 8% polyacrylamide SDS gel prior to transfer to nitrocellulose and blocking in 5% (w/v) dried milk protein. The nitrocellulose blots were then incubated for 2 hours at room temperature with the immune serum of rabbit YE627 following pre-incubation of the anti-serum with either peptide 4 (A) or the peptide against which the antiserum was raised, peptide 5 (B). Such pre-incubations were done by mixing the anti-serum with a 1 mg/ml solution of the peptide in the ratio 1:1, and leaving for 1 hour, as described previously (Tang *et al.*, 1993). Following incubation of the nitrocellulose with the primary antibody, unhybridised antibodies were removed by successive washing, the blots were incubated in HRP-conjugated anti-rabbit IgG, and detection was performed by enhanced chemiluminescence. The arrow indicates the immuno-reactive species which was detected despite the pre-incubation with the specific peptide.

A



B

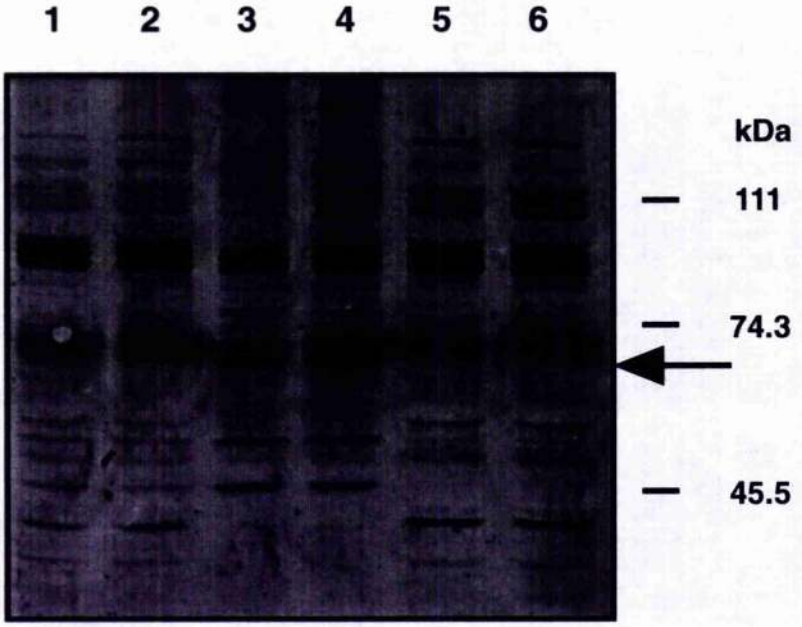


Figure A.4. Immunoblot of COS cell extracts using as primary antibody either pre-immune serum or an antiserum raised against peptide 5 of the glucagon receptor

From COS-7 cells which were either glucagon receptor cDNA-transfected (lanes 1, 3 and 5) or mock-transfected (lanes 2, 4 and 6), 100 μg of protein of crude lysate (lanes 1 and 2), membranes (lanes 3 and 4) or cytosol (lanes 5 and 6) were boiled in Laemmli sample buffer and subjected to electrophoresis on an 8% polyacrylamide SDS gel. Subsequently, the proteins were transferred to nitrocellulose and blocking was performed by incubation in 5% (w/v) dried milk protein. The nitrocellulose blots were then incubated for 2 hours at room temperature with either immune (A) or pre-immune (B) serum of rabbit YE627, diluted 1 in 100. Following the removal of unhybridised antibodies by successive washing, the blots were incubated in HRP-conjugated anti-rabbit IgG, and detection performed by enhanced chemiluminescence. The arrow indicates the immuno-reactive species discussed in the text, which was detected more strongly by the immune than the pre-immune serum, but was equally intense in the extracts of the transfected and control COS cells.

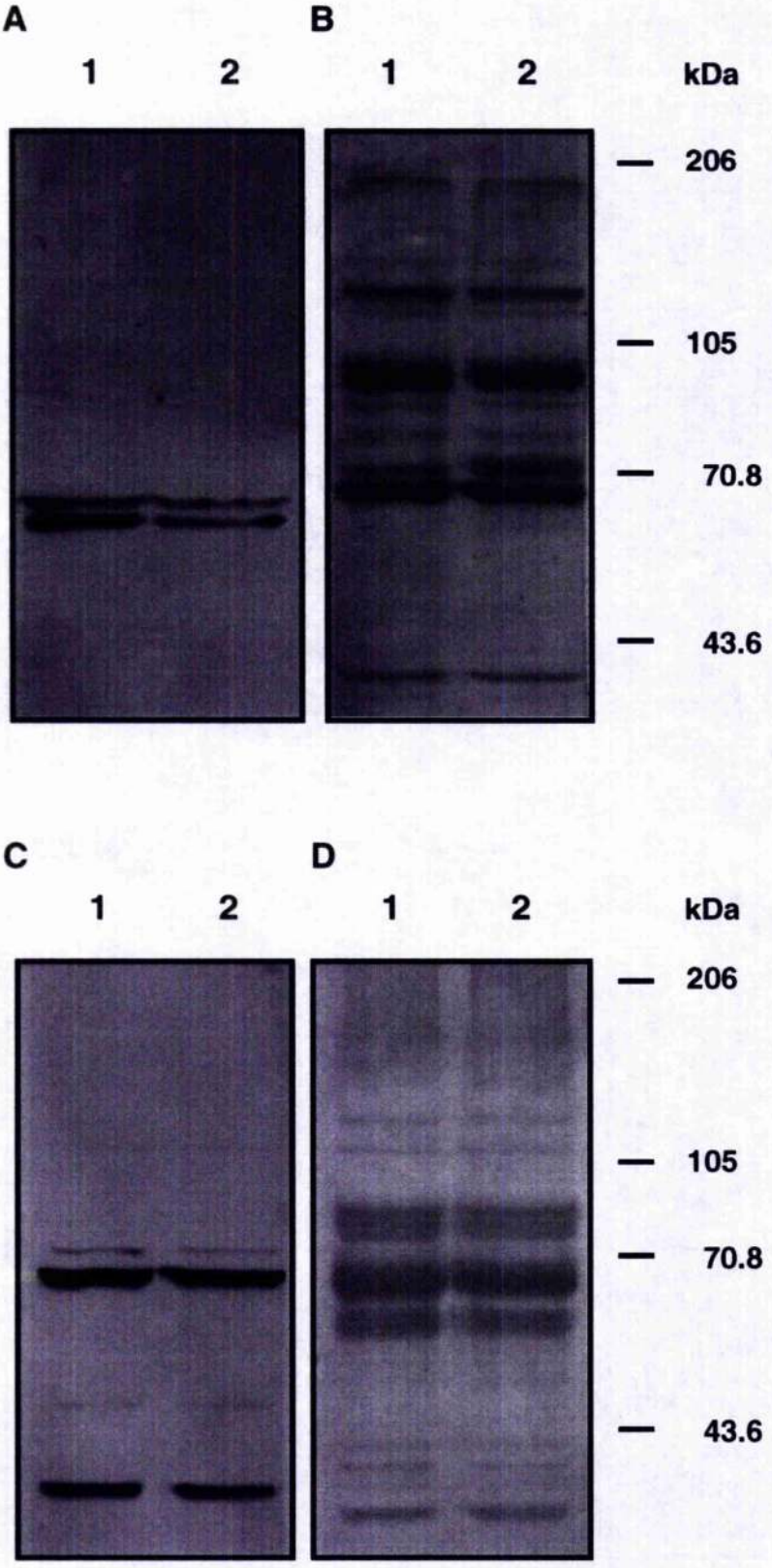

A
B
1
2
1
2
kDa
206
105
70.8
43.6
C
D
1
2
1
2
kDa
206
105
70.8
43.6

Figure A.5. Immunoblots of COS cell extracts using as primary antibodies, antisera YE621, YE622, YE625 and YE628

From COS-7 cells which were either mock-transfected (lanes 1) or glucagon receptor cDNA-transfected (lanes 2), 100 μg of protein of crude lysate was boiled in Laemmli sample buffer and subjected to electrophoresis on an 8% polyacrylamide SDS gel. Subsequently, the proteins were transferred to nitrocellulose and blocking was performed by incubation in 5% (w/v) dried milk protein. The nitrocellulose blots were then incubated for 2 hours at room temperature with antiserum from rabbit YE621 (A), YE622 (B), YE625 (C) or YE628 (D), diluted 1 in 100. These antisera had been raised against GR peptides 2, 2, 4 and 5, respectively. Following the removal of unhybridised antibodies by successive washing, the blots were incubated in HRP-conjugated anti-rabbit IgG, and detection performed by enhanced chemiluminescence. In each case, the immuno-reactive species were observed to be of equal intensity in the extracts of the transfected and control COS cells.

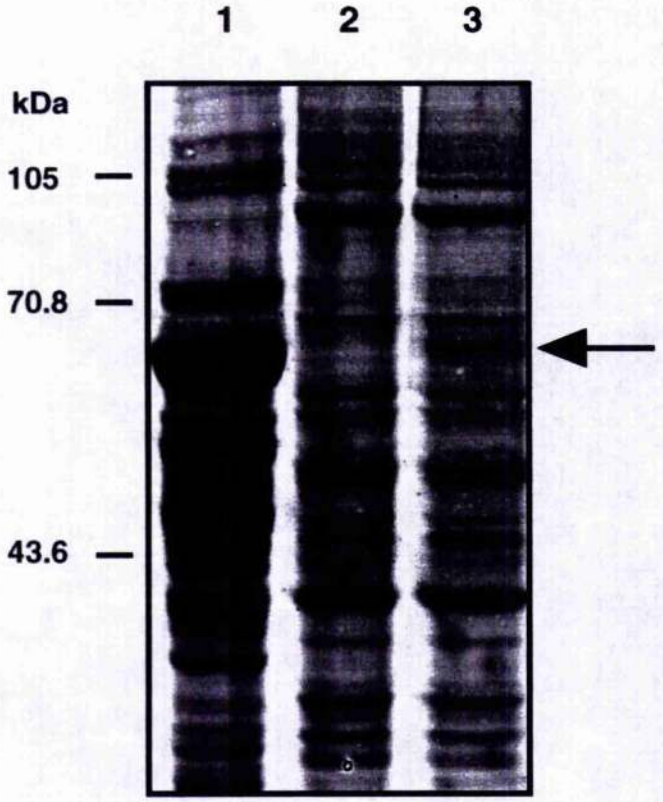
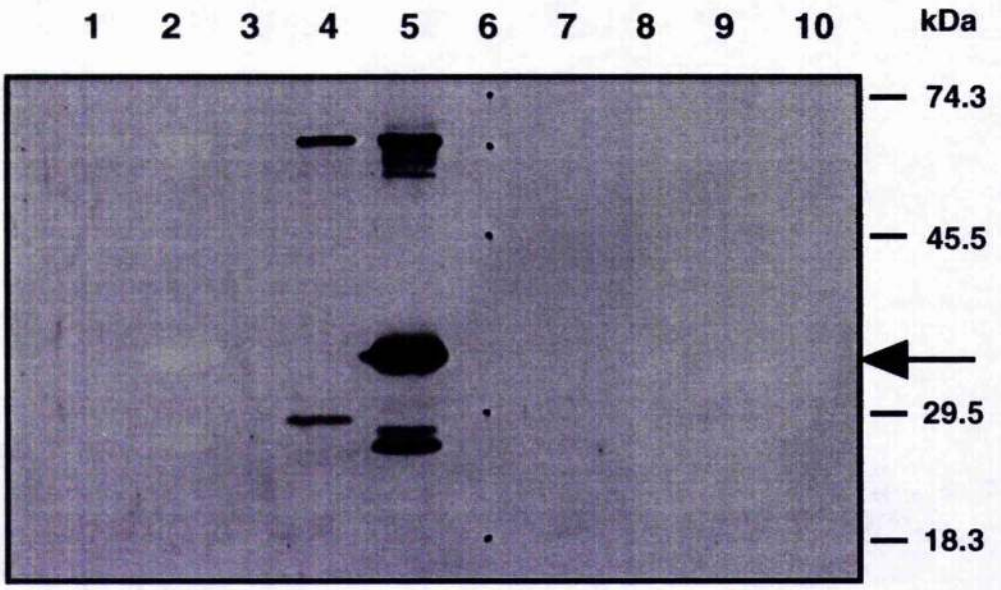

1
2
3
kDa
105
70.8
43.6

Figure A.6. Protein-staining of hepatocyte membranes and COS cell extracts, confirming the expression of transfected glucagon receptor cDNA

From crude lysates of COS-7 cells which had been either mock-transfected (lanes 2) or transfected with the glucagon receptor cDNA (lane 3), and from a plasma membrane preparation of rat hepatocytes (lane 1), 100 μg of protein was boiled in Laemmli sample buffer and subjected to electrophoresis on an 8% polyacrylamide SDS gel. Subsequently, the proteins were transferred to nitrocellulose, which was then rinsed with water, and stained with Ponceau solution. The arrow indicates the only protein species to be detected in transfected but not in control COS cells.

A


1 2 3 4 5 6 7 8 9 10
kDa
74.3
45.5
29.5
18.3

B

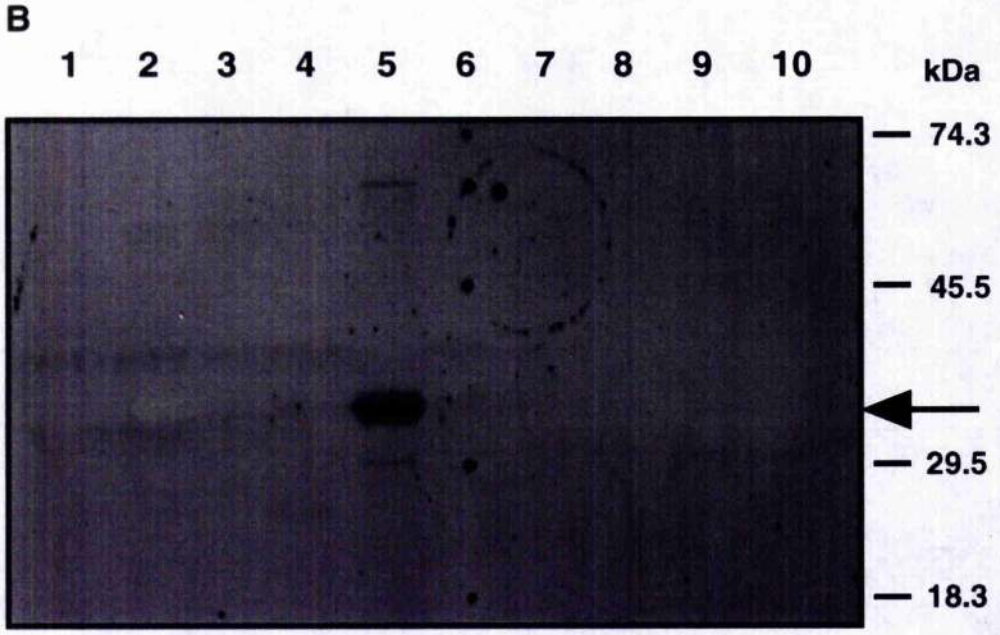

1 2 3 4 5 6 7 8 9 10
kDa
74.3
45.5
29.5
18.3

Figure A.7. Immunoblots of hepatocyte and COS cell extracts using as primary antibody an antiserum raised against a GST-GR fusion protein

A 12% polyacrylamide SDS gel was loaded with samples of hepatocyte membranes (lane 1), transfected (lane 2) and control (lane 3) COS-7 cells lysates, purified GST (lane 4) and GST-GR fusion protein (lane 5), the P_2 fraction of control (lane 7) and transfected COS cells (lane 8), and the P_1 fraction of control (lane 9) and transfected cells (lane 10). In each case an aliquot containing 100 μg of protein (but only 30 μg of the purified GST proteins) was boiled in Laemmli sample buffer prior to loading. 10 μg of protein molecular weight markers was also loaded (lane 6). Following electrophoresis, the proteins were transferred to nitrocellulose and blocking was performed by incubation in 5% (w/v) dried milk protein. The nitrocellulose blots were then incubated for 2 hours at room temperature with GST-purified antiserum from rabbit Ig7 (A) or Ig8 (B), diluted 1 in 200. Following the removal of unhybridised antibodies by successive washing, the blots were incubated in HRP-conjugated anti-rabbit IgG, and detection performed by enhanced chemiluminescence. The arrows indicate the 34 kDa GST-GR species discussed in the text, which was detected much more strongly than the 28 kDa GST protein species, by both antisera.

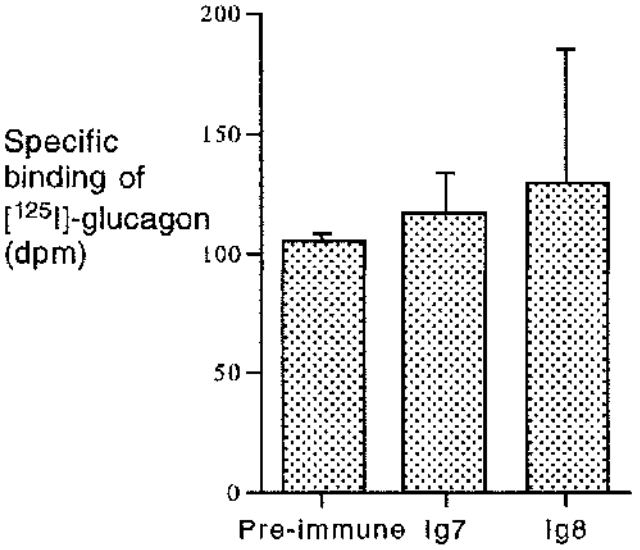

Specific binding of [125I]-glucagon (dpm)
200
150
100
50
0
Pre-immune
Ig7
Ig8
Antiserum used for immunoprecipitation

Figure A.8. [^{125}I]-glucagon specific binding following immunoprecipitation from hepatocyte membranes using the antisera raised against a GST-GR fusion protein

The source material for the specific immunoprecipitation of [^{125}I]-glucagon binding activity was a hepatocyte membrane fraction (1.75 mg of starting material) solubilised by CHAPS detergent. The solubilisation mixture comprised 25 mM HEPES pH 7.5, 137 mM NaCl, 2 mM EDTA pH 8.0, 6 mM CHAPS and 1x protease inhibitor "cocktail" (see section 2.3.3.). After a period of 30 min on ice, during which the constituents were agitated every 10 min, any unsolubilised membranes were pelleted by centrifugation at 245,000 g_{av} for 30 min at 4 °C. Antiserum (either pre-immune serum, or the Ig7 or Ig8 antiserum raised against the GST-GR fusion protein) was added to each sample of the supernatant at a dilution of 1:50 and the samples briefly vortexed before being left overnight at 4 °C in order to allow conjugation to occur. Pansorbin was then added to a final concentration of 2% w/v, and the resultant suspension gently mixed at 4 °C for 2 h. The preparations were then centrifuged at 14,000g for 2 min and the pellets were resuspended in the immunoprecipitation buffer (25 mM HEPES pH 7.5, 137 mM NaCl, 2 mM EDTA pH 8.0 and 1x protease inhibitors) before being recentrifuged as before. The resultant pellets were then washed twice more.

The final pellet was resuspended in binding assay buffer and assayed immediately for specific glucagon binding as described previously (Houslay *et al.*, 1977). Briefly, 40 µl of each immunoprecipitate was incubated in a final reaction volume of 100 µl containing 0.1% (w/v) BSA, 1 mM-EDTA, 20 mM-Tris.HCl pH 7.5 (binding buffer), and 0.2 nM [^{125}I]-glucagon. Non-specific binding was determined by the addition of 1 µM unlabelled glucagon. Incubations were undertaken for 20 min at 32 °C. The reactions were terminated by the addition of 3 ml ice-cold binding buffer and the diluted samples immediately filtered through 0.45-µm cellulose acetate filters which had been soaked in 10% BSA overnight and washed with 2 ml ice-cold binding buffer. The tubes were rinsed twice with 3 ml ice-cold binding buffer and the rinses filtered. The filters were then washed finally with 6 ml ice-cold binding buffer and the radioactivity bound to them was measured using a γ counter.

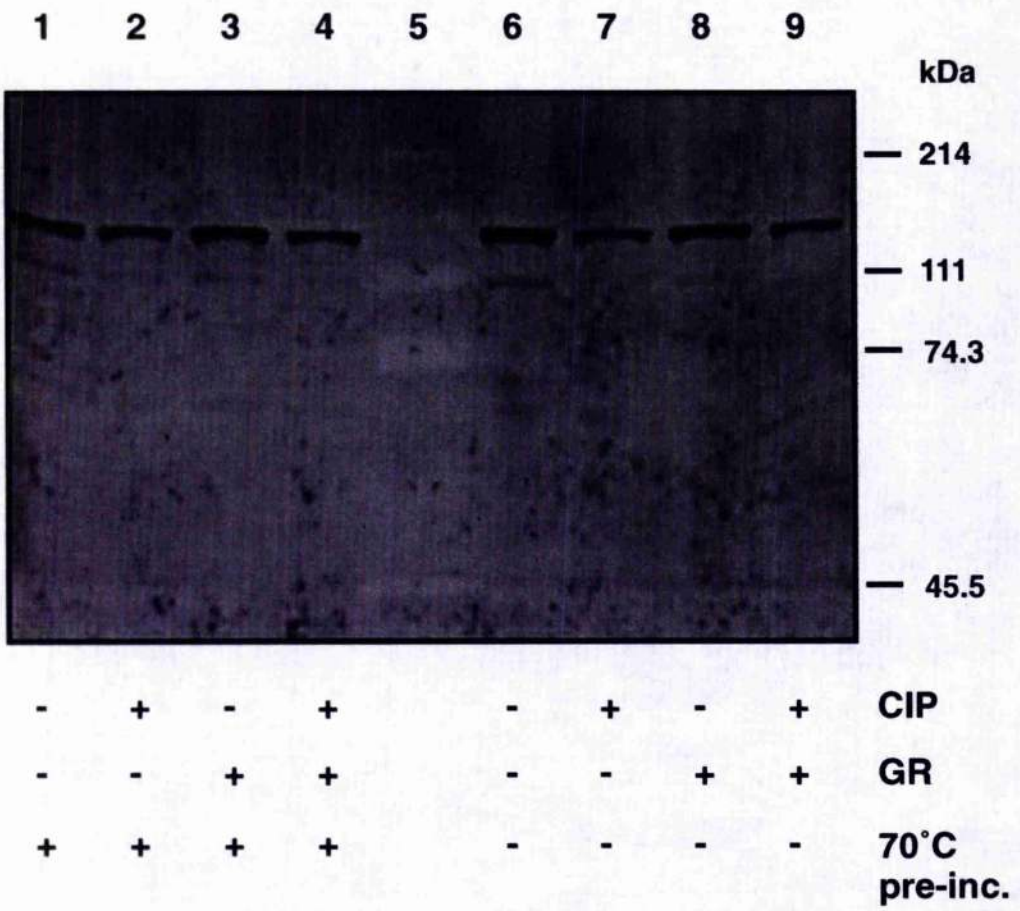

1 2 3 4 5 6 7 8 9
kDa
214
111
74.3
45.5
- + - + - + - +
CIP
- - + + - - + +
GR
+ + + + - - - -
70°C
pre-inc.

Figure A.9. Immunoblots of COS cell membrane fractions following phosphatase treatment using an antiserum raised against a GST-GR fusion protein

200 µg of membrane proteins prepared from either untransfected (lanes 1, 2, 6 and 7) or GR cDNA-transfected COS cells (lanes 3, 4, 8 and 9) were treated in a total volume of 100 µl with 20 units of alkaline phosphatase for 1 hour (lanes 2, 4, 7 and 9) or in buffer alone (lanes 1, 3, 6 and 8). This was done following a 15 min pre-incubation at 70 °C to inactivate any kinases isolated with the membranes (lanes 1-4), or on ice (lanes 6-9).

After boiling in Laemmli sample buffer, these samples were loaded on an 8% polyacrylamide SDS gel. 10 µg of protein molecular weight markers was loaded in lane 5. Following electrophoresis, the proteins were transferred to nitrocellulose and blocking was performed by incubation in 5% (w/v) dried milk protein. The nitrocellulose blots were then incubated for 2 hours at room temperature with GST-purified antiserum from rabbit Ig8, diluted 1 in 200. Following the removal of unhybridised antibodies by successive washing, the blots were incubated in HRP-conjugated anti-rabbit IgG, and detection performed by enhanced chemiluminescence. A similar blot was also probed with the antiserum from rabbit Ig7, but no immunoreactive species were detected.

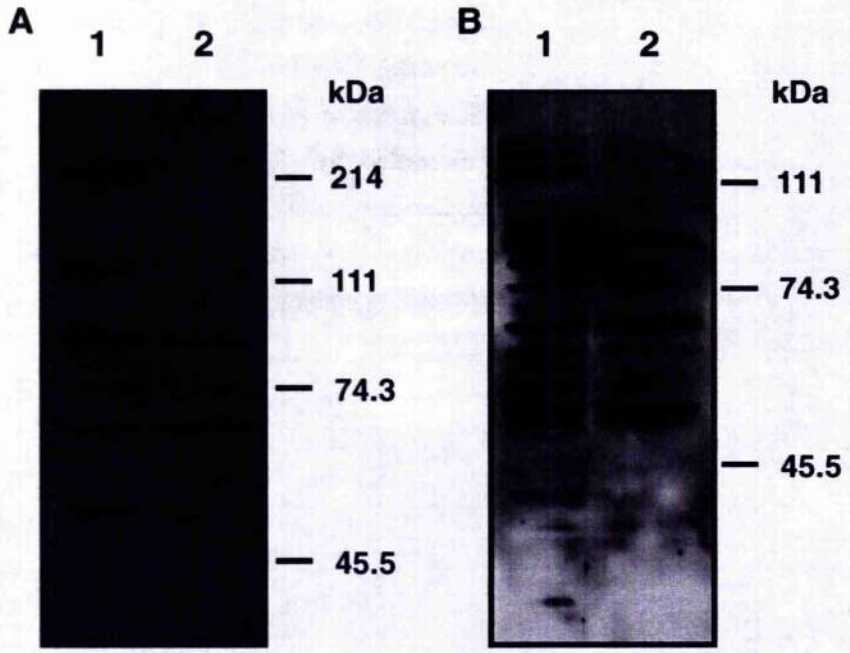

A
1
2
kDa
214
111
74.3
45.5
B
1
2
kDa
111
74.3
45.5

Figure A.10. Immunoblots of COS cell extracts using antisera raised against re-conjugated peptide 4 and peptide 5 of the glucagon receptor

From COS-7 cells which were either glucagon receptor cDNA-transfected (lanes 2) or mock-transfected (lanes 1), 100 μg of protein of crude lysate was boiled in Laemmli sample buffer and subjected to electrophoresis on an 8% polyacrylamide SDS gel. Subsequently, the proteins were transferred to nitrocellulose and blocking was performed by incubation in 5% (w/v) dried milk protein. The nitrocellulose blots were then incubated for 2 hours at room temperature with an antiserum raised against either re-conjugated peptide 4 (A) or peptide 5 (B) of the glucagon receptor, diluted 1 in 100. Following the removal of unbybridised antibodies by successive washing, the blots were incubated in HRP-conjugated anti-rabbit IgG, and detection performed by enhanced chemiluminescence.

REFERENCES

Abou-Samra, A.-B., Juppner, H., Force, T., Freeman, M. W., Kong, X. F., Schipani, E., Urena, P., Richards, J., Bonventre, J. V., Potts, J. T., Kronenberg, H. M., and Segre, G. V. (1992). Expression cloning of a common receptor for parathyroid hormone and parathyroid hormone-related peptide from rat osteoblast-like cells. Proc Natl Acad Sci USA *89*, 2732-2736.

Abrahamsen, N., Lundgren, K., and Nishimura, E. (1995). Regulation of glucagon receptor mRNA in cultured primary rat hepatocytes by glucose and cAMP. J Biol Chem *270*, 15853-15857.

Aderem, A. (1992). The MARCKS brothers: a family of protein kinase C substrates. Cell *71*, 713-716.

Adie, E. J., and Milligan, G. (1994). Regulation of basal adenylate cyclase activity in neuroblastoma x glioma hybrid, NG108-15, cells transfected to express the human beta2 adrenoceptor: Evidence for empty receptor stimulation of the adenylate cyclase cascade. Biochem J *303*, 803-808.

Aihara, H., Asaoka, Y., Yoshida, K., and Nishizuka, Y. (1991). Sustained activation of protein kinase C is essential to HL-60 cell differentiation to macrophage. Proc Natl Acad Sci USA *88*, 11062-6.

Al-Molish, M. I., and Dubes, G. R. (1973). The kinetics of DEAE-dextran-induced cell sensitization to transfection. J Gen Virol *18*, 189-193.

Albers, K., and Fuchs, E. (1987). The expression of mutant epidermal keratin cDNAs transfected in simple epithelial and squamous cell carcinoma lines. J Cell Biol *105*, 791-806.

Ambrose, C., James, M., Barnes, G., Lin, C., Bates, G., Altherr, M., Duyao, M., Groot, N., Church, D., Wasmuth, J. J., Lehrach, H., Houseman, D., Buckler, A., Gusella, J. F., and MacDonald, M. E. (1993). A novel G protein-coupled receptor kinase gene cloned from 4p16.3. Human Molecular Genetics *1*, 697-703.

Anderson, W. B., and Jaworski, C. J. (1979). Isoproterenol-induced desensitization of adenylate cyclase responsiveness in a cell-free system. J Biol Chem *254*, 4596-4601.

Angelos, K. L., Ramachandran, C., and Walsh, D. A. (1987). Subunit phosphorylation and activation of phosphorylase kinase in perfused rat hearts. J Biol Chem *262*, 3219-3226.

Antonny, B., and Chabre, M. (1992). Characterisation of the aluminium and beryllium fluoride species which activate transducin. Analysis of the binding and dissociation kinetics. J Biol Chem *267*, 6710-6718.

Arner, P., Pollare, T., Lithell, H., and Livingston, J. N. (1987). Defective insulin receptor tyrosine kinase in human skeletal muscle in obesity and type 2 (non-insulin-dependent) diabetes mellitus. Diabetologia *30*, 437-40.

Asaoka, Y., Nakamura, S., Yoshida, K., and Nishizuka, Y. (1992). Protein kinase C, calcium and phospholipid degradation. Trends Biochem Sci *17*, 414-7.

Asaoka, Y., Oka, M., Yoshida, K., and Nishizuka, Y. (1991). Metabolic rate of membrane-permeant diacylglycerol and its relation to human resting T-lymphocyte activation. Proc Natl Acad Sci USA *88*, 8681-5.

Bakalyar, H. A., and Reed, R. R. (1990). Identification of a specialized adenylyl cyclase that may mediate odorant detection. Science *250*, 1403-1406.

Baldwin, J. M. (1994). Structure and function of receptors coupled to G proteins. Curr Opin Cell Biol *6*, 180-90.

Baron, A. D., Schaeffer, L., Shragg, P., and Kolterman, O. G. (1987). Role of hyperglucagonemia in maintenance of increased rates of hepatic glucose output in type II diabetics. Diabetes *36*, 274-283.

Barritt, G. J., and Hughes, B. P. (1991). The nature and mechanism of activation of the hepatocyte receptor- activated Ca^{2+} inflow system. Cellular Signalling *3*, 283-292.

Bates, M. D., Caron, M. G., and Raymond, J. R. (1991). Desensitization of DA1 dopamine receptors coupled to adenylyl cyclase in opossum kidney cells. Am J Phys *260*, F937-F945.

Beavo, J. A., and Reifsnyder, D. H. (1990). Primary sequence of cyclic nucleotide phosphodiesterase isozymes and the design of selective inhibitors. Trends Pharmacol Sci *11*, 150-5.

Beebe, S. J., Oyen, O., Sandberg, M., Froysa, A., Hansson, V., and Jahnsen, T. (1990). Molecular cloning of a tissue-specific protein kinase (C gamma) from human testis-representing a third isoform for the catalytic subunit of cAMP-dependent protein kinase. Mol Endocrinol *4*, 465-475.

Beebe, S. J., Salomonsky, P., Jahnsen, T., and Li, Y. (1992). The C gamma subunit is a unique isozyme of the cAMP-dependent protein kinase. J Biol Chem *267*, 25505-25512.

Begin-Heick, N. (1992). Alpha-subunits of Gs and Gi in adipocyte plasma membranes of genetically diabetic (db/db) mice. Am J Phys *263*, C121-C129.

Bell, R. M., and Burns, D. J. (1991). Lipid activation of protein kinase C. J Biol Chem *266*, 4661-4.

Benovic, J. L., DeBlasi, A., Stone, W. C., Caron, M. G., and Lefkowitz, R. J. (1989). Beta-adrenergic receptor kinase: primary structure delineates a multigene family. Science *246*, 235-40.

Benovic, J. L., and Gomez, J. (1993). Molecular cloning and expression of GRK6. A new member of the G protein-coupled receptor kinase family. J Biol Chem *268*, 19521-7.

Benovic, J. L., Onorato, J. J., Arriza, J. L., Stone, W. C., Lohse, M., Jenkins, N. A., Gilbert, D. J., Copeland, N. G., Caron, M. G., and Lefkowitz, R. J. (1991). Cloning, expression, and chromosomal localization of beta-adrenergic receptor kinase 2. A new member of the receptor kinase family. J Biol Chem *266*, 14939-46.

Benovic, J. L., Pike, L. J., Cerione, R. A., Staniszewski, C., Yoshimasa, T., Codina, J., Caron, M. G., and Lefkowitz, R. J. (1985). Phosphorylation of the mammalian beta-adrenergic receptor by cyclic AMP-dependent protein kinase. Regulation of the rate of receptor phosphorylation and dephosphorylation by agonist occupancy and effects on coupling of the receptor to the stimulatory guanine nucleotide regulatory protein. J Biol Chem *260*, 7094-7101.

Berridge, M. J., and Irvine, R. F. (1984). Inositol trisphosphate, a novel second messenger in cellular signal transduction. Nature *312*, 315-21.

Berry, M. N., and Friend, D. S. (1969). High-yield preparation of isolated rat liver parenchymal cells. A biochemical and fine structural study. J Cell Biol *43*, 506-520.

Berti, L., Mosthaf, L., Kroder, G., Kellerer, M., Tippmer, S., Mushack, J., Seffer, E., Seedorf, K., and Haring, H. (1994). Glucose-induced translocation of protein kinase C isoforms in rat-1 fibroblasts is paralleled by inhibition of the insulin receptor tyrosine kinase. J Biol Chem *269*, 3381-3386.

Best, J. D., Judzewitsch, R. G., Pfeiffer, M. A., Beard, J. C., B., H. J., and Porte, D. J. (1982). The effect of chronic sulfonylurea therapy on hepatic glucose production in non-insulin-dependent diabetes. Diabetes *31*, 333-338.

Bigay, J., Faurobert, E., Franco, M., and Chabre, M. (1994). Roles of lipid modifications of transducin subunits in their GDP- dependent association and membrane binding. Biochemistry (USA) *33*, 14081-14090.

Billah, M. M., and Anthes, J. C. (1990). The regulation and cellular functions of phosphatidylcholine hydrolysis. Biochem J *269*, 281-91.

Birnbaumer, L., Abramowitz, J., and Brown, A. M. (1990a). Receptor-effector coupling by G proteins. Biochim Biophys Acta *1031*, 163-224.

Birnbaumer, L., Abramowitz, J., Yatani, A., Okabe, K., Mattera, R., Graf, R., Sanford, J., Codina, J., and Brown, A. M. (1990b). Roles of G proteins in coupling of receptors to ionic channels and other effector systems. Crit Rev Biochem Mol Biol *25*, 225-44.

Birnbaumer, L., Pohl, S. L., and Rodbell, M. (1971). The glucagon-sensitive adenyl cyclase system in plasma membranes of rat liver. II. Comparison between glucagon- and fluoride-stimulated activities. J Biol Chem *246*, 1857-1860.

Blackmore, P. F., and Exton, J. H. (1986). Studies on the hepatic calcium-mobilizing activity of aluminum fluoride and glucagon. Modulation by cAMP and phorbol myristate acetate. J Biol Chem *261*, 11056-11063.

Blackshear, P. J. (1993). The MARCKS family of cellular protein kinase C substrates. J Biol Chem *268*, 1501-4.

Bocckino, S. B., Blackmore, P. F., and Exton, J. H. (1985). Stimulation of 1,2-diacylglycerol accumulation in hepatocytes by vasopressin, epinephrine, and angiotensin II. J Biol Chem *260*, 14201-14207.

Bokoch, G. M. (1993). Biology of the Rap proteins, members of the ras superfamily of GTP-binding proteins. Biochem J *289*, 17-24.

Bollag, G. E., Roth, R. A., Beaudoin, J., Mochly-Rosen, D., and Koshland, D. E. J. (1986). Protein kinase C directly phosphorylates the insulin receptor in vitro and reduces its protein-tyrosine kinase activity. Proc Natl Acad Sci USA *83*, 5822-4.

Bourne, H. R. (1995). Team blue sees red. Nature *376*, 727-729.

Bouvier, M., Collins, S., O'Dowd, B. F., Campbell, P. T., De Blasi, A., Kobilka, B. K., MacGregor, C., Irons, G. P., Caron, M. G., and Lefkowitz, R. J. (1989). Two distinct pathways for cAMP-mediated down-regulation of the beta2- adrenergic receptor. Phosphorylation of the receptor and regulation of its mRNA level. J Biol Chem *264*, 16786-16792.

Bouvier, M., Leeb-Lundberg, L. M. F., Benovic, J. L., Caron, M. G., and Lefkowitz, R. J. (1987). Regulation of adrenergic receptor function by phosphorylation. II. Effects of agonist occupancy on phosphorylation of alpha1- and beta2- adrenergic receptors by protein kinase C and the cyclic AMP-dependent protein kinase. J Biol Chem *262*, 3106-3113.

Bradford, M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry *72*, 248-254.

Brandt, D. R., and Ross, E. M. (1986). Catecholamine-stimulated GTPase cycle - multiple sites of regulation by beta-adrenergic-receptor and Mg studies in reconstituted receptor-g8 vesicles. J Biol Chem *261*, 1656-1664.

Brass, L. F. (1992). Homologous desensitization of HEL cell thrombin receptors. Distinguishable roles for proteolysis and phosphorylation. J Biol Chem *267*, 6044-6050.

Braun, S., Tolkovsky, A. M., and Levitzki, A. (1982). Mechanism of control of the turkey erythrocyte beta-adrenoceptor dependent adenylate-cyclase by guanyl nucleotides. A minimum model. Journal of Cyclic Nucleotide Research *8*, 133-147.

Brown, B. L., Ekins, R. P., and Albano, J. M. P. (1972). Adv Cyclic Nucleotide Res *2*, 25-40.

Buggy, J., Hull, J., and Yoo-Warren, H. (1995a). Isolation and structural analysis of the 5' flanking region of the gene encoding the human glucagon receptor. Biochemical and Biophysical Research Communications *208*, 339-344.

Buggy, J. J., Livingston, J. N., Rabin, D. U., and Yoo-Warren, H. (1995b). Glucagon/Glucagon-like peptide 1 receptor chimeras reveal domains that determine specificity of glucagon binding. J Biol Chem *270*, 7474-7478.

Burcelin, R., Li, J., and Charron, M. J. (1995). Cloning and sequence analysis of the murine glucagon receptor-encoding gene. Gene *164*, 305-310.

Bushfield, M., Griffiths, S. L., Murphy, G. J., Pyne, N. J., Knowler, J. T., Milligan, G., Parker, P. J., Mollner, S., and Houslay, M. D. (1990a). Diabetes-induced alterations in the expression, functioning and phosphorylation state of the inhibitory guanine nucleotide regulatory protein G(i)-2 in hepatocytes. Biochem J *271*, 365-372.

Bushfield, M., Murphy, G. J., Lavan, B. E., Parker, P. J., Hruby, V. J., Milligan, G., and Houslay, M. D. (1990b). Hormonal regulation of G(i)2 alpha-subunit phosphorylation in intact hepatocytes. Biochem J *268*, 449-457.

Bushfield, M., Pyne, N. J., and Houslay, M. D. (1990c). Changes in the phosphorylation state of the inhibitory guanine- nucleotide-binding protein G(i)-2 in hepatocytes from lean (Fa/Fa) and obese (fa/fa) Zucker rats. Eur J Biochem *192*, 537-542.

Buss, J. E., Mumby, S. M., Casey, P. J., Gilman, A. G., and Sefton, B. M. (1987). Myristoylated alpha-subunits of guanine nucleotide-binding regulatory proteins. Proc Natl Acad Sci USA *84*, 7493-7497.

Butcher, R. W., and Sutherland, E. W. (1962). Adenosine 3'5'-phosphate in biological materials. J Biol Chem *237*, 1244-1250.

Bygrave, F. L., and Benedetti, A. (1993). Calcium: its modulation in liver by cross-talk between the actions of glucagon and calcium-mobilizing agonists. Biochem J *296*, 1-14.

Bygrave, F. L., Gamberucci, A., Fulceri, R., and Benedetti, A. (1993). Evidence that stimulation of plasma-membrane Ca^{2+} inflow is an early action of glucagon and dibutyryl cyclic AMP in rat hepatocytes. Biochem J *292*, 19-22.

Cacace, A. M., Guadagno, S. N., Krauss, R. S., Fabbro, D., and Weinstein, I. B. (1993). The epsilon isoform of protein kinase C is an oncogene when overexpressed in rat fibroblasts. Oncogene *8*, 2095-104.

Cacace, A. M., Ueffing, M., Philipp, A., Han, E. K.-H., Kolch, W., and Weinstein, I. B. (1996). PKC epsilon functions as an oncogene by enhancing activation of the Raf kinase. Oncogene *13*, 2517-2526.

Cali, J. J., Zwaagstra, J. C., Mons, N., Cooper, D. M. F., and Krupinski, J. (1994). Type VIII adenylyl cyclase. A Ca^{2+}/calmodulin-stimulated enzyme expressed in discrete regions of rat brain. J Biol Chem *269*, 12190-12195.

Camps, M., Carozzi, A., Schnabel, P., Scheer, A., Parker, P. J., and Gierschik, P. (1992). Isozyme-selective stimulation of phospholipase C-beta2 by G protein $\beta\gamma$ subunits. Nature *360*, 684-686.

Cano, E., and Mahadevan, L. C. (1995). Parallel signal processing among mammalian MAPKs. Trends Biochem Sci *20*, 117-122.

Caro, J. F., Ittoop, O., Pories, W. J., Meelheim, D., Flickinger, E. G., Thomas, F., Jenquin, M., Silverman, J. F., Khazanie, P. G., and Sinha, M. K. (1986). Studies on the mechanism of insulin resistance in the liver from humans with non-insulin-dependent diabetes. Insulin action and binding in isolated hepatocytes, insulin receptor structure, and kinase activity. J Clin Invest *78*, 249-58.

Caro, J. F., Jenquin, M., and Long, S. (1992). Effects of phorbol esters on insulin receptor function and insulin action in hepatocytes: evidence for heterogeneity. Mol Cell Biochem *109*, 115-8.

Caro, J. F., Sinha, M. K., Raju, S. M., Ittoop, O., Pories, W. J., Flickinger, E. G., Meelheim, D., and Dohm, G. L. (1987). Insulin receptor kinase in human skeletal muscle from obese subjects with and without noninsulin dependent diabetes. J Clin Invest *79*, 1330-7.

Carruthers, C. J. L., Unson, C. G., Kim, H. N., and Sakmar, T. P. (1994). Synthesis and expression of a gene for the rat glucagon receptor. Replacement of an aspartic acid in the extracellular domain prevents glucagon binding. J Biol Chem *269*, 29321-29328.

Carty, D. J., Padrell, E., Codina, J., Birnbaumer, L., Hildebrandt, J. D., and Iyengar, R. (1990). Distinct guanine nucleotide binding and release properties of the three G(i) proteins. J Biol Chem *265*, 6268-6273.

Cassel, D., and Pfeuffer, T. (1978). Mechanisms of chlolera-toxin action: covalent modification of guanyl nucleotide-binding protein of the adenylate cyclase system. Proc Natl Acad Sci USA *75*, 2669-2673.

Cassel, D., and Selinger, Z. (1978). Mechanism of adenylate cyclase activation through the beta-adrenergic receptor: catecholamine-induced displacement of bound GDP by GTP. Proc Natl Acad Sci USA *75*, 4155.

Cazaubon, S., Bornancin, F., and Parker, P. J. (1994). Threonine-497 is a critical site for permissive activation of protein kinase C-alpha. Biochem J *301*, 443-448.

Cazaubon, S. M., and Parker, P. J. (1993). Identification of the phosphorylated region responsible for the permissive activation of protein kinase C. J Biol Chem *268*, 17559-17563.

Chabre, O., Conklin, B. R., Lin, H. Y., Lodish, H. F., Wilson, E., Ives, H. E., Catanzariti, L., Hemmings, B. A., and Bourne, H. R. (1992). A recombinant calcitonin receptor independently stimulates 3',5'-cyclic adenosine monophosphate and Ca^{2+}/inositol phosphate signaling pathways. Mol Endocrinol *6*, 551-6.

Charest, R., Blackmore, P. F., Berthon, B., and Exton, J. H. (1983). Changes in free cytosolic calcium in hepatocytes following alpha1- adrenergic stimulation. Studies on Quin-2-loaded hepatocytes. J Biol Chem *258*, 8769-8773.

Chen, C. K., Inglese, J., Lefkowitz, R. J., and Hurley, J. B. (1995a). Ca^{2+}-dependent interaction of recoverin with rhodopsin kinase. J Biol Chem *270*, 18060-18066.

Chen, J., DeVivo, M., Dingus, J., Harry, A., Li, J., Sui, J., Carty, D. J., Blank, J. L., Exton, J. H., Stoffel, R. H., Inglese, J., Lefkowitz, R. J., Logothetis, D. E., Hildebrandt, J. D., and Iyengar, R. (1995b). A region of adenylyl cyclase 2 critical for regulation by G protein $\beta\gamma$ subunits. Science *268*, 1166-1169.

Chen, K.-H., Widen, S. G., Wilson, S. H., and Huang, K.-P. (1990). Characterization of the 5'-flanking region of the rat protein kinase C gamma gene. J Biol Chem *265*, 19961-5.

Cheng, S., Chang, S. Y., Gravitt, P., and Respess, R. (1994). Long PCR. Nature *369*, 684-5.

Chidiac, P., Hebert, T. E., Valiquette, M., Dennis, M., and Bouvier, M. (1994). Inverse agonist activity of beta-adrenergic antagonists. Mol Pharmacol *45*, 490-499.

Chin, J. E., Dickens, M., Tavare, J. M., and Roth, R. A. (1993). Overexpression of protein kinase C isoenzymes alpha, beta I, gamma, and epsilon in cells overexpressing the insulin receptor. Effects on receptor phosphorylation and signaling. J Biol Chem *268*, 6338-47.

Choi, E.-J., Wong, S. T., Dittman, A. H., and Storm, D. R. (1993). Phorbol ester stimulation of the type I and type III adenylyl cyclases in whole cells. Biochemistry *32*, 1891-4.

Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate- phenol-chloroform extraction. Analytical Biochemistry *162*, 156-159.

Chou, Q., Russell, M., Birch, D. E., Raymond, J., and Bloch, W. (1992). Prevention of pre-PCR mis-priming and primer dimerization improves low-copy-number amplifications. Nucleic Acids Res *20*, 1717-1723.

Christoffersen, T., and Berg, T. (1974). Glucagon control of cyclic AMP accumulation in isolated intact rat liver parenchymal cells in vitro. Biochim Biophys Acta *338*, 408-417.

Christophe, J. (1995). Glucagon receptors: From genetic structure and expression to effector coupling and biological responses. Biochim Biophys Acta *1241*, 45-57.

Chuang, T. T., LeVine, H., and De Blasi, A. (1995). Phosphorylation and activation of beta-adrenergic receptor kinase by protein kinase C. J Biol Chem *270*, 18660-18665.

Claret, F.-X., Hibi, M., Dhut, S., Toda, T., and Karin, M. (1996). A new group of conserved coactivators that increase the specificity of AP-1 transcription factors. Nature *383*, 453-456.

Clark, R. B., Friedman, J., Dixon, R. A. F., and Strader, C. D. (1989). Identification of a specific site required for rapid heterologous desensitization of the beta-adrenergic receptor by cAMP-dependent protein kinase. Mol Pharmacol *36*, 343-348.

Clark, R. B., Kunkel, M. W., Friedman, J., Goka, T. J., and Johnson, J. A. (1988). Activation of cAMP-dependent protein-kinase is required for heterologous desensitization of adenylyl cyclase in s49 wild-type lymphoma-cells. Proc Natl Acad Sci USA *85*, 1442-1446.

Cockcroft, S., and Thomas, G. M. H. (1992). Inositol-lipid-specific phospholipase C isoenzymes and their differential regulation by receptors. Biochem J *288*, 1-14.

Coghlan, V. M., Langeberg, L. K., Fernandez, A., Lamb, N. J. C., and Scott, J. D. (1994). Cloning and characterisation of AKAP 95, a nuclear protein that associates with the regulatory subunit of type II cAMP-dependent protein kinase. J Biol Chem *269*, 7658-7665.

Coghlan, V. M., Perrino, B. A., Howard, M., Langeberg, L. K., Hicks, J. B., Gallatin, W. M., and Scott, J. D. (1995). Association of protein kinase A and protein phosphatase 2B with a common anchoring protein. Science *267*, 108-111.

Coleman, D. E., Berghuis, A. M., Lee, E., Linder, M. E., Gilman, A. G., and Sprang, S. R. (1994). Structures of active conformations of $G_i$alpha1 and the mechanism of GTP hydrolysis. Science *265*, 1405-1412.

Considine, R. V., Nyce, M. R., Allen, L. E., Morales, L. M., Triester, S., Serrano, J., Colberg, J., Jacoby, S. L., and Caro, J. F. (1995). Protein kinase C is increased in the liver of humans and rats with non- insulin-dependent diabetes mellitus: An alteration not due to hyperglycemia. J Clin Invest *95*, 2938-2944.

Cook, S. J., Briscoe, C. P., and Wakelam, M. J. O. (1991). The regulation of phospholipase D activity and its role in *sn*-1,2-diradylglycerol formation in bombesin- and phorbol 12-myristate 13-acetate-stimulated Swiss 3T3 cells. Biochem J *280*, 431-438.

Cooper, D. M. F., Mons, N., and Karpen, J. W. (1995). Adenylyl cyclases and the interaction between calcium and cAMP signalling. Nature *374*, 421-424.

Cooper, D. R., Khalakdina, A., and Watson, J. E. (1993). Chronic effects of glucose on insulin signaling in A-10 vascular smooth muscle cells. Arch Biochem Biophys *302*, 490-8.

Corbin, J. D., Sugden, P. H., Lincoln, T. M., and Keely, S. L. (1977). Compartmentalization of adenosine 3':5'-monophosphate and adenosine 3':5'-monophosphate-dependent protein kinase in heart tissue. J Biol Chem *252*, 3855-3861.

Coso, O. A., Teramoto, H., Simonds, W. F., and Gutkind, J. S. (1996). Signaling from G protein-coupled receptors to c-Jun kinase involves βγ subunits of heterotrimeric G proteins acting on a Ras and Rac1-dependent pathway. J Biol Chem *271*, 3963-3966.

Davis, R. J. (1994). MAPKs: new JNK expands the group. Trends Biochem Sci *19*, 470-3.

Debernardi, M. A., Munshi, R., Yoshimura, M., Cooper, D. M. F., and Brooker, G. (1993). Predominant expression of type-VI adenylate-cyclase in C6-2B rat glioma-cells may account for inhibition of cyclic-AMP accumulation by calcium. Biochem J *293*, 325-328.

Degtyarev, M. Y., Spiegel, A. M., and Jones, T. L. Z. (1993). Increased palmitoylation of the G(s) protein alpha subunit after activation by the beta-adrenergic receptor or cholera toxin. J Biol Chem *268*, 23769-23772.

Dekker, L. V., McIntyre, P., and Parker, P. J. (1993). Mutagenesis of the regulatory domain of rat protein kinase C-eta. A molecular basis for restricted histone kinase activity. J Biol Chem *268*, 19498-19504.

Dekker, L. V., and Parker, P. J. (1994). Protein kinase C - a question of specificity. Trends Biochem Sci *19*, 73-7.

DeRubertis, F. R., and Craven, P. (1976). Reduced sensitivity of the hepatic adenylate cyclase-cyclic AMP system to glucagon during sustained hormonal stimulation. J Clin Invest *57*, 435-443.

Dieterich, S., Herget, T., Link, G., Bottinger, H., Pfizenmaier, K., and Johannes, F. J. (1996). In vitro activation and substrates of recombinant baculovirus expressed human protein kinase C mu. FEBS Lett *381*, 183-187.

Dimitrijevic, S. M., Ryves, W. J., Parker, P. J., and Evans, F. J. (1995). Characterization of phorbol ester binding to protein kinase C isotypes. Mol Pharmacol *48*, 259-267.

Dix, C. J., and Cooke, B. A. (1982). Resensitization of lutropin-desensitized tumour Leydig-cell adenylate cyclase with human erythrocyte membranes. Biochem J *204*, 613-616.

Dix, C. J., Schumacher, M., and Cooke, B. A. (1982). Desensitization of tumour Leydig cells by lutropin: Evidence for uncoupling of the lutropin receptor from the guanine nucleotide- binding protein. Biochem J *202*, 739-745.

Dixon, R. A. F., Kobilka, B. K., Strader, D. J., Benovic, J. L., Dohlman, H. G., Frielle, T., Bolanowski, M. A., Bennett, C. D., Rands, E., Diehl, R. E., Mumford, R. A., Slater, E. E., Sigal, I. S., Caron, M. G., Lefkowitz, R. J., and Strader, C. D. (1986). Cloning of the gene and cDNA for mammalian beta-adrenergic receptor and homology with rhodopsin. Nature *321*, 75-79.

Dizhoor, A. M., Chen, C. K., Olshevskaya, E., Sinelnikova, V. V., Phillipov, P., and Hurley, J. B. (1993). Role of the acylated amino terminus of recoverin in Ca^{2+}-dependent membrane interaction. Science *259*, 829-832.

Donnelly, R., and Connell, J. M. C. (1992). Insulin resistance and blood pressure. Br J Hosp Med *47*, 9-11.

Downward, J., Graves, J. D., Warne, P. H., Rayter, S., and Cantrell, D. A. (1990). Stimulation of p21(ras) upon T-cell activation. Nature *346*, 719-723.

Doyle, K. (1996). Protocols and Applications Guide (Madison: Promega).

Dunlay, R., and Hruska, K. (1990). PTH receptor coupling to phospholipase C is an alternate pathway of signal transduction in bone and kidney. Am J Phys *258*, F223-F231.

Duronio, V., and Jacobs, S. (1990). The effect of protein kinase-C inhibition on insulin receptor phosphorylation. Endocrinology *127*, 481-7.

Dutil, E. M., Keranen, L. M., DePaoliRoach, A. A., and Newton, A. C. (1994). In vivo regulation of protein kinase C by trans-phosphorylation followed by autophosphorylation. J Biol Chem *269*, 29359-29362.

England, P. J. (1975). Correlation between contraction and phosphorylation of the inhibitory subunit of troponin in perfused rat heart. FEBS Lett *50*, 57-60.

England, P. J. (1976). Studies on the phosphorylation of the inhibitory subunit of troponin during modification of contraction in perfused rat heart. Biochem J *160*, 295-304.

England, R. D., Jenkins, W. T., Flanders, K. C., and Gurd, R. S. (1983). Noncooperative receptor interactions of glucagon and eleven analogues: inhibition of adenylate cyclase. Biochemistry *22*, 1722-1728.

Exton, J. H., Corbin, J. G., and Park, C. R. (1969). Control of gluconeogenesis in liver. IV. Differential effects of fatty acids and glucagon on ketogenesis and gluconeogenesis in the perfused rat liver. J Biol Chem *244*, 4095-4102.

Exton, J. H., Mallette, L. E., Jefferson, L. S., Wong, E. H. A., Friedmann, N., and Park, C. R. (1970). Role of adenosine 3', 5'-monophosphate in the control of gluconeogenesis. Am J Clin Nutr *23*, 993-1003.

Faure, M., Voyno-Yasenetskaya, T. A., and Bourne, H. R. (1994). cAMP and beta gamma subunits of heterotrimeric G proteins stimulate the mitogen-activated protein kinase pathway in COS-7 cells. J Biol Chem *269*, 7851-7854.

Faux, M. C., and Scott, J. D. (1996). Molecular glue: kinase anchoring and scaffold proteins. Cell *85*, 9-12.

Feinstein, P. G., Schrader, K. A., Bakalyar, H. A., Tang, W. J., Krupinski, J., Gilman, A. G., and Reed, R. R. (1991). Molecular cloning and characterization of a Ca^{2+}/calmodulin- insensitive adenylyl cyclase from rat brain. Proc Natl Acad Sci USA *88*, 10173-10177.

Feramisco, J. R., Glass, D. B., and Krebs, E. G. (1980). Optimal spatial requirements for the location of basic residues in peptide substrates for the cyclic AMP-dependent protein kinase. J Biol Chem *255*, 4240-424245.

Firsov, D., Bellanger, A. C., Marsy, S., and Elalouf, J. M. (1995). Quantitative RT-PCR analysis of calcitonin receptor messenger-RNAs in the rat nephron. Am J Phys *38*, F 702-F 709.

Fong, T. M., Yu, H., Huang, R. R. C., and Strader, C. D. (1992). The extracellular domain of the neurokinin-1 receptor is required for high-affinity binding of peptides. Biochemistry *31*, 11806-11811.

Fredericks, Z. L., Pitcher, J. A., and Lefkowitz, R. J. (1996). Identification of the G-protein-coupled receptor kinase phosphorylation sites in the human beta2-adrenergic receptor. J Biol Chem *271*, 13796-13803.

Freidenberg, G. R., Henry, R. R., Klein, H. H., Reichart, D. R., and Olefsky, J. M. (1987). Decreased kinase activity of insulin receptors from adipocytes of non-insulin-dependent diabetic subjects. J Clin Invest *79*, 240-50.

Fung, B. K.-K., Hurley, J. B., and Stryer, L. (1981). Flow of information in the light-triggered cyclic nucleotide cascade of vision. Proc Natl Acad Sci USA *78*, 152-156.

Fung, B. K. K., and Stryer, L. (1980). Photolyzed rhodopsin catalyses the exchange of GTP for bound GDP in retinal rod outer segments. Proc Natl Acad Sci USA *77*, 2500.

Gao, B., and Gilman, A. G. (1991). Cloning and expression of a widely distributed (type IV) adenylyl cyclase. Proc Natl Acad Sci USA *88*, 10178-10182.

Garcia-Higuera, I., and Mayor, F. J. (1992). Rapid agonist-induced beta-adrenergic receptor kinase translocation in C6 glioma cells. FEBS Lett *302*, 61-64.

Garcia-Higuera, I., Penela, P., Murga, C., Egea, G., Bonay, P., Benovic, J. L., and Mayor, F. J. (1994). Association of the regulatory beta-adrenergic receptor kinase with rat liver microsomal membranes. J Biol Chem *269*, 1348-1355.

Garcia-Sainz, J. A., Mendlovic, F., and Martinez-Olmedo, M. A. (1985). Effects of phorbol esters on alpha1-adrenergic-mediated and glucagon-mediated actions in isolated rat hepatocytes. Biochem J *228*, 277-280.

Garritsen, A., Van Galen, P. J. M., and Simonds, W. F. (1993). The N-terminal coiled-coil domain of beta is essential for gamma association: A model for G-protein $\beta\gamma$ subunit interaction. Proc Natl Acad Sci USA *90*, 7706-7710.

Gawler, D., Milligan, G., Spiegel, A. M., Unson, C. G., and Houslay, M. D. (1987). Abolition of the expression of inhibitory guanine nucleotide regulatory protein Gi activity in diabetes. Nature *327*, 229-32.

Gibson, T. J., Hyvonen, M., Musacchio, A., Saraste, M., and Birney, E. (1994). PH domain: The first anniversary. Trends Biochem Sci *19*, 349-353.

Gill, D. M., and Meren, R. (1978). ADP-ribosylation of membrane proteins catalyzed by cholera toxin: basis of the activation of adenylate cyclase. Proc Natl Acad Sci USA *75*, 3050-3054.

Gilman, A. G. (1987). G proteins: Transducers of receptor-generated signals. Annu Rev Biochem *56*, 615-649.

Glantz, S. B., Li, Y., and Rubin, C. S. (1993). Characterisation of distinct tethering and intracellular targeting domains in AKAP75, a protein that links cAMP-dependent protein kinase IIb to the cytoskeleton. J Biol Chem *268*, 12796-12804.

Gluzman, Y. (1981). SV40-transformed simian cells support the replication of early SV40 mutants. Cell *23*, 175-182.

Gorn, A. H., Lin, H. Y., Yamin, M., Auron, P. E., Flannery, M. R., Tapp, D. R., Manning, C. A., Lodish, H. F., Krane, S. M., and Goldring, S. R. (1992). Cloning, characterization, and expression of a human calcitonin receptor from an ovarian carcinoma cell line. J Clin Invest *90*, 1726-1735.

Graham, F. L., Harrison, T., and Williams, J. (1978). Defective transforming capacity of adenovirus type 5 host-range mutants. Virology *86*, 10-21.

Graham, F. L., Smiley, J., Russell, W. C., and Nairn, R. (1977). Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J Gen Virol *36*, 59-72.

Grand, R. J. A., Turnell, A. S., and Grabham, P. W. (1996). Cellular consequences of thrombin-receptor activation. Biochem J *313*, 353-368.

Grassie, M. A., McCallum, J. F., Guzzi, F., Magee, A. I., Milligan, G., and Parenti, M. (1994). The palmitoylation status of the G-protein $G_0$1alpha regulates its avidity of interaction with the plasma membrane. Biochem J *302*, 913-920.

Griffiths, S. L., Knowler, J. T., and Houslay, M. D. (1990). Diabetes-induced changes in guanine-nucleotide-regulatory-protein mRNA detected using synthetic oligonucleotide probes. Eur J Biochem *193*, 367-374.

Gschwendt, M., Kittstein, W., and Marks, F. (1991). Protein kinase C activation by phorbol esters: do cysteine-rich regions and pseudosubstrate motifs play a role? Trends Biochem Sci *16*, 167-9.

Gudermann, T., Birnbaumer, M., and Birnbaumer, L. (1992). Evidence for dual coupling of the murine luteinizing hormone receptor to adenylyl cyclase and phosphoinositide breakdown and Ca^{2+} mobilization. Studies with the cloned murine luteinizing hormone receptor expressed in L cells. J Biol Chem *267*, 4479-88.

Gurr, J. A., and Ruh, T. A. (1980). Desensitization of primary cultures of adult rat liver parenchymal cells to stimulation of adenosine 3',5'-monophosphate production by glucagon and epinephrine. Endocrinology *107*, 1309-1319.

Hadcock, J. R., and Malbon, C. C. (1991). Regulation of receptor expression by agonists: Transcriptional and post-transcriptional controls. Trends in Neurosciences *14*, 242-247.

Hadcock, J. R., Wang, H.-Y., and Malbon, C. C. (1989). Agonist-induced destabilization of beta-adrenergic receptor mRNA. Attenuation of glucocorticoid-induced up-regulation of beta- adrenergic receptors. J Biol Chem *264*, 19928-19933.

Hamm, H. E., Deretic, D., Arendt, A., Hargrave, P. A., Koenig, B., and Hofmann, K. P. (1988). Site of G protein binding to rhodopsin mapped with synthetic peptides from the alpha subunit. Science *241*, 832-835.

Hamm, H. E., and Gilchrist, A. (1996). Heterotrimeric G proteins. Current Opinion in Cell Biology *8*, 189-196.

Han, J., Lee, J. D., Bibbs, L., and Ulevitch, R. J. (1994). A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. Science *265*, 808-81.

Hanks, S. K., and Hunter, T. (1995). The eukaryotic protein kinase superfamily: Kinase (catalytic) domain structure and classification. FASEB Journal *9*, 576-596.

Hanks, S. K., and Quinn, A. M. (1991). Protein kinase catalytic domain sequence database: identification of conserved features of primary structure and classification of family members. Methods in Enzymology *200*, 38-62.

Hargrave, P. A., and Hamm, H. E. (1994). Regulation of visual transduction. In Regulation of cellular signal transduction pathways by desensitization and amplification, D. R. Sibley and M. D. Houslay, eds. (Chichester: John Wiley & Sons), pp. 25-67.

Harlan, J. E., Hajduk, P. J., Yoon, H. S., and Fesik, S. W. (1994). Pleckstrin homology domains bind to phosphatidylinositol-4,5- bisphosphate. Nature *371*, 168-170.

Harrison, T., Graham, F., and Williams, J. (1977). Host-range mutants of adenovirus type 5 defective for growth in HeLa cells. Virology *77*, 319-329.

Hausdorff, W. P., Bouvier, M., O'Dowd, B. F., Irons, G. P., Caron, M. G., and Lefkowitz, R. J. (1989). Phosphorylation sites on two domains of the beta2-adrenergic receptor are involved in distinct pathways of receptor desensitization. J Biol Chem *264*, 12657-12665.

Hausdorff, W. P., Caron, M. G., and Lefkowitz, R. J. (1990). Turning off the signal: Desensitization of beta-adrenergic receptor function. FASEB Journal *4*, 2881-2889.

Hausken, Z. E., Coghlan, V. M., Schafer-Hastings, C. A., Reimann, E. M., and Scott, J. D. (1994). Type II regulatory subunit (RII) of the cAMP-dependent protein kinase interaction with A-kinase anchor proteins requires isoleucines 3 and 5. J Biol Chem *269*, 24245-24251.

Hell, J. W., Appleyard, S. M., Yokoyama, C. T., Warner, C., and Catterall, W. A. (1994). Differential phosphorylation of two size forms of the N-type calcium channel alpha1 subunit which have different COOH termini. J Biol Chem *269*, 7390-7396.

Helms, J. B. (1995). Role of heterotrimeric GTP binding proteins in vesicular protein transport: Indications for both classical and alternative G protein cycles. FEBS Lett *369*, 84-88.

Henderson, R., and Unwin, P. N. T. (1975). Three dimensional model of the purple membrane obtained by electron microscopy. Nature *257*, 28-32.

Herbert, J. M., Augereau, J. M., Gleye, J., and Maffrand, J. P. (1990). Chelerythrine is a potent and specific inhibitor of protein kinase C. Biochemical and Biophysical Research Communications *172*, 993-999.

Heurich, R. O., Buggy, J. J., Vandenberg, M. T., and Rossomando, A. J. (1996). Glucagon induces a rapid and sustained phosphorylation of the human glucagon receptor in chinese-hamster ovary cells. Biochemical and Biophysical Research Communications *220*, 905-910.

Heyworth, C. M., and Houslay, M. D. (1983). Challenge of hepatocytes by glucagon triggers a rapid modulation of adenylate cyclase activity in isolated membranes. Biochem J *214*, 93-98.

Heyworth, C. M., Wallace, A. V., and Houslay, M. D. (1983). Insulin and glucagon regulate the activation of 2 distinct membrane-bound cyclic AMP phosphodiesterases in hepatocytes. Biochem J *214*, 99-110.

Heyworth, C. M., Whetton, A. D., Kinsella, A. R., and Houslay, M. D. (1984). The phorbol ester, TPA inhibits glucagon-stimulated adenylate cyclase activity. FEBS Lett *170*, 38-42.

Heyworth, C. M., Wilson, S. P., Gawler, D. J., and Houslay, M. D. (1985). The phorbol ester TPA prevents the expression of both glucagon desensitization and the glucagon-mediated block of insulin stimulation of the peripheral plasma-membrane cyclic-AMP phosphodiesterase in rat hepatocytes. FEBS Lett *187*, 196-200.

Higgins, J. B., and Casey, P. J. (1994). In vitro processing of recombinant G protein gamma subunits. Requirements for assembly of an active βγ complex. J Biol Chem *269*, 9067-9073.

Hirata, K., Kuroda, R., Sakoda, T., Katayama, M., Inoue, N., Suematsu, M., Kawashima, S., and Yokoyama, M. (1995). Inhibition of endothelial nitric oxide synthase activity by protein kinase C. Hypertension *25*, 180-5.

Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989). Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene *77*, 51-9.

Hoey, M., and Houslay, M. D. (1990). Identification and selective inhibition of four distinct soluble forms of cyclic nucleotide phosphodiesterase activity from kidney. Biochem Pharmacol *40*, 193-202.

Hoffman, J. M., Ishizuka, T., and Farese, R. V. (1991). Interrelated effects of insulin and glucose on diacylglycerol-protein kinase-C signalling in rat adipocytes and solei muscle in vitro and in vivo in diabetic rats. Endocrinology *128*, 2937-48.

Hosoda, K., Fitzgerald, L. R., Vaidya, V. A., Feussner, G. K., Fishman, P. H., and Duman, R. S. (1995). Regulation of beta2-adrenergic receptor mRNA and gene transcription in rat C6 glioma cells: Effects of agonist, forskolin, and protein synthesis inhibition. Mol Pharmacol *48*, 206-211.

House, C., and Kemp, B. E. (1987). Protein kinase C contains a pseudosubstrate prototype in its regulatory domain. Science *238*, 1726-8.

Houslay, M. D. (1990). Altered expression and functioning of guanine nucleotide binding regulatory proteins during growth, transformation, differentiation and in pathological states. In G-proteins as Mediators of Cellular Signalling Processes, M. D. Houslay and G. Milligan, eds. (London: John Wiley), pp. 197-230.

Houslay, M. D. (1991). 'Crosstalk': a pivotal role for protein kinase C in modulating relationships between signal transduction pathways. Eur J Biochem *195*, 9-27.

Houslay, M. D. (1992). G-protein linked receptors: a family probed by molecular cloning and mutagenesis procedures. Clin Endocrinol *36*, 525-34.

Houslay, M. D. (1994). Protein kinase C and the modulation of G-protein-controlled adenylyl cyclase and other signal transmission systems. In Regulation of cellular signal transduction pathways by desensitization and amplification, D. R. Sibley and M. D. Houslay, eds. (Chichester: John Wiley & Sons), pp. 129-168.

Houslay, M. D., Dipple, I., and Elliott, K. R. F. (1980). Guanosine 5'-triphosphate and guanosine 5'-(beta gamma-imido)triphosphate effect a collision coupling mechanism between the glucagon receptor and catalytic unit of adenylate cyclase. Biochem J *186*, 649-658.

Houslay, M. D., and Elliott, K. R. F. (1979). Cholera toxin mediated activation of adenylate cyclase in intact rat hepatocytes. FEBS Lett *104*, 359-363.

Houslay, M. D., and Elliott, K. R. F. (1981). Is the receptor-mediated endocytosis of cholera toxin a pre-requisite for its activation of adenylate cyclase in intact rat hepatocytes? FEBS Lett *128*, 289-292.

Houslay, M. D., Ellory, J. C., Smith, G. A., Hesketh, T. R., Stein, J. M., Warren, G. B., and Metcalfe, J. C. (1977). Exchange of partners in glucagon receptor-adenylate cyclase complexes. Physical evidence for the independent, mobile receptor model. Biochim Biophys Acta *467*, 208-219.

Houslay, M. D., Gawler, D. J., Milligan, G., and Wilson, A. (1989). Multiple defects occur in the guanine nucleotide regulatory protein system in liver plasma membranes of obese (fa/fa) but not lean (Fa/Fa) Zucker rats: loss of functional Gi and abnormal Gs function. Cellular Signalling *1*, 9-22.

Houslay, M. D., Metcalfe, J. C., Warren, G. B., Hesketh, T. R., and Smith, G. A. (1976). The glucagon receptor of rat liver plasma membrane can couple to adenylate cyclase without activating it. Biochim Biophys Acta *436*, 489-494.

Hsieh, J. C., Jurutka, P. W., Galligan, M. A., Terpening, C. M., Haussler, C. A., Samuels, D. S., Shimizu, Y., Shimizu, N., and Haussler, M. R. (1991). Human vitamin D receptor is selectively phosphorylated by protein kinase C on serine 51, a residue crucial to its trans-activation function. Proc Natl Acad Sci USA *88*, 9315-9319.

Hug, H., and Sarre, T. F. (1993). Protein kinase C isoenzymes: divergence in signal transduction? Biochem J *291*, 329-43.

Huggins, J. P., and England, P. J. (1983). Sarcolemmal phospholamban is phosphorylated in isolated rat hearts perfused with isoprenaline. FEBS Lett *163*, 297-302.

Hunter, T. (1995). Protein kinases and phosphatases: the Yin and Yang of protein phosphorylation and signaling. Cell *80*, 225-236.

Huston, E., Pooley, L., Julien, P., Scotland, G., McPhee, I., Sullivan, M., Bolger, G., and Houslay, M. D. (1996). The human cyclic AMP-specific phosphodiesterase PDE-46 (HSPDE4A4B) expressed in transfected COS7 cells occurs as both particulate and cytosolic species that exhibit distinct kinetics of inhibition by the antidepressant rolipram. J Biol Chem *271*, 31334-31344.

Ido, M., Sekiguchi, K., Kikkawa, U., and Nishizuka, Y. (1987). Phosphorylation of the EGF receptor from A431 epidermoid carcinoma cells by 3 distinct types of protein kinase-C. FEBS Lett *219*, 215-218.

Inglese, J., Freedman, N. J., Koch, W. J., and Lefkowitz, R. J. (1993). Structure and mechanism of the G protein-coupled receptor kinases. J Biol Chem *268*, 23735-8.

Inglese, J., Koch, W. J., Touhara, K., and Lefkowitz, R. J. (1995). G beta gamma interactions with PH domains and Ras-MAPK signaling pathways. Trends Biochem Sci *20*, 151-6.

Inoguchi, T., Battan, R., Handler, E., Sportsman, J. R., Heath, W., and King, G. L. (1992). Preferential elevation of protein kinase C isoform beta II and diacylglycerol levels in the aorta and heart of diabetic rats: differential reversibility to glycemic control by islet cell transplantation. Proc Natl Acad Sci USA *89*, 11059-63.

Inoguchi, T., Xia, P., Kunisaki, M., Higashi, S., Feener, E. P., and King, G. L. (1994). Insulin's effect on protein kinase C and diacylglycerol induced by diabetes and glucose in vascular tissues. Am J Phys *267*, E369-E379.

Inoue, M., Kishimoto, A., Takai, Y., and Nishizuka, Y. (1977). Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues, II: proenzyme and its activation by calcium-dependent protease from rat brain. J Biol Chem *252*, 7610-6.

Ishihara, T., Nakamura, S., Kaziro, Y., Takahashi, T., Takahashi, K., and Nagata, S. (1991). Molecular cloning and expression of a cDNA encoding the secretin receptor. EMBO Journal *10*, 1635-1641.

Ishihara, T., Shigemoto, R., Mori, K., Takahashi, K., and Nagata, S. (1992). Functional expression and tissue distribution of a novel receptor for vasoactive intestinal polypeptide. Neuron *8*, 811-819.

Ishikawa, Y., Katsushika, S., Chen, L., Halnon, N. J., Kawabe, J. I., and Homcy, C. J. (1992). Isolation and characterization of a novel cardiac adenylylcyclase cDNA. J Biol Chem *267*, 13553-13557.

Iwanij, V., and Vincent, A. C. (1990). Characterization of the glucagon receptor and its functional domains using monoclonal antibodies. J Biol Chem *265*, 21302-8.

Iyengar, R., and Herberg, J. T. (1984). Structural analysis of the hepatic glucagon receptor. Identification of a guanine nucleotide-sensitive hormone-binding region. J Biol Chem *259*, 5222-5229.

Jacobowitz, O., Chen, J., Premont, R. T., and Iyengar, R. (1993). Stimulation of specific types of Gs-stimulated adenylyl cyclases by phorbol ester treatment. J Biol Chem *268*, 3829-32.

James, G., and Olson, E. (1992). Deletion of the regulatory domain of protein kinase C alpha exposes regions in the hinge and catalytic domains that mediate nuclear targeting. J Cell Biol *116*, 863-74.

James, S. R., Vaziri, C., Walker, T. R., Milligan, G., and Downes, C. P. (1994). The turkey erythrocyte beta-adrenergic receptor couples to both adenylate cyclase and phospholipase C via distinct G-protein alpha subunits. Biochem J *304*, 359-64.

Jaspan, J. B., Polonsky, K. S., Lewis, M., Pensler, J., Pugh, W., Moosa, A. R., and Rubenstein, A. H. (1981). Hepatic metabolism of glucagon in the dog: contribution of the liver to the overall metabolic disposal of glucagon. Am J Phys *240*, E233-244.

Jelinek, L. J., Lok, S., Rosenberg, G. B., Smith, R. A., Grant, F. J., Biggs, S., Bensch, P. A., Kuijper, J. L., Sheppard, P. O., Sprecher, C. A., O'Hara, P. J., Foster, D., Walker, K. M., Chen, L. H. J., McKernan, P. A., and Kindsvogel, W. (1993). Expression cloning and signaling properties of the rat glucagon receptor. Science *259*, 1614-6.

Jelsema, C. L., and Axelrod, J. (1987). Stimulation of phospholipase A2 activity in bovine rod outer segments by the $\beta\gamma$ subunits of transducin and its inhibition by the alpha subunit. Proc Natl Acad Sci USA *84*, 3623-3627.

Johannes, F.-J., Prestle, J., Dieterich, S., Oberhagemann, P., Link, G., and Pfizenmaier, K. (1995). Characterization of activators and inhibitors of protein kinase C-mu. Eur J Biochem *227*, 303-307.

Johannes, F. J., Prestle, J., Eis, S., Oberhagemann, P., and Pfizenmaier, K. (1994). PKCu is a novel, atypical member of the protein kinase C family. J Biol Chem *269*, 6140-8.

Johnson, J. A., Clark, R. B., Friedman, J., Dixon, R. A. F., and Strader, C. D. (1990). Identification of a specific domain in the beta-adrenergic receptor required for phorbol ester-induced inhibition of catecholamine- stimulated adenylyl cyclase. Mol Pharmacol *38*, 289-293.

Johnson, M. E. M., Das, N. M., Butcher, F. R., and Fain, J. N. (1972). The regulation of gluconeogenesis in isolated rat liver cells by glucagon, insulin, dibutyryl cyclic adenosine monophosphate and fatty acids. J Biol Chem *247*, 3229-3235.

Jones, T. L. Z., Simonds, W. F., Merendino Jj, Jr., Brann, M. R., and Spiegel, A. M. (1990). Myristoylation of an inhibitory GTP-binding protein alpha subunit is essential for its membrane attachment. Proc Natl Acad Sci USA *87*, 568-572.

Juppner, H., Abou-Samra, A.-B., Freeman, M., Kong, X. F., Schipani, E., Richards, J., Kolakowski L. F., J., Hock, J., Potts J. T., J., Kronenberg, H. M., and Segre, G. V. (1991). A G protein-linked receptor for parathyroid hormone and parathyroid hormone-related peptide. Science *254*, 1024-1026.

Kagaya, A., Mikuni, M., Kusumi, I., Yamamoto, H., and Takahashi, K. (1990). Serotonin-induced acute desensitization of serotonin2 receptors in human platelets via a mechanism involving protein kinase C. Journal of Pharmacology and Experimental Therapeutics *255*, 305-311.

Kalman, V. K., Erdman, R. A., Maltese, W. A., and Robishaw, J. D. (1995). Regions outside of the CAAX motif influence the specificity of prenylation of G protein gamma subunits. J Biol Chem *270*, 14835-14841.

Karin, M., and Smeal, T. (1992). Control of transcription factors by signal transduction pathways: the beginning of the end. Trends Biochem Sci *17*, 418-22.

Katada, T., Bokoch, G. M., Northup, J. K., Ui, M., and Gilman, A. G. (1984a). The inhibitory guanine nucleotide-binding regulatory component of adenylate-cyclase. Properties and function of the purified protein. J Biol Chem *259*, 3568-3577.

Katada, T., Gilman, A. G., Watanabe, Y., and et, a. (1985). Protein kinase C phosphorylates the inhibitory guanine-nucleotide- binding regulatory component and apparently suppressed its function in hormonal inhibition of adenylate cyclase. Eur J Biochem *151*, 431-437.

Katada, T., Northup, J. K., Bokoch, G. M., Ui, M., and Gilman, A. G. (1984b). The inhibitory guanine nucleotide-binding regulatory component of adenylate-cyclase. Subunit dissociation and guanine nucleotide-dependent hormonal inhibition. J Biol Chem *259*, 3578-3585.

Katada, T., and Ui, M. (1982a). ADP ribosylation of the specific membrane-protein of C6 cells by islet-activating protein associated with modification of adenylate cyclase activity. J Biol Chem *257*, 7210-7216.

Katada, T., and Ui, M. (1982b). Direct modification of the membrane adenylate-cyclase system by islet-activating protein due to ADP-ribosylation of a membrane protein. Proc Natl Acad Sci USA *79*, 3129-3133.

Katsushika, S., Chen, L., Kawabe, J. I., Nilakantan, R., Halnon, N. J., Homcy, C. J., and Ishikawa, Y. (1992). Cloning and characterization of a sixth adenylyl cyclase isoform: Types V and VI constitute a subgroup within the mammalian adenylyl cyclase family. Proc Natl Acad Sci USA *89*, 8774-8778.

Kawabe, J. I., Ebina, T., Toya, Y., Oka, N., Schwencke, C., Duzic, E., and Ishikawa, Y. (1996). Regulation of type V adenylyl cyclase by PMA-sensitive and - insensitive protein kinase C isoenzymes in intact cells. FEBS Lett *384*, 273-276.

Kawabe, J. I., Iwami, G., Ebina, T., Ohno, S., Katada, T., Ueda, Y., Homcy, C. J., and Ishikawa, Y. (1994). Differential activation of adenylyl cyclase by protein kinase C isoenzymes. J Biol Chem *269*, 16554-16558.

Kemp, B. E., Graves, D. J., Benjamini, E., and Krebs, E. G. (1977). Role of multiple basic residues in determining the substrate specificity of cyclic AMP-dependent protein kinase. J Biol Chem *252*, 4888-4894.

Kemp, B. E., and Pearson, R. B. (1990). Protein kinase recognition sequence motifs. Trends Biochem Sci *15*, 342-6.

Kikkawa, U., Takai, Y., Minakuchi, R., Inohara, S., and Nishizuka, Y. (1982). Calcium-activated, phospholipid-dependent protein kinase from rat brain. Sub-cellular distribution, purification, and properties. J Biol Chem *257*, 3341-3348.

Kilgour, E. (1993). A role for inositol-glycan mediators and G-proteins in insulin action. Cellular Signalling *5*, 97-105.

Kim, D., Lewis, D. L., Graziadei, L., Neer, E. J., BarSagi, D., and Clapham, D. E. (1989). G-protein $\beta\gamma$ subunits activate the cardiac muscarinic K^+- channel via phospholipase A2. Nature *337*, 557-560.

Kisselev, O. G., Ermolaeva, M. V., and Gautam, N. (1994). A farnesylated domain in the G protein gamma subunit is a specific determinant of receptor coupling. J Biol Chem *269*, 21399-21402.

Klauck, T. M., Faux, M. C., Labudda, K., Langeberg, L. K., Jaken, S., and Scott, J. D. (1996). Coordination of three signaling enzymes by AKAP79, a mammalian scaffold protein. Science *271*, 1589-1592.

Knighton, D. R., Zheng, J., Ten Eyck, L. F., Ashford, V. A., Xuong, N., Taylor, S. S., and Sowadski, J. M. (1991). Crystal structure of the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. Science *253*, 407-414.

Kohl, N. E., Diehl, R. E., Schaber, M. D., Rands, E., Soderman, D. D., He, B., Moores, S. L., Pompliano, D. L., FerroNovick, S., Powers, S., Thomas, K. A., and Gibbs, J. B. (1991). Structural homology among mammalian and Saccharomyces cerevisiae isoprenyl-protein transferases. J Biol Chem *266*, 18884-18888.

Koide, H., Ogita, K., Kikkawa, U., and Nishizuka, Y. (1992). Isolation and characterization of the epsilon subspecies of protein kinase C from rat brain. Proc Natl Acad Sci USA *89*, 1149-53.

Kolch, W., Heidecker, G., Kochs, G., Hummel, R., Vahidi, H., Mischak, H., Finkenzeller, G., Marme, D., and Rapp, U. R. (1993). Protein kinase C alpha activates RAF-1 by direct phosphorylation. Nature *364*, 249-52.

Kosugi, S., Okajima, F., Ban, T., Hidaka, A., Shenker, A., and Kohn, L. D. (1992). Mutation of alanine 623 in the third cytoplasmic loop of the rat thyrotropin (TSH) receptor results in a loss in the phosphoinositide but not cAMP signal induced by TSH and receptor autoantibodies. J Biol Chem *267*, 24153-6.

Kozak, M. (1986). Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell *44*, 283-292.

Krapivinsky, G., Krapivinsky, L., Wickman, K., and Clapham, D. E. (1995). G-$\beta\gamma$ binds directly to the G protein-gated K channel, I(KACh). J Biol Chem *270*, 29059-29062.

Krebs, E. G., and Beavo, J. A. (1979). Phosphorylation-dephosphorylation of enzymes. Annu Rev Biochem *48*, 923-959.

Kreis, T. E. (1986). Microinjected antibodies against the cytoplasmic domain of vesicular stomatitis virus glycoprotein block its transport to the cell surface. EMBO Journal *5*, 931-941.

Krupinski, J., Coussen, F., Bakalyar, H. A., Tang, W. J., Feinstein, P. G., Orth, K., Slaughter, C., Reed, R. R., and Gilman, A. G. (1989). Adenylyl cyclase amino acid sequence: Possible channel- or transporter-like structure. Science *244*, 1558-1564.

Kubo, K., Ohno, S., and Suzuki, K. (1987). Nucleotide sequence of the 3' portion of a human gene for protein kinase C beta I/beta II. Nucleic Acids Res *15*, 7179-80.

Kunapuli, P., and Benovic, J. L. (1993). Cloning and expression of GRK5: a member of the G protein-coupled receptor kinase family. Proc Natl Acad Sci USA *90*, 5588-92.

Kurose, H., and Lefkowitz, R. J. (1994). Differential desensitization and phosphorylation of three cloned and transfected alpha2-adrenergic receptor subtypes. J Biol Chem *269*, 10093-10099.

Kwatra, M. M., Schwinn, D. A., Schreurs, J., Blank, J. L., Kim, C. M., Benovic, J. L., Krause, J. E., Caron, M. G., and Lefkowitz, R. J. (1993). The substance P receptor, which couples to G(q/11), is a substrate of beta- adrenergic receptor kinase 1 and 2. J Biol Chem *268*, 9161-9164.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *222*, 680-682.

Lambright, D. G., Noel, J. P., Hamm, H. E., and Sigler, P. B. (1994). Structural determinants for activation of the alpha-subunit of a heterotrimeric G protein. Nature *369*, 621-628.

Lambright, D. G., Sondek, J., Bohm, A., Skiba, N. P., Hamm, H. E., and Sigler, P. B. (1996). The 2.0 A crystal structure of a heterotrimeric G protein. Nature *379*, 311-319.

Langhans-Rajasekaran, S. A., Wan, Y., and Huang, X. Y. (1995). Activation of Tsk and Btk tyrosine kinases by G protein $\beta\gamma$ subunits. Proc Natl Acad Sci USA *92*, 8601-8605.

Lavan, B. E., Lakey, T., and Houslay, M. D. (1989). Resolution of soluble cyclic nucleotide phosphodiesterase isoenzymes, from liver and hepatocytes, identifies a novel IBMX insensitive form. Biochem Pharmacol *38*, 4123-4136.

Leach, K. L., James, M. L., and Blumberg, P. M. (1983). Characterization of a specific phorbol ester aporeceptor in mouse brain cytosol. Proc Natl Acad Sci USA *80*, 4208-12.

Lee, C., Murakami, T., and Simonds, W. F. (1995). Identification of a discrete region of the G protein gamma subunit conferring selectivity in βγ complex formation. J Biol Chem *270*, 8779-8784.

Lefkowitz, R. J. (1996). G protein-coupled receptors and receptor kinases: From molecular biology to potential therapeutic applications. Nature Biotechnology *14*, 283-286.

Leurs, R., Smit, M. J., Bast, A., and Timmerman, H. (1991). Homologous histamine H1 receptor desensitization results in reduction of H1 receptor agonist efficacy. Eur J Pharmacol *196*, 319-322.

Lev, S., Moreno, H., Martinez, R., Canoll, P., Peles, E., Musacchio, J. M., Plowman, G. D., Rudy, B., and Schlessinger, J. (1995). Protein tyrosine kinase PYK2 involved in Ca^{2+}-induced regulation of ion channel and MAP kinase functions. Nature *376*, 737-745.

Levitzki, A. (1988). From epinephrine to cyclic AMP. Science *241*, 800-806.

Lewin, B. (1985). Genes II, 2nd Edition (New York: Wiley).

Lewis, R. E., Cao, L., Perregaux, D., and Czech, M. P. (1990). Threonine 1336 of the human insulin receptor is a major target for phosphorylation by protein kinase C. Biochemistry *29*, 1807-13.

Liggett, S. B., and Lefkowitz, R. J. (1994). Adrenergic receptor-coupled adenylyl cyclase systems: regulation of receptor function by phosphorylation, sequestration and downregulation. In Regulation of cellular signal transduction pathways by desensitization and amplification, D. R. Sibley and M. D. Houslay, eds. (Chichester: John Wiley & Sons), pp. 71-98.

Limbird, L. E., Gill, D. M., and Lefkowitz, R. J. (1980). Agonist-promoted coupling of the beta-adrenergic receptor with the guanine nucleotide regulatory protein of the adenylate cyclase system. Proc Natl Acad Sci USA *77*, 775-779.

Lin, H. Y., Harris, T. L., Flannery, M. S., Aruffo, A., Kaji, E. H., Gorn, A., Kolakowski L. F., J., Lodish, H. F., and Goldring, S. R. (1991). Expression cloning of an adenylate cyclase-coupled calcitonin receptor. Science *254*, 1022-1024.

Lin, S. C., Lin, C. R., Gukovsky, I., Lusis, A. J., Sawchenko, P. E., and Rosenfeld, M. G. (1993). Molecular basis of the *little* mouse phenotype and implications for cell type-specific growth. Nature *364*, 208-213.

Liscovitch, M., and Cantley, L. C. (1994). Lipid second messengers. Cell *77*, 329-334.

Livingstone, C., MacDonald, C., Willett, B., and Houslay, M. D. (1994). Analysis of the adenylate cyclase signalling system, and alterations induced by culture with insulin, in a novel SV40-DNA-immortalized hepatocyte cell line (P9 cells). Biochem J *300*, 835-42.

Livingstone, C., McLellan, A. R., McGregor, M. A., Wilson, A., Connell, J. M. C., Small, M., Milligan, G., Paterson, K. R., and Houslay, M. D. (1991). Altered G-protein expression and adenylate cyclase activity in platelets of non-insulin-dependent diabetic (NIDDM) male subjects. Biochim Biophys Acta *1096*, 127-133.

Logothetis, D. E., Kurachi, Y., Galper, J., and et, a. (1987). The βγ subunits of GTP-binding proteins activate the muscarinic K^+ channel in heart. Nature *325*, 321-326.

Lohse, M. J. (1990). Quantitation of receptor desensitization by an operational model of agonism. J Biol Chem *265*, 3210-3211.

Lohse, M. J. (1993). Molecular mechanisms of membrane receptor desensitization. Biochim Biophys Acta *1179*, 171-88.

Lohse, M. J., Andexinger, S., Pitcher, J., Trukawinski, S., Codina, J., Faure J, P., Caron, M. G., and Lefkowitz, R. J. (1992). Receptor-specific desensitization with purified proteins. Kinase dependence and receptor specificity of beta-arrestin and arrestin in the beta2- adrenergic receptor and rhodopsin systems. J Biol Chem *267*, 8558-8564.

Lohse, M. J., Benovic, J. L., Caron, M. G., and Lefkowitz, R. J. (1990). Multiple pathways of rapid beta2-adrenergic receptor desensitization. Delineation with specific inhibitors. J Biol Chem *265*, 3202-3211.

Lohse, M. J., Krasel, C., Winstel, R., and Mayor, F. (1996). G-protein-coupled receptor kinases. Kidney International *49*, 1047-1052.

Lok, S., Kuijper, J. L., Jelinek, L. J., Kramer, J. M., Whitmore, T. E., Sprecher, C. A., Mathewes, S., Grant, F. J., Biggs, S. H., Rosenberg, G. B., Sheppard, P. O., O'Hara, P. J., Foster, D. C., and Kindsvogel, W. (1994). The human glucagon receptor encoding gene: structure, cDNA sequence and chromosomal localization. Gene *140*, 203-9.

Lopez-Ilasaca, M., Crespo, P., Pellici, P. G., Gutkind, J. S., and Wetzker, R. (1997). Linkage of G protein-coupled receptors to the MAPK signaling pathway through PI 3-kinase gamma. Science *275*, 394-397.

Lorenz, W., Inglese, J., Palczewski, K., Onorato, J. J., Caron, M. G., and Lefkowitz, R. J. (1991). The receptor kinase family: Primary structure of rhodopsin kinase reveals similarities to the beta-adrenergic receptor kinase. Proc Natl Acad Sci USA *88*, 8715-8719.

Loudon, R. P., and Benovic, J. L. (1994). Expression, purification, and characterization of the G protein-coupled receptor kinase GRK6. J Biol Chem *269*, 22691-7.

Luo, J., and Weinstein, I. B. (1993). Calcium-dependent activation of protein kinase C. The role of the C2 domain in divalent cation selectivity. J Biol Chem *268*, 23580-23584.

Luo, Z., Shafit-Zagardo, B., and Erlichman, J. (1990). Identification of the MAP2- and P75-binding domain in the regulatory subunit (RIIbeta) of type II cAMP-dependent protein kinase. Cloning and expression of the cDNA for bovine brain RIIbeta. J Biol Chem *265*, 21804-21810.

Lustig, K. D., Conklin, B. R., Herzmark, P., Taussig, R., and Bourne, H. R. (1993). Type II adenylylcyclase integrates coincident signals from G(s), G(i), and G(q). J Biol Chem *268*, 13900-13905.

MacNeil, D. J., Occi, J. L., Hey, P. J., Strader, C. D., and Graziano, M. P. (1994). Cloning and expression of a human glucagon receptor. Biochem Biophys Res Commun *198*, 328-34.

Marchmont, R. J., and Houslay, M. D. (1980). Insulin controls the cyclic AMP-dependent phosphorylation of integral and peripheral proteins associated with the rat liver plasma membrane. FEBS Lett *118*, 18-24.

Marquardt, B., Frith, D., and Stabel, S. (1994). Signalling from TPA to MAP kinase requires protein kinase C, raf and MEK: reconstitution of the signalling pathway in vitro. Oncogene *9*, 3213-8.

Mauger, J. P., Poggioli, J., and Claret, M. (1985). Synergistic stimulation of the calcium influx in rat hepatocytes by glucagon and the calcium-linked hormones vasopressin and angiotensin II. J Biol Chem *260*, 1635-1642.

Maurice, D. H., Waldo, G. L., Morris, A. J., Nicholas, R. A., and Harden, T. K. (1993). Identification of G alpha11 as the phospholipase C-activating G- protein of turkey erythrocytes. Biochem J *290*, 765-770.

Mayor, F. J., Benovic, J. L., Caron, M. G., and Lefkowitz, R. J. (1987). Somatostatin induces translocation of the beta-adrenergic receptor kinase and desensitizes somatostatin receptors in S49 lymphoma cells. J Biol Chem *262*, 6468-6471.

McCallum, J. F., Wise, A., Grassie, M. A., Magee, A. I., Guzzi, F., Parenti, M., and Milligan, G. (1995). The role of palmitoylation of the guanine nucleotide binding protein G11alpha in defining interaction with the plasma membrane. Biochem J *310*, 1021-1027.

Meek, D. W., and Street, A. J. (1992). Nuclear protein phosphorylation and growth control. Biochem J *287*, 1-15.

Menard, L., Ferguson, S. S. G., Barak, L. S., Bertrand, L., Premont, R. T., Colapietro, A.-M., Lefkowitz, R. J., and Caron, M. G. (1996). Members of the G protein-coupled receptor kinase family that phosphorylate the beta2-adrenergic receptor facilitate sequestration. Biochemistry *35*, 4155-4160.

Milligan, G., Bond, R. A., and Lee, M. (1995a). Inverse agonism: Pharmacological curiosity or potential therapeutic strategy? Trends Pharmacol Sci *16*, 10-13.

Milligan, G., Carr, C., Gould, G. W., Mullaney, I., and Lavan, B. E. (1991). Agonist-dependent, cholera toxin-catalyzed ADP-ribosylation of pertussis toxin-sensitive G-proteins following transfection of the human alpha2-C10 adrenergic receptor into rat 1 fibroblasts: Evidence for the direct interaction of a single receptor with two pertussis toxin-sensitive G-proteins G(i)2 and G(i)3. J Biol Chem *266*, 6447-6455.

Milligan, G., Parenti, M., and Magee, A. I. (1995b). The dynamic role of palmitoylation in signal transduction. Trends Biochem Sci *20*, 181-186.

Mine, T., Kojima, I., and Ogata, E. (1988). Evidence of cyclic AMP-independent action of glucagon on calcium mobilization in rat hepatocytes. Biochim Biophys Acta *970*, 166-171.

Mixen, M. B., Lee, E., Coleman, D. E., Berghuis, A. M., Gilman, A. G., and Sprang, S. R. (1995). Tertiary and quaternary structural changes in $G_i$alpha1 induced by GTP hydrolysis. Science *270*, 954-960.

Mons, N., and Cooper, D. M. F. (1995). Adenylate cyclases: critical foci in neuronal signaling. Trends In Neurosciences *18*, 536-542.

Moriarty, T. M., Padrell, E., Carty, D. J., Omri, G., Landau, E. M., and Iyengar, R. (1990). G_o protein as signal transducer in the pertussis toxin-sensitive phosphatidylinositol pathway. Nature *343*, 79-82.

Morris, A. J., Rudge, S. A., Mahlum, C. E., and Jenco, J. M. (1995a). Regulation of phosphoinositide-3-kinase by G protein $\beta\gamma$ subunits in a rat osteosarcoma cell line. Mol Pharmacol *48*, 532-539.

Morris, N. J., Bushfield, M., and Houslay, M. D. (1996). Streptozotocin-induced diabetes elicits the phosphorylation of hepatocyte alpha-Gi-2 at the protein kinase C site but not at the protein kinase A-controlled site. Biochem J *315*, 417-420.

Morris, N. J., Young, P., and Houslay, M. D. (1995b). Insulin inhibits the phosphorylation of alpha-Gi-2 in intact hepatoyctes. Biochem J *308*, 693-696.

Mosior, M., Kim, J., Chung, L., Wu, H., and McLaughlin, S. (1990). A molecular mechanism for the binding of protein kinase-C to membranes. Biophysical Journal *57*, A 463-A 463.

Moss, J., and Vaughan, M. (1977). Mechanism of action of choleragen. Evidence for ADP-ribosyltransferase activity with arginine as an acceptor. J Biol Chem *252*, 2455-2457.

Moxham, C. M., and Malbon, C. C. (1996). Insulin action impaired by deficiency of the G-protein subunit $G_i$alpha2. Nature *379*, 840-844.

Mullaney, I., Carr, I. C., and Milligan, G. (1996). Analysis of inverse agonism at the delta opioid receptor after expression in Rat 1 fibroblasts. Biochem J *315*, 227-234.

Müller, G., Ayoub, M., Storz, P., Rennecke, J., Fabbro, D., and Pfizenmaier, K. (1995). PKC zeta is a molecular switch in signal transduction of TNF-alpha, bifunctionally regulated by ceramide and arachidonic acid. EMBO Journal *14*, 1961-1969.

Muller, H. K., Kellerer, M., Ermel, B., Muhlhofer, A., Obermaier-Kusser, B., Vogt, B., and Haring, H. U. (1991). Prevention by protein kinase C inhibitors of glucose-induced insulin-receptor tyrosine kinase resistance in rat fat cells. Diabetes *40*, 1440-8.

Mumby, S. M., Heukeroth, R. O., Gordon, J. I., and Gilman, A. G. (1990). G-protein alpha-subunit expression, myristoylation, and membrane association in COS cells. Proc Natl Acad Sci USA *87*, 728-732.

Munro, S., and Pelham, H. R. B. (1984). Use of peptide tagging to detect proteins expressed from cloned genes: deletion mapping functional domains of Drosophila hsp70. EMBO Journal *3*, 3087-3093.

Munro, S., and Pelham, H. R. B. (1986). An hsp70-like protein in the ER: identity with the 78 kd glucose-regulated protein and immunoglobulin heavy chain binding protein. Cell *46*, 291-300.

Munro, S., and Pelham, H. R. B. (1987). A C-terminal signal prevents secretion of luminal ER proteins. Cell *48*, 899-907.

Murga, C., Ruiz-Gomez, A., Garcia-Higuera, I., Kim, C. M., Benovic, J. L., and Mayor, F. J. (1996). High affinity binding of beta-adrenergic receptor kinase to microsomal membranes. Modulation of the activity of bound kinase by heterotrimeric G protein activation. J Biol Chem *271*, 985-994.

Murphy, G. J., Gawler, D. J., Milligan, G., Wakelam, M. J., Pyne, N. J., and Houslay, M. D. (1989). Glucagon desensitization of adenylate cyclase and stimulation of inositol phospholipid metabolism does not involve the inhibitory guanine nucleotide regulatory protein Gi, which is inactivated upon challenge of hepatocytes with glucagon. Biochem J *259*, 191-7.

Murphy, G. J., and Houslay, M. D. (1988). Resensitization of hepatocyte glucagon-stimulated adenylate cyclase can be inhibited when cAMP phosphodiesterase inhibitors are used to elevate intracellular cAMP concentrations to supraphysiological values. Biochem J *249*, 543-547.

Murphy, G. J., Hruby, V. J., Trivedi, D., Wakelam, M. J. O., and Houslay, M. D. (1987). The rapid desensitization of glucagon-stimulated adenylate cyclase is a cyclic AMP-independent process that can be mimicked by hormones which stimulate inositol phospholipid metabolism. Biochem J *243*, 39-46.

Murthy, K. S., and Makhlouf, G. M. (1995). Adenosine A1 receptor-mediated activation of phospholipase C-beta3 in intestinal muscle: Dual requirement for alpha and $\beta\gamma$ subunits of G_i3. Mol Pharmacol *47*, 1172-1179.

Musacchio, A., Gibson, T., Rice, P., Thompson, J., and Saraste, M. (1993). The PH domain: A common piece in the structural patchwork of signalling proteins. Trends Biochem Sci *18*, 343-348.

Nakanishi, H., Brewer, K. A., and Exton, J. H. (1993). Activation of the zeta isozyme of protein kinase C by phosphatidylinositol 3,4,5-trisphosphate. J Biol Chem *268*, 13-6.

Nakanishi, H., and Exton, J. H. (1992). Purification and characterization of the zeta isoform of protein kinase C from bovine kidney. J Biol Chem *267*, 16347-16354.

Nantel, F., Bonin, H., Emorine, L. J., Zilberfarb, V., Strosberg, A. D., Bouvier, M., and Marullo, S. (1993). The human beta3-adrenergic receptor is resistant to short term agonist-promoted desensitization. Mol Pharmacol *43*, 548-555.

Neer, E. J. (1995). Heterotrimeric G proteins: Organizers of transmembrane signals. Cell *80*, 249-257.

Neer, E. J., Schmidt, C. J., Nambudripad, R., and Smith, T. F. (1994). The ancient regulatory-protein family of WD-repeat proteins. Nature *371*, 297-300.

Newlands, C., and Houslay, M. D. (1991). Treatment of intact hepatocytes with synthetic diacyl glycerols mimics the ability of glucagon to cause the desensitization of adenylate cyclase. FEBS Lett *289*, 129-32.

Newton, A. C., and Koshland, D. E. J. (1989). High cooperativity, specificity, and multiplicity in the protein kinase C-lipid interaction. J Biol Chem *264*, 14909-15.

Nigg, E. A., Schafer, G., Hilz, H., and Eppenberger, H. (1985). Cyclic-AMP-dependent protein kinase type II is associated with the Golgi complex and with centrosomes. Cell *41*, 1039-1051.

Niino, Y. S., Ohno, S., and Suzuki, K. (1992). Positive and negative regulation of the transcription of the human protein kinase C beta gene. J Biol Chem *267*, 6158-63.

Nishizuka, Y. (1984). The role of protein kinase C in cell surface signal transduction and tumour promotion. Nature *308*, 693-8.

Nishizuka, Y. (1988). The molecular heterogeneity of protein kinase C and its implications for cellular regulation. Nature *334*, 661-5.

Nishizuka, Y. (1995). Protein kinase C and lipid signaling for sustained cellular responses. FASEB Journal *9*, 484-496.

Noda, C., Shinjyo, F., Tomomura, A., Kato, S., Nakamura, T., and Ichihara, A. (1984). Mechanism of heterologous desensitization of the adenylate cyclase system by glucagon in primary cultures of adult rat hepatocytes. J Biol Chem *259*, 7747-7754.

Noel, J. P., Hamm, H. E., and Sigler, P. B. (1993). The 2.2 A crystal structure of transducin-alpha complexed with GTPgammaS. Nature *366*, 654-663.

Nussenzveig, D. R., Thaw, C. N., and Gershengorn, M. C. (1994). Inhibition of inositol phosphate second messenger formation by intracellular loop one of a human calcitonin receptor. Expression and mutational analysis of synthetic receptor genes. J Biol Chem *269*, 28123-28129.

O'Brien, R. M., and Granner, D. K. (1991). Regulation of gene expression by insulin. Biochem J *278*, 609-619.

O'Dowd, B. F., Hnatowich, M., Caron, M. G., Lefkowitz, R. J., and Bouvier, M. (1989). Palmitoylation of the human beta2-adrenergic receptor. Mutation of Cys341 in the carboxyl tail leads to an uncoupled nonpalmitoylated form of the receptor. J Biol Chem *264*, 7564-7569.

O'Dowd, B. F., Hnatowich, M., Regan, J. W., Leader, W. M., Caron, M. G., and Lefkowitz, R. J. (1988). Site-directed mutagenesis of the cytoplasmic domains of the human beta2-adrenergic receptor. Localization of regions involved in G protein-receptor coupling. J Biol Chem *263*, 15985-15992.

Obeid, L. M., Blobe, G. C., Karolak, L. A., and Hannun, Y. A. (1992). Cloning and characterization of the major promoter of the human protein kinase C beta gene. Regulation by phorbol esters. J Biol Chem *267*, 20804-10.

Obermaier-Kusser, B., White, M. F., Pongratz, D. E., Su, Z., Ermel, B., Muhlbacher, C., and Haring, H. U. (1989). A defective intramolecular autoactivation cascade may cause the reduced kinase activity of the skeletal muscle insulin receptor from patients with non-insulin-dependent diabetes mellitus. J Biol Chem *264*, 9497-504.

Ogita, K., Miyamoto, S., Yamaguchi, K., Koide, H., Fujisawa, N., Kikkawa, U., Sahara, S., Fukami, Y., and Nishizuka, Y. (1992). Isolation and characterization of delta-subspecies of protein kinase C from rat brain. Proc Natl Acad Sci USA *89*, 1592-6.

Ohno, S., Kawasaki, H., Imajoh, S., Suzuki, K., Inagaki, M., Yokokura, H., Sakoh, T., and Hidaka, H. (1987). Tissue-specific expression of three distinct types of rabbit protein kinase C. Nature *325*, 161-6.

Ohshima, Y., and Gotoh, Y. (1987). Signals for the selection of a splice site in pre-mRNA. Computer analysis of splice junction sequences and like sequences. J Mol Biol *195*, 247-259.

Okamoto, T., Murayama, Y., Hayashi, Y., Inagaki, M., Ogata, E., and Nishimoto, I. (1991). Identification of a Gs activator region of the beta2-adrenergic receptor that is autoregulated via protein kinase A-dependent phosphorylation. Cell *67*, 723-730.

Oliveira, L., Paiva, A. C. M., Sander, C., and Vriend, G. (1994). A common step for signal transduction in G protein-coupled receptors. Trends Pharmacol Sci *15*, 170-172.

Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Igarashi, K., and Nishizuka, Y. (1989). Protein kinase C zeta subspecies from rat brain: its structure, expression, and properties. Proc Natl Acad Sci USA *86*, 3099-103.

Onorato, J. J., Palczewski, K., Regan, J. W., Caron, M. G., Lefkowitz, R. J., and Benovic, J. L. (1991). Role of acidic amino acids in peptide substrates of the beta-adrenergic receptor kinase and rhodopsin kinase. Biochemistry *30*, 5118-5125.

Oppermann, M., Freedman, N. J., Alexander, R. W., and Lefkowitz, R. J. (1996). Phosphorylation of the type 1A angiotensin II receptor by G protein-coupled receptor kinases and protein kinase C. J Biol Chem *271*, 13266-13272.

Orlandi, P. A., Curran, P. K., and Fishman, P. H. (1993). Brefeldin A blocks the response of cultured cells to cholera toxin. Implications for intracellular trafficking in toxin action. J Biol Chem *268*, 12010-12016.

Orr, J. W., and Newton, A. C. (1994). Intrapeptide regulation of protein kinase C. J Biol Chem *269*, 8383-7.

Osada, S.-I., Mizuno, K., Saido, T. C., Akita, Y., Suzuki, K., Kuroki, T., and Ohno, S. (1990). A phorbol ester receptor/protein kinase, nPKC eta, a new member of the protein kinase C family predominantly expressed in lung and skin. J Biol Chem *265*, 22434-40.

Osada, S.-I., Mizuno, K., Saido, T. C., Suzuki, K., Kuroki, T., and Ohno, S. (1992). A new member of the protein kinase C family, nPKC theta, predominantly expressed in skeletal muscle. Mol Cell Biol *12*, 3930-8.

Palczewski, K., Buczylko, J., Kaplan, M. W., Polans, A. S., and Crabb, J. W. (1991). Mechanism of rhodopsin kinase activation. J Biol Chem *266*, 12949-12955.

Palczewski, K., Ohguro, H., Premont, R. T., and Inglese, J. (1995). Rhodopsin kinase autophosphorylation. Characterization of site specific mutations. J Biol Chem *270*, 15294-15298.

Palmer, S., and Wakelam, M. J. O. (1990). Mass measurement of inositol 1,4,5-trisphosphate using a specific binding assay. In Methods in Inositide Research, R. F. Irvine, ed. (New York: Raven Press), pp. 127-134.

Paterson, J. M., Smith, S. M., Harmar, A. J., and Antoni, F. A. (1995). Control of a novel adenylyl cyclase by calcineurin. Biochemical and Biophysical Research Communications *214*, 1000-1008.

Pears, C., Schaap, D., and Parker, P. J. (1991). The regulatory domain of protein kinase C-epsilon restricts the catalytic domain specificity. Biochem J *276*, 257-260.

Pears, C., Stabel, S., Cazaubon, S., and Parker, P. J. (1992). Studies on the phosphorylation of protein kinase C-alpha. Biochem J *283*, 515-8.

Pelham, H. R. B., Hardwick, K. G., and Lewis, M. J. (1988). Sorting of soluble ER proteins in yeast. EMBO Journal *7*, 1757-1762.

Pettitt, T. R., Zaqqa, M., and Wakelam, M. J. O. (1994). Epidermal growth factor stimulates distinct diradylglycerol species generation in Swiss 3T3 fibroblasts: evidence for a potential phosphatidylcholine-specific phospholipase C-catalysed pathway. Biochem J *298*, 655-660.

Phillips, W. J., and Cerione, R. A. (1992). Rhodopsin/transducin interactions. I. Characterization of the binding of the transducin-$\beta\gamma$ subunit complex to rhodopsin using fluorescence spectroscopy. J Biol Chem *267*, 17032-17039.

Pilkis, S. J., Claus, T. H., Johnson, R. A., and Park, C. R. (1975). Hormonal control of cyclic 3':5'-AMP levels and gluconeogenesis in isolated hepatocytes from fed rats. J Biol Chem *250*, 6328-6336.

Pilkis, S. J., El-Maghrabi, M. R., Coven, B., Claus, T. H., Tager, H. S., Steiner, D. F., Kleim, P. S., and Heinrikson, R. L. (1980). Phosphorylation of rat hepatic fructose-1,6-bisphosphatase and pyruvate kinase. J Biol Chem *255*, 2770-2775.

Pilkis, S. J., Exton, J. H., Johnson, R. A., and Park, C. R. (1974). Effects of glucagon on cyclic AMP and carbohydrate metabolism in livers from diabetic rats. Biochim Biophys Acta *343*, 250-267.

Pillay, T. S., Whittaker, J., and Siddle, K. (1990). Phorbol ester-induced downregulation of protein kinase C potentiates insulin receptor tyrosine autophosphorylation: evidence for a major constitutive role in insulin receptor regulation. Biochem Soc Trans *18*, 494-5.

Pin, J. P., Joly, C., Heinemann, S. F., and Bockaert, J. (1994). Domains involved in the specificity of G protein activation in phospholipase C-coupled metabotropic glutamate receptors. EMBO Journal *13*, 342-348.

Pippig, S., Andexinger, S., and Lohse, M. J. (1995). Sequestration and recycling of beta2-adrenergic receptors permit receptor resensitization. Mol Pharmacol *47*, 666-676.

Pitcher, J., Lohse, M. J., Codina, J., Caron, M. G., and Lefkowitz, R. J. (1992a). Desensitization of the isolated beta2-adrenergic receptor by beta-adrenergic receptor kinase, cAMP-dependent protein kinase, and protein kinase C occurs via distinct molecular mechanisms. Biochemistry *31*, 3193-3197.

Pitcher, J. A., Inglese, J., Higgins, J. B., Arriza, J. L., Casey, P. J., Kim, C., Benovic, J. L., Kwatra, M. M., Caron, M. G., and Lefkowitz, R. J. (1992b). Role of βγ subunits of G proteins in targeting the beta- adrenergic receptor kinase to membrane-bound receptors. Science *257*, 1264-1267.

Pitcher, J. A., Payne, E. S., Csortos, C., DePaoli-Roach, A. A., and Lefkowitz, R. J. (1995a). The G-protein-coupled receptor phosphatase: a protein phosphatase type 2A with a distinct subcellular distribution and substrate specificity. Proc Natl Acad Sci USA *92*, 8343-8347.

Pitcher, J. A., Touhara, K., Payne, E. S., and Lefkowitz, R. J. (1995b). Pleckstrin homology domain-mediated membrane association and activation of the beta-adrenergic receptor kinase requires coordinate interaction with G(beta-gamma) subunits and lipid. J Biol Chem *270*, 11707-11710.

Pittner, R. A., and Fain, J. N. (1991). Activation of membrane protein kinase C by glucagon and Ca-mobilizing hormones in cultured rat hepatocytes. Role of phosphatidylinositol and phosphatidylcholine hydrolysis. Biochem J *277*, 371-8.

Plas, C., and Nunez, J. (1975). Glycogenolytic response to glucagon of cultured fetal hepatocytes. Refractoriness following prior exposure to glucagon. J Biol Chem *250*, 5304-5311.

Pohl, S. L., Birnbaumer, L., and Rodbell, M. (1971). The glucagon-sensitive adenyl cyclase system in plasma membranes of rat liver. I. Properties. J Biol Chem *246*, 1849-1856.

Ponte, P., Ng, S. Y., Engel, J., Gunning, P., and Kedes, L. (1984). Evolutionary conservation in the untranslated regions of actin mRNAs: DNA sequence of a human beta-actin cDNA. Nucleic Acids Res *12*, 1687-1696.

Premont, R. T., Chen, J., Ma, H. W., Ponnapalli, M., and Iyengar, R. (1992). Two members of a widely expressed subfamily of hormone-stimulated adenylyl cyclases. Proc Natl Acad Sci USA *89*, 9809-9813.

Premont, R. T., Inglese, J., and Lefkowitz, R. J. (1995). Protein kinases that phosphorylate activated G protein-coupled receptors. FASEB Journal *9*, 175-82.

Premont, R. T., and Iyengar, R. (1988). Glucagon-induced desensitization of adenylyl cyclase in primary cultures of chick hepatocytes - evidence for multiple pathways. J Biol Chem *263*, 16087-16095.

Premont, R. T., Koch, W. J., Inglese, J., and Lefkowitz, R. J. (1994). Identification, purification, and characterization of GRK5, a member of the family of G protein-coupled receptor kinases. J Biol Chem *269*, 6832-41.

Premont, R. T., Matsuoka, I., Mattei, M. G., Pouille, Y., Defer, N., and Hanoune, J. (1996). Identification and characterization of a widely expressed form of adenylyl cyclase. J Biol Chem *271*, 13900-13907.

Pronin, A. N., and Gautam, N. (1992). Interaction between G-protein beta and gamma subunit types is selective. Proc Natl Acad Sci USA *89*, 6220-6224.

Pumiglia, K. M., LeVine, H., Haske, T., Habib, T., Jove, R., and Decker, S. J. (1995). A direct interaction between G-protein $\beta\gamma$ subunits and the Raf-1 protein kinase. J Biol Chem *270*, 14251-14254.

Pyne, N. J., Murphy, G. J., Milligan, G., and Houslay, M. D. (1989). Treatment of intact hepatocytes with either the phorbol ester TPA or glucagon elicits the phosphorylation and functional inactivation of the inhibitory guanine nucleotide regulatory protein G(i). FEBS Lett *243*, 77-82.

Quest, A. F. G., Bardes, E. S. G., and Bell, R. M. (1994). A phorbol ester binding domain of protein kinase C gamma. J Biol Chem *269*, 2953-2960.

Ramachandran, C., Goris, J., Waelkens, E., Merlevede, W., and Walsh, D. A. (1987). The interrelationship between cAMP-dependent alpha- and beta- subunit phosphorylation in the regulation of phosphorylase kinase activity. Studies using subunit specific phosphatases. J Biol Chem *262*, 3210-3218.

Ranganathan, R., and Stevens, C. F. (1995). Arrestin binding determines the rate of inactivation of the G protein-coupled receptor rhodopsin in vivo. Cell *81*, 841-8.

Rarick, H. M., Artemyev, N. O., and Hamm, H. E. (1992). A site on rod G protein alpha subunit that mediates effector activation. Science *256*, 1031-1033.

Rees, D. C., DeAntonio, L., and Eisenberg, D. (1989). Hydrophobic organization of membrane proteins. Science *245*, 510-513.

Refsnes, M., Johansen, E. J., and Christoffersen, T. (1989). Glucagon-induced refractoriness of hepatocyte adenylate cyclase. Comparison of homologous and heterologous components and evidence against a role of cAMP. Pharmacol Toxicol *64*, 397-403.

Rhee, S. G., and Choi, K. D. (1992). Regulation of inositol phospholipid-specific phospholipase C isozymes. J Biol Chem *267*, 12393-12396.

Riordan, J. R., Rommens, J. M., Kerem, B. S., Alon, N., Rozmahel, R., Grzelczak, Z., Zielinski, J., Lok, S., Plavsic, N., Chou, J. L., Drumm, M. L., Iannuzzi, M. C., Collins, F. S., and Tsui, L. C. (1989). Identification of the cystic fibrosis gene: Cloning and characterization of complementary DNA. Science *245*, 1066-1073.

Roach, P. J. (1991). Multisite and hierarchal protein phosphorylation. J Biol Chem *266*, 14139-14142.

Rodbell, M. (1980). The role of hormone receptors and GTP-regulatory proteins in membrane transduction. Nature *284*, 17-22.

Rodbell, M., Birnbaumer, L., Pohl, S. L., and Krans, H. M. J. (1971a). The glucagon-sensitive adenyl cyclase system in plasma membranes of rat liver. V. An obligatory role of guanyl nucleotides in glucagon action. J Biol Chem *246*, 1877-1882.

Rodbell, M., Krans, H. M. J., Pohl, S. L., and Birnbaumer, L. (1971b). The glucagon-sensitive adenyl cyclase system in plasma membranes of rat liver. III. Binding of glucagon: method of assay and specificity. J Biol Chem *246*, 1861-1871.

Rodbell, M., Krans, H. M. J., Pohl, S. L., and Birnbaumer, L. (1971c). The glucagon-sensitive adenyl cyclase system in plasma membranes of rat liver. IV. Effects of guanyl nucleotides on binding of ^{125}I-glucagon. J Biol Chem *246*, 1872-1876.

Rodbell, M., Lin, M. C., and Salomon, Y. (1974). Evidence for interdependent action of glucagon and nucleotides on the hepatic adenylate cyclase system. J Biol Chem *249*, 59-65.

Rojas, F. J., and Birnbaumer, L. (1985). Regulation of glucagon receptor binding. Lack of effect of Mg and preferential role for GDP. J Biol Chem *260*, 7829-7835.

Rooney, T. A., Hager, R., and Thomas, A. P. (1991). Beta-adrenergic receptor-mediated phospholipase C activation independent of cAMP formation in turkey erythrocyte membranes. J Biol Chem *266*, 15068-15074.

Ross, E. M., Howlett, A. C., Ferguson, K. M., and Gilman, A. G. (1978). Reconstitution of hormone-sensitive adenylate cyclase activity with resolved components of the enzyme. J Biol Chem *253*, 6401-6412.

Roth, N. S., Campbell, P. T., Caron, M. G., Lefkowitz, R. J., and Lohse, M. J. (1991). Comparative rates of desensitization of beta-adrenergic receptors by the beta-adrenergic receptor kinase and the cyclic AMP-dependent protein kinase. Proc Natl Acad Sci USA *88*, 6201-6204.

Rozengurt, E., Sinnett-Smith, J., Van Lint, J., and Valverde, A. M. (1995). Protein kinase D (PKD): A novel target for diacylglycerol and phorbol esters. Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis *333*, 153-160.

Rubin, C. S., Erlichman, J., and Rosen, O. R. (1972). Cyclic adenosine 3',5'-monophosphate-dependent protein kinase of human erythrocyte membranes. J Biol Chem *247*, 6135-6139.

Sadoshima, J., and Izumo, S. (1996). The heterotrimeric Gq protein-coupled angiotensin II receptor activates p21ras via the tyrosine kinase-Shc-Grb2-Sos pathway in cardiac myocytes. EMBO Journal *15*, 775-787.

Saiki, R. K. (1989). The design and optimization of the PCR. In PCR Technology, H. A. Erlich, ed. (New York: Stockton Press), pp. 7-16.

Sakuta, H., Sekiguchi, M., Okamoto, K., and Sakai, Y. (1991). Desensitization of endogenous angiotensin II receptors in Xenopus oocytes: A role of protein kinase C. Eur J Pharmacol *208*, 41-47.

Salavatori, S., Damiani, E., Barhanin, J., Furlan, S., Giovanni, S., and Margreth, A. (1990). Co-localization of the dihydropyridine receptor and the cyclic AMP- binding subunit of an intrinsic protein kinase to the junctional membrane of the transverse tubules of skeletal muscle. Biochem J *267*, 679-687.

Samama, P., Cotecchia, S., Costa, T., and Lefkowitz, R. J. (1993). A mutation-induced activated state of the beta2-adrenergic receptor. Extending the ternary complex model. J Biol Chem *268*, 4625-4636.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular cloning - a laboratory manual, Second Edition, N. Ford, ed.: Cold Spring Harbor Laboratory Press).

Sanchez, I., Hughes, R. T., Mayer, B. J., Yee, K., Woodgett, J. R., Avruch, J., Kyriakis, J. M., and Zon, L. I. (1994). Role of SAPK/ERK kinase-1 in the stress-activated pathway regulating transcription factor c-Jun. Nature *372*, 794-8.

Sando, J. J., and Young, M. C. (1983). Identification of high-affinity phorbol ester receptor in cytosol of EL4 thymoma cells: Requirement for calcium, magnesium, and phospholipids. Proc Natl Acad Sci USA *80*, 2642-6.

Santos, A., and Blazquez, E. (1982). Direct evidence of a glucagon-dependent regulation of the concentration of glucagon receptors in the liver. Eur J Biochem *121*, 671-677.

Savage, A., Zeng, L., and Houslay, M. D. (1995). A role for protein kinase C-mediated phosphorylation in eliciting glucagon desensitization in rat hepatocytes. Biochem J *307*, 281-5.

Schaap, D., Hsuan, J., Totty, N., and Parker, P. J. (1990). Proteolytic activation of protein kinase C-epsilon. Eur J Biochem *191*, 431-5.

Schertler, G. F. X., Villa, C., and Henderson, R. (1993). Projection structure of rhodopsin. Nature *362*, 770-772.

Schmidt, C. J., Thomas, T. C., Levine, M. A., and Neer, E. J. (1992). Specificity of G protein beta and gamma subunit interactions. J Biol Chem *267*, 13807-13810.

Scott, J. D., and Carr, D. W. (1992). Subcellular localisation of type II cAMP-dependent protein kinase. News in Physiological Sciences *7*, 143-148.

Scott, J. D., Stofko, R. E., McDonald, J. R., Comer, J. D., Vitalis, E. A., and Mangili, J. (1990). Type II regulatory subunit dimerization determines the subcellular localization of the cAMP-dependent protein kinase. J Biol Chem *265*, 21561-21566.

Seabra, M. C., Reiss, Y., Casey, P. J., Brown, M. S., and Goldstein, J. L. (1991). Protein farnesyltransferase and geranylgeranyltransferase share a common alpha subunit. Cell *65*, 429-434.

Seamon, K. B., Padgett, W., and Daley, J. W. (1981). Forskolin: Unique diterpene activator of adenylate cyclase in membranes and in intact cells. Proc Natl Acad Sci USA *78*, 3363-3367.

Seed, B. (1987). An LFA-3 cDNA encodes a phospholipid-linked membrane protein homologous to its receptor CD2. Nature *329*, 840-842.

Shakur, Y., Pryde, J. G., and Houslay, M. D. (1993). Engineered deletion of the unique N-terminal domain of the cyclic AMP-specific phosphodiesterase RD1 prevents plasma membrane association and the attainment of enhanced thermostability without altering its sensitivity to inhibition by rolipram. Biochem J *292*, 677-686.

Showers, M. O., and Maurer, R. A. (1986). A cloned bovine cDNA encodes an alternate form of the catalytic subunit of cAMP-dependent protein kinase. J Biol Chem *261*, 16288-16291.

Sibley, D. R., Nambi, P., Peters, J. R., and Lefkowitz, R. J. (1984). Phorbol diesters promote beta-adrenergic receptor phosphorylation and adenylate cyclase desensitization in duck erythrocytes. Biochemical and Biophysical Research Communications *121*, 973-979.

Sinha, M. K., Pories, W. J., Flickinger, E. G., Meelheim, D., and Caro, J. F. (1987). Insulin-receptor kinase activity of adipose tissue from morbidly obese humans with and without NIDDM. Diabetes *36*, 620-5.

Sistare, F. D., Picking, R. A., and Haynes R. C., J. (1985). Sensitivity of the response of cytosolic calcium in Quin-2-loaded rat hepatocytes to glucagon, adenine nucleosides, and adenine nucleotides. J Biol Chem *260*, 12744-12747.

Slater, S. J., Kelly, M. B., Taddeo, F. J., Rubin, E., and Stubbs, C. D. (1994). Evidence for discrete diacylglycerol and phorbol ester activator sites on protein kinase C. Differences in effects of 1-alkanol inhibition, activation by phosphatidylethanolamine and calcium chelation. J Biol Chem *269*, 17160-5.

Smit, M. J., Bloemers, S. M., Leurs, R., Tertoolen, L. G. J., Bast, A., De Laat, S. W., and Timmerman, H. (1992). Short-term desensitization of the histamine H1 receptor in human HeLa cells: Involvement of protein kinase C dependent and independent pathways. Br J Pharmacol *107*, 448-455.

Smit, M. J., Leurs, R., Alewijnse, A. E., Blauw, J., Van Nieuw Amerongen, G. P., Van de Vrede, Y., Roovers, E., and Timmerman, H. (1996). Inverse agonism of histamine H2 antagonists accounts for upregulation of spontaneously active histamine H2 receptors. Proc Natl Acad Sci USA *93*, 6802-6807.

Smrcka, A. V., and Sternweis, P. C. (1993). Regulation of purified subtypes of phosphatidylinositol-specific phospholipase C beta by G protein alpha and βγ subunits. J Biol Chem *268*, 9667-9674.

Sneader, W. (1996). Drug prototypes and their exploitation (Chichester: Wiley).

Soldati, T., and Perriard, J.-C. (1991). Intracompartmental sorting of essential myosin light chains: molecular dissection and in vivo monitoring by epitope tagging. Cell *66*, 277-289.

Sondek, J., Bohm, A., Lambriht, D. G., Hamm, H. E., and Sigler, P. B. (1996). Crystal structure of a G protein βγ dimer at 2.1 A resolution. Nature *379*, 369-374.

Sondek, J., Lambright, D. G., Noel, J. P., Hamm, H. E., and Sigler, P. B. (1994). GTPase mechanism of G proteins from the 1.7-A crystal structure of transducin alpha GDP AIF4-. Nature *372*, 276-279.

Sonne, O., Berg, T., and Christoffersen, T. (1978). Binding of 125I-labeled glucagon and glucagon-stimulated accumulation of adenosine 3':5'-monophosphate in isolated intact rat hepatocytes. Evidence for receptor heterogeneity. J Biol Chem *253*, 3203-3210.

Spence, S., Rena, G., Sweeney, G., and Houslay, M. D. (1995). Induction of Ca^{2+}/calmodulin-stimulated cyclic AMP phosphodiesterase (PDE1) activity in Chinese hamster ovary cells (CHO) by phorbol 12- myristate 13-acetate and by the selective overexpression of protein kinase C isoforms. Biochem J *310*, 975-982.

Stehnobittel, L., Krapivinsky, G., Krapivinsky, L., Perezterzic, C., and Clapham, D. E. (1995). The G protein $\beta\gamma$ subunit transduces the muscarinic receptor signal for Ca^{2+} release in Xenopus oocytes. J Biol Chem *270*, 30068-30074.

Sternweis, P. C. (1994). The active role of beta gamma in signal transduction. Curr Opin Cell Biol *6*, 198-203.

Sternweis, P. C., and Smrcka, A. V. (1992). Regulation of phospholipase C by G proteins. Trends Biochem Sci *17*, 502-6.

Stock, J. B. (1994). Adaptive responses in bacterial chemotaxis. In Regulation of cellular signal transduction pathways by desensitization and amplification, D. R. Sibley and M. D. Houslay, eds. (Chichester: John Wiley & Sons), pp. 3-24.

Stoffel, R. H., Randall, R. R., Premont, R. T., Lefkowitz, R. J., and Inglese, J. (1994). Palmitoylation of G protein-coupled receptor kinase, GRK6. Lipid modification diversity in the GRK family. J Biol Chem *269*, 27791-4.

Strader, C. D., Candelore, M. R., Hill, W. S., Sigal, I. S., and Dixon, R. A. F. (1989). Identification of two serine residues involved in agonist activation of the beta-adrenergic receptor. J Biol Chem *264*, 13572-13578.

Strader, C. D., Fong, T. M., Graziano, M. P., and Tota, M. R. (1995). The family of G-protein-coupled receptors. FASEB Journal *9*, 745-754.

Strader, C. D., Fong, T. M., Tota, M. R., Underwood, D., and Dixon, R. A. F. (1994). Structure and function of G protein-coupled receptors. Annu Rev Biochem *63*, 101-132.

Strader, C. D., Sigal, I. S., Rios Candelore, M., Rands, E., Hill, W. S., and Dixon, R. A. F. (1988). Conserved aspartic acid residues 79 and 113 of the beta-adrenergic receptor have different roles in receptor function. J Biol Chem *263*, 10267-10271.

Strasser, R. H., Benovic, J. L., Caron, M. G., and Lefkowitz, R. J. (1986). Beta-agonist- and prostaglandin E1-induced translocation of the beta-adrenergic receptor kinase: Evidence that the kinase may act on multiple adenylate cyclase-coupled receptors. Proc Natl Acad Sci USA *83*, 6362-6366.

Strassheim, D., Palmer, T. M., Milligan, G., and Houslay, M. D. (1991). Alterations in G-protein expression and the hormonal regulation of adenylate cyclase in the adipocytes of obese (fa/fa) Zucker rats. Biochem J *276*, 197-202.

Stroop, S. D., Kuestner, R. E., Serwold, T. F., Chen, L., and Moore, E. E. (1995). Chimeric human calcitonin and glucagon receptors reveal two dissociable calcitonin interaction sites. Biochemistry *34*, 1050-1057.

Sunahara, R. K., Dessauer, C. W., and Gilman, A. G. (1996). Complexity and diversity of mammalian adenylyl cyclases. Annu Rev Pharmacol Toxicol *36*, 461-480.

Svoboda, M., Ciccarelli, E., Tastenoy, M., Cauvin, A., Stievenart, M., and Christophe, J. (1993a). Small introns in a hepatic cDNA encoding a new glucagon-like peptide 1-type receptor. Biochem Biophys Res Commun *191*, 479-86.

Svoboda, M., Ciccarelli, E., Tastenoy, M., Robbercecht, P., and Christophe, J. (1993b). A cDNA construct allowing the expression of rat hepatic glucagon receptors. Biochem Biophys Res Commun *192*, 135-42.

Takai, Y., Sasaki, T., Tanaka, K., and Nakanishi, H. (1995). Rho as a regulator of the cytoskeleton. Trends Biochem Sci *20*, 227-231.

Takayama, S., White, M. F., and Kahn, C. R. (1988). Phorbol ester-induced serine phosphorylation of the insulin receptor decreases its tyrosine kinase activity. J Biol Chem *263*, 3440-7.

Tang, E. K. Y., and Houslay, M. D. (1992). Glucagon, vasopressin and angiotensin all elicit a rapid, transient increase in hepatocyte protein kinase C activity. Biochem J *283*, 341-6.

Tang, E. Y., Parker, P. J., Beattie, J., and Houslay, M. D. (1993). Diabetes induces selective alterations in the expression of protein kinase C isoforms in hepatocytes. FEBS Lett *326*, 117-123.

Tang, W.-J., and Gilman, A. G. (1991). Type-specific regulation of adenylyl cyclase by G-protein beta-gamma subunits. Science *254*, 1500-1503.

Tang, W. J., and Gilman, A. G. (1995). Construction of a soluble adenylyl cyclase activated by Gs alpha and forskolin. Science *268*, 1769-1772.

Tang, W. J., Krupinski, J., and Gilman, A. G. (1991). Expression and characterization of calmodulin-activated (type I) adenylylcyclase. J Biol Chem *266*, 8595-8603.

Tang, W. J., Stanzel, M., and Gilman, A. G. (1995). Truncation and alanine-scanning mutants of type I adenylyl cyclase. Biochemistry *34*, 14563-14572.

Taussig, R., and Gilman, A. G. (1995). Mammalian membrane-bound adenylyl cyclases. J Biol Chem *270*, 1-4.

Taussig, R., Quarmby, L. M., and Gilman, A. G. (1993). Regulation of purified type I and type II adenylylcyclases by G protein $\beta\gamma$ subunits. J Biol Chem *268*, 9-12.

Taylor, S. S. (1989). cAMP-dependent protein kinase. Model for an enzyme family. J Biol Chem *264*, 8443-8446.

Thompson, W. J., and Appleman, M. M. (1971). Multiple cyclic nucleotide phosphodiesterase activities in rat brain. Biochemistry *10*, 311-316.

Thompson, W. J., Terasaki, W. L., Epstein, P. M., and Strada, S. J. (1979). Assay of cyclic nucleotide phosphodiesterase and resolution of multiple molecular forms of the enzyme. In Advances in cyclic nucleotide Research, P. G. G. Brooker, G.A. Robison, ed. (New York: Raven Press), pp. 69-92.

Thorens, B. (1992). Expression cloning of the pancreatic beta cell receptor for the gluco-incretin hormone glucagon-like peptide 1. Proc Natl Acad Sci USA *89*, 8641-8645.

Toda, T., Shimanuki, M., and Yanagida, M. (1993). Two novel protein kinase C-related genes of fission yeast are essential for cell viability and implicated in cell shape control. EMBO Journal *12*, 1987-1995.

Torchia, J., Yi, Q., and Sen, A. K. (1994). Carbachol-stimulated phosphorylation of the Na-K-Cl cotransporter of avian salt gland. Requirement for Ca^{2+} and PKC activation. J Biol Chem *269*, 29778-29784.

Touhara, K., Hawes, B. E., Van Biesen, T., and Lefkowitz, R. J. (1995). G protein βγ subunits stimulate phosphorylation of Shc adapter protein. Proc Natl Acad Sci USA *92*, 9284-9287.

Touhara, K., Inglese, J., Pitcher, J. A., Shaw, G., and Lefkowitz, R. J. (1994). Binding of G protein βγ subunits to pleckstrin homology domains. J Biol Chem *269*, 10217-10220.

Treisman, R. (1992). The serum response element. Trends Biochem Sci *17*, 423-6.

Tsukada, S., Simon, M. I., Witte, O. N., and Katz, A. (1994). Binding of βγ subunits of heterotrimeric G proteins to the PH domain of Bruton tyrosine kinase. Proc Natl Acad Sci USA *91*, 11256-11260.

Tung, J.-S., Daugherty, B. L., O'Neill, L., Law, S. W., Han, J., and Mark, G. E. (1989). PCR amplification of specific sequences from a cDNA library. In PCR Technology, H. A. Erlich, ed. (New York: Stockton Press), pp. 99-104.

Uhler, M. D., Carmichael, D. F., Lee, D. C., Chrivia, J. C., Krebs, E. G., and McKnight, G. S. (1986a). Isolation of cDNA clones coding for the catalytic subunit of mouse cAMP-dependent protein kinase. Proc Natl Acad Sci USA *83*, 1300-1304.

Uhler, M. D., Chrivia, J. C., and McKnight, G. S. (1986b). Evidence for a second isoform of the catalytic subunit of cAMP-dependent protein kinase. J Biol Chem *261*, 15360-15363.

Unson, C. G., Cypess, A. M., Kim, H. N., Goldsmith, P. K., Carruthers, C. J. L., Merrifield, R. B., and Sakmar, T. P. (1995). Characterization Of Deletion and Truncation Mutants Of the Rat Glucagon Receptor - 7 Transmembrane Segments Are Necessary For Receptor Transport to the Plasma-Membrane and Glucagon Binding. J Biol Chem *270*, 27720-27727.

Unson, C. G., Cypess, A. M., Wu, C.-R., Goldsmith, P. K., Merrifield, R. B., and Sakmar, T. P. (1996). Antibodies against specific extracellular epitopes of the glucagon receptor block glucagon binding. Proc Natl Acad Sci USA *93*, 310-315.

Unson, C. G., Gurzenda, E. M., and Merrifield, R. B. (1989). Biological activities of des-his1[glu9]glucagon amide, a glucagon antagonist. Peptides *10*, 1171-1177.

Valverde, A. M., Sinnett-Smith, J., Van Lint, J., and Rozengurt, E. (1994). Molecular cloning and characterization of protein kinase D: A target for diacylglycerol and phorbol esters with a distinctive catalytic domain. Proc Natl Acad Sci USA *91*, 8572-8576.

Van Biesen, T., Hawes, B. E., Luttrell, D. K., Krueger, K. M., Touhara, K., Porfiri, E., Sakaue, M., Luttrell, L. M., and Lefkowitz, R. J. (1995). Receptor-tyrosine-kinase and G beta-gamma-mediated MAP kinase activation by a common signalling pathway. Nature *376*, 781-784.

Van Eldik, L. J., Zendegui, J. G., Marshak, D. R., and Watterson, D. M. (1982). Calcium-binding proteins and the molecular basis of calcium action. International Review of Cytology *77*, 1-61.

Van Lint, J., Sinnett-Smith, J., and Rozengurt, E. (1995). Expression and characterization of PKD, a phorbol ester and diacylglycerol-stimulated serine protein kinase. J Biol Chem *270*, 1455-1461.

Vaziri, C., and Downes, C. P. (1992). G-protein-mediated activation of turkey erythrocyte phospholipase C by beta-adrenergic and P(2y)-purinergic receptors. Biochem J *284*, 917-922.

Völkel, H., Beitz, E., Klumpp, S., and Schultz, J. E. (1996). Cloning and expression of a bovine adenylyl cyclase type VII specific to the retinal pigment epithelium. FEBS Lett *378*, 245-249.

Von Zastrow, M., and Kobilka, B. K. (1992). Ligand-regulated internalization and recycling of human beta2- adrenergic receptors between the plasma membrane and endosomes containing transferrin receptors. J Biol Chem *267*, 3530-3538.

Voyno-Yasenetskaya, T., Conklin, B. R., Gilbert, R. L., Hooley, R., Bourne, H. R., and Barber, D. L. (1994). Galpha13 stimulates Na-H exchange. J Biol Chem *269*, 4721-4724.

Wakelam, M. J. O., Murphy, G. J., Hruby, V. J., and Houslay, M. D. (1986). Activation of two signal-transduction systems in hepatocytes by glucagon. Nature *323*, 68-71.

Wall, M. A., Coleman, D. E., Lee, E., IniguezLluhi, J. A., Posner, B. A., Gilman, A. G., and Sprang, S. R. (1995). The structure of the G protein heterotrimer $G_i$alpha1beta1gamma2. Cell *83*, 1047-1058.

Walsh, D. A., Clippinger, M. S., Sivaramakrishnan, S., and McCullough, T. E. (1979). Cyclic adenosine monophosphate dependent and independent phosphorylation of sarcolemma membrane proteins in perfused rat heart. Biochemistry *18*, 871-877.

Walsh, D. A., and Van Patten, S. M. (1994). Multiple pathway signal transduction by the cAMP-dependent protein kinase. FASEB Journal *8*, 1227-1236.

Watson, P. A., Krupinski, J., Kempinski, A. M., and Frankenfield, C. D. (1994). Molecular cloning and characterization of the type VII isoform of mammalian adenylyl cyclase expressed widely in mouse tissues and in S49 mouse lymphoma cells. J Biol Chem *269*, 28893-28898.

Wayman, G. A., Hinds, T. R., and Storm, D. R. (1995). Hormone stimulation of type III adenylyl cyclase induces Ca^{2+} oscillations in HEK-293 cells. J Biol Chem *270*, 24108-24115.

Wedegaertner, P. B., Wilson, P. T., and Bourne, H. R. (1995). Lipid modifications of trimeric G proteins. J Biol Chem *270*, 503-506.

Wei, J., Wayman, G., and Storm, D. R. (1996). Phosphorylation and inhibition of type III adenylyl cyclase by calmodulin-dependent protein kinase II in vivo. J Biol Chem *271*, 24231-24235.

Wetsel, W. C., Khan, W. A., Merchenthaler, I., Rivera, H., Halpern, A. E., Phung, H. M., Negro-Vilar, A., and Hannun, Y. A. (1992). Tissue and cellular distribution of the extended family of protein kinase C isoenzymes. J Cell Biol *117*, 121-33.

Whipps, D. E., Armston, A. E., Pryor, H. J., and Halestrap, A. P. (1987). Effects of glucagon and calcium on the metabolism of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5- bisphosphate in isolated rat hepatocytes and plasma membranes. Biochem J *241*, 835-845.

Whisnant, R. E., Gilman, A. G., and Dessauer, C. W. (1996). Interaction of the two cytosolic domains of mammalian adenylyl cyclase. Proc Natl Acad Sci USA *93*, 6621-6625.

White, M. F., and Kahn, C. R. (1994). The insulin signalling system. J Biol Chem *269*, 1-4.

Widmann, C., Dolci, W., and Thorens, B. (1996). Desensitization and phosphorylation of the glucagon-like peptide-1 (GLP-1) receptor by GLP-1 and 4-phorbol 12-myristate 13-acetate. Mol Endocrinol *10*, 62-75.

Wilks, A. F. (1989). Two putative protein-tyrosine kinases identified by application of the polymerase chain reaction. Proc Natl Acad Sci USA *86*, 1603-1607.

Williams, B. (1995). Glucose-induced vascular smooth muscle dysfunction - the role of protein kinase C. J Hypertens *13*, 477-486.

Winkel, G. K., Sardet, C., Pouyssegur, J., and Ives, H. E. (1993). Role of cytoplasmic domain of the Na^+/H^+ exchanger in hormonal activation. J Biol Chem *268*, 3396-3400.

Wolf, B. A., Williamson, J. R., Easom, R. A., Chang, K., Sherman, W. R., and Turk, J. (1991). Diacylglycerol accumulation and microvascular abnormalities induced by elevated glucose levels. J Clin Invest *87*, 31-8.

Wu, Z., Wong, S. T., and Storm, D. R. (1993). Modification of the calcium and calmodulin sensitivity of the type I adenylyl cyclase by mutagenesis of its calmodulin binding domain. J Biol Chem *268*, 23766-23768.

Xanthopoulos, K. G., and Mirkovitch, J. (1993). Gene regulation in rodent hepatocytes during development, differentiation and disease. Eur J Biochem *216*, 353-60.

Yan, M., Dai, T., Deak, J. C., Kyriakis, J. M., Zon, L. I., Woodgett, J. R., and Templeton, D. J. (1994). Activation of stress-activated protein kinase by MEKK1 phosphorylation of its activator SEK1. Nature *372*, 798-800.

Yarfitz, S., and Hurley, J. B. (1994). Transduction mechanisms of vertebrate and invertebrate photoreceptors. J Biol Chem *269*, 14329-14332.

Yee, R., and Liebman, P. A. (1978). Light-activated phosphodiesterase of the rod outer segment. Kinetics and parameters of activation and deactivation. J Biol Chem *253*, 8902.

Yoo-Warren, H., Willse, A. G., Hancock, N., Hull, J., McCaleb, M., and Livingston, J. N. (1994). Regulation of rat glucagon receptor expression. Biochemical and Biophysical Research Communications *205*, 347-353.

Yoon, H. S., Hajduk, P. J., Petros, A. M., Olejniczak, E. T., Meadows, R. P., and Fesik, S. W. (1994). Solution structure of a pleckstrin-homology domain. Nature *369*, 672-5.

Yoshimura, M., and Cooper, D. M. F. (1992). Cloning and expression of a Ca^{2+}-inhibitable adenylyl cyclase from NCB- 20 cells. Proc Natl Acad Sci USA *89*, 6716-6720.

Yoshimura, M., and Cooper, D. M. F. (1993). Type-specific stimulation of adenylyl cyclase by protein kinase C. J Biol Chem *268*, 4604-4607.

Zhang, G., Liu, Y., Ruoho, A. E., and Hurley, J. H. (1997). Stucture of the adenylyl cyclase catalytic core. Nature *386*, 247-253.

Zhang, J., Zhang, J., Benovic, J. L., Sugai, M., Wetzker, R., Gout, I., and Rittenhouse, S. E. (1995). Sequestration of a G-protein βγ subunit or ADP-ribosylation of Rho can inhibit thrombin-induced activation of platelet phosphoinositide 3-kinases. J Biol Chem *270*, 6589-6594.

Zhu, X., Gilbert, S., Birnbaumer, M., and Birnbaumer, L. (1994). Dual signaling potential is common among Gs-coupled receptors and dependent on receptor density. Mol Pharmacol *46*, 460-9.

Zugaza, J. L., Sinnett-Smith, J., and Rozengurt, E. (1996). Protein Kinase D (PKD) activation in intact cells through a protein kinase C-dependent signal transduction pathway. EMBO Journal *15*, 6220-6230.

APPENDIX B

Amino acid sequence of the rat hepatic glucagon receptor

```
MLLTQLHCPY LLLLLVVLSC LPKAPSAQVM DFLFEKWKLY SDQCHHNLSL          50
                                                      *

LPPPTELVCN RTFDKYSCWP DTPPNTTANI SCPWYLPWYH KVQHRLVFKR         100
          *                      *    *

CGPDGQWVRG PRGQSWRDAS QCQMDDDEIE VQKGVAKMYS SYQVMYTVGY         150
                                                    TM1

SLSLGALLLA LVILLGLRKL HCTRNYIHGN LFASFVLKAG SVLVIDWLLK         200
                                  TM2

TRYSQKIGDD LSVSVWLSDG AVAGCRVATV IMQYGIIANY CWLLVEGVYL         250
                                  TM3

YSLLSITTFS EKSFFSLYLC IGWGSPLLFV IPWVVVKCLF ENVQCWTSND         300
            TM4

NMGFWWILRI PVLLAILINF FIFVRIIHLL VAKLRAHQMH YADYKFRLAR         350
          TM5

STLTLIPLLG VHEVVFAFVT DEHAQGTLRS TKLFFDLFFS SFQGLLVAVL         400
TM6                                 TM7

YCFLNKEVQA ELLRRWRRWQ EGKALQEERM ASSHGSHMAP AGTCHGDPCE         450
                                          #

KLQLMSAGSS SGTGCEPSAK TSLASSLPRL ADSPT                         485
```

The peptide sequence of the rat hepatic glucagon receptor (Genbank Accession No. L04796) is shown. The amino acids contained within the seven putative transmembrane domains (TM1-7) are underlined. The four sites of asparagine-linked glycosylation in the amino-terminal domain are indicated with asterisks while the putative PKD phosphorylation site at serine 432 in the carboxy-terminal tail is indicated by a hash symbol.