The Regulation Of Adenylate Cyclase Activity In Murine Models Of Type II Diabetes Mellitus

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"The history of modern knowledge is concerned in no small degree with man's attempt to escape from his previous concepts. Within the present century we have seen physics liberated from the cramped philosophy of a rigid causality to the more fluid concept of probability. We are now witnessing a similar liberation of medical thought by the substitution of 'syndromes' for 'disease entities'...The 'syndrome' has its philosophical basis not in specific disease factors but in a chain of physiological processes, interference with which at any point produces the same impairment of bodily function. The same syndrome may thus arise from many different causes. This newer view inspires a more catholic concept of aetiology and renders pointless many existing controversies. But the revision of medical thought entailed by its application has hardly begun."

Professor H.P. Himsworth, M.D.,
in an address to the Royal College of
Physicians, March 1949.

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ABBREVIATIONS

The abbreviations used throughout this thesis are in accordance with the recommendations set out in "Instructions To Authors", *Biochem. J.* (1985) **225**, 1-26, with the following additions:-

A_1	Inhibitory adenosine receptor
ACTH	Adrenocorticotropic hormone
AHO	Albright Hereditary Osteodystrophy
BB/Wor	Bio-Breeding diabetic rats
BSA	Bovine serum albumin
C	Catalytic unit of adenylate cyclase
cDNA	Complimentary DNA
CGRP	Calcitonin gene-related peptide
ΔChIR	Human insulin receptor in which the fourteen C-terminal amino acids have been removed
CHO	Chinese hamster ovary
CTC	Copper-tartrate-carbonate
CTx	Cholera toxin
D_2	Inhibitory dopamine receptor
db	"Diabetes" gene
DTT	Dithiothreitol
EC_{50}	Ligand concentration at which half-maximal stimulation is achieved
EF-Tu	Bacterial elongation factor-Tu
EGF	Epidermal growth factor
fa	"Fatty" gene
GAP	GTPase activating protein
GH	Growth hormone

GHRH	Growth hormone-releasing hormone
GLUT	Facilitative glucose transporter
Gpp(NH)p	Guanylyl 5'-[βγ-imido]diphosphate
G-protein	Guanine nucleotide-binding regulatory protein (individual G-proteins defined in text)
GTPγS	Guanosine 5'-[3-O-thio] triphosphate
5-HT	5-hydroxytryptamine
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
IAPP	Islet amyloid polypeptide (amylin)
IAP	Islet-activating protein (pertussis toxin)
IC_{50}	Ligand concentration at which half-maximal inhibition is achieved
IDDM	Insulin-dependent diabetes mellitus
IGF-I	Insulin-like growth factor-I
IgG	Immunoglobulin G
k_{cat}	Catalytic rate constant
k_{on}	Rate constant for hormone receptor-mediated stimulation of adenylate cyclase activity
KRH	Krebs-Ringer-Henseleit solution
LDL	Low density lipoprotein
MAP-2	Microtubule-associated protein-2
mRNA	Messenger RNA
NEM	N-ethylmaleimide
NZO	New Zealand Obese mice
ob	"Obese" gene
OGTT	Oral Glucose Tolerance Test
$p21^{ras}$	Product from the *ras* family of genes coding for 21 kDa GTP-binding proteins

P_{2y}	Inhibitory purinergic receptor
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDE	Cyclic nucleotide phosphodiesterase
PDGF	Platelet-derived growth factor
PGE_1	Prostaglandin E_1
PHP-Ia	Pseudohypoparathyroidism type Ia
PIA	N^6-phenylisopropyladenosine
PKA	Cyclic AMP-dependent protein kinase
PKC	Ca^{2+}- and phospholipid-dependent protein kinase
POS	Phospho-oligosaccharide
PPHP	Pseudo-pseudohypoparathyroidism
PTx	Pertussis toxin (islet-activating protein)
R_i	Inhibitory receptor of adenylate cyclase
R_s	Stimulatory receptor of adenylate cyclase
SDS	Sodium dodecylsulphate
SDS-PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoresis
Spec. Ac.	Specific activity
stz	Streptozotocin
TEMED	N, N, N', N' tetramethylethylenediamine
TGF-α	Transforming growth factor-α
Tris	Tris(hydroxymethyl)aminomethane
TSH	Thyroid-stimulating hormone
c-*fos*, c-*myc*	Proto-oncogenic proteins which form part of a growth factor-regulated transcription initiation complex
TPA	Tetraphorbol acetate

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SUMMARY

It is now well-established that many hormones regulate the metabolism of target cells by binding to receptors which can interact with specific members of a family of guanine nucleotide-binding regulatory proteins (G-proteins) which can in turn control the activity of various intracellular effector enzymes and ion channels. One such effector enzyme is adenylate cyclase which is controlled by stimulatory (G_s) and inhibitory (G_i) proteins. Both of these proteins are heterotrimers consisting of α-, β- and γ-subunits, with the α-subunits conferring the abilities to interact with particular receptors and effectors. There are at least three members of the G_iα-subunit family of proteins, termed G_iα-1, G_iα-2 and G_iα-3. The functional significance of this multiplicity is unknown although it has been suggested that G_iα-2 may be responsible for mediating the inhibition of adenylate cyclase activity in certain systems and that each of these proteins can regulate K^+ channel opening.

Several reports have demonstrated that the regulation of adenylate cyclase activity is altered in membranes prepared from liver and adipose tissue isolated from animals which exhibit insulin resistance associated with diabetes mellitus and/or obesity. In particular, the functioning of G_i has been shown to be attenuated, as assessed by measuring the ability of the non-hydrolysable GTP analogue Gpp(NH)p (guanylyl 5'-[β,γ-imido]diphosphate) to inhibit forskolin-stimulated adenylate cyclase activity. However, subsequent work in both liver and adipocyte membranes from chemically-induced Type I diabetic rats suggests that this lesion is restricted to an attenuation in the guanine nucleotide-dependent, receptor-independent inhibition of adenylate cyclase as receptor-dependent inhibitory effects are still present.

Liver plasma membranes prepared from genetically diabetic (*db/db*) mice expressed reduced levels of G_iα-2, G_iα-3 and β-subunits compared with membranes prepared from lean animals. However, these changes were

associated with only a partial (40%) reduction in the ability of Gpp(NH)p to inhibit forskolin-stimulated adenylate cyclase activity. There was no alteration in the ability of either glucagon or NaF to stimulate adenylate cyclase activity, although the maximal stimulation elicited by the β-adrenergic receptor agonist isoproterenol was reduced by approximately 40% in membranes from diabetic animals compared with those from lean animals. However, membranes from diabetic animals were more sensitive to stimulation by increasing concentrations of Gpp(NH)p than those from lean animals. Also, whilst there was no alteration in the expression of either of the two forms of $G_s\alpha$-subunits present in liver plasma membranes from diabetic animals compared with those from lean animals, membranes from the former were better substrates for cholera toxin-catalysed ADP-ribosylation compared with those from lean animals. In addition, the ability of pertussis toxin to catalyse the ADP-ribosylation of $G_i\alpha$-subunits was severely attenuated in membranes from diabetic animals compared with those from lean animals. It is proposed that a reduced concentration of βγ-subunits in membranes from diabetic animals, following from the reduced levels of β-subunits, alters the equilibrium between holomeric and dissociated G-protein subunits such that dissociation is favoured: this would account for both the enhanced sensitivity to activation by Gpp(NH)p and the enhanced cholera toxin-catalysed labelling of $G_s\alpha$-subunits in membranes from diabetic animals in the absence of any alteration in the expression of $G_s\alpha$-subunits between lean and diabetic animals. This may also explain why the reduction in pertussis toxin-catalysed labelling of $G_i\alpha$-subunits was slightly greater than the reductions in expression of $G_i\alpha$-2 and $G_i\alpha$-3.

Crude adipocyte membranes from diabetic (*db/db*) mice exhibited drastically reduced responses to increasing concentrations of isoproterenol, glucagon and secretin compared with membranes from lean animals. However, the response of adenylate cyclase to stimulation by both TSH and the wasp

venom mastoparan were similar using membranes from either lean or diabetic animals. The range of differences suggest the existence of specific modifications in either individual receptor number or function. Consistent with the lesion lying at the level of individual receptors rather than the G-protein system was the absence of any difference in the expression of the two forms of $G_s\alpha$-subunits between membranes from lean and diabetic animals, or any difference in their functioning. This was deduced from observations showing that the maximal fold activation above basal activity attained using NaF, which activates adenylate cyclase *via* G_s, was the same in membranes from both lean and diabetic animals. With respect to inhibitory regulation, an enhanced maximal response to the A_1 adenosine receptor agonist PIA was noted, although the maximal inhibitory responses to PGE_1 and nicotinic acid were unaltered.

Adipocyte membranes from genetically Type II diabetic CBA/Ca mice exhibited an enhanced stimulatory response upon incubation with increasing concentrations of isoproterenol compared with that seen using membranes from lean animals. However, the response to glucagon, which produced a potent stimulation of adenylate cyclase activity in membranes from lean animals, was abolished in membranes from obese animals. In contrast, secretin elicited a similar increase in adenylate cyclase activity using membranes from either set of animals. Whilst the activity of adenylate cyclase was markedly increased in membranes from obese animals when assessed in the presence of either $MnCl_2$ or forskolin, NaF- and GTP-stimulated adenylate cyclase activities were identical, as were the levels of expression of the two forms of $G_s\alpha$-subunits were similar in membranes from both sets of animals: these anomalies are discussed with regard to a possible molecular mechanism. Guanine nucleotide-mediated receptor-independent 'tonic' inhibitory effects were present in membranes from lean and obese animals, as determined by measuring the abilities of Gpp(NH)p and GTP to inhibit forskolin- and isoproterenol-stimulated adenylate cyclase activities. However, whereas the ability of GTP to inhibit isoproterenol-

stimulated adenylate cyclase activity was severely reduced in membranes from obese animals compared with those from lean animals, Gpp(NH)p was capable of inhibiting forskolin-stimulated adenylate cyclase activity by a similar degree in both lean and diabetic animals. A_1 adenosine receptor-mediated inhibition of forskolin-stimulated adenylate cyclase activity was retained, and even slightly enhanced, in membranes from obese animals, although the maximal inhibitions elicited by PGE_1 and nicotinc acid were unaltered. These changes in inhibitory regulation were accompanied by small reductions in the expression of $G_i\alpha$-1, $G_i\alpha$-2 and $G_i\alpha$-3, in the absence of any alteration in the expression of β-subunits. It is suggested that a reduced 'tonic' inhibitory functioning of G_i in adipocyte membranes from obese animals may account for the enhanced responsiveness of adenylate cyclase to stimulation by isoproterenol compared with membranes from lean animals. However, this functional defect would appear to be distinct from that which has been found in other rodent models of obesity, and possible molecular mechanisms by which it may arise are discussed.

CHAPTER 1

Introduction

1.1 DIABETES MELLITUS & INSULIN RESISTANCE

1.1.1 INTRODUCTION

Diabetes mellitus is a complex disorder characterized by alterations in carbohydrate, lipid and protein metabolism resulting from a deficiency of insulin or of its cellular metabolic actions. As a function of time, these metabolic alterations lead to chronic developmental changes which manifest themselves as the classical side-effects of diabetes. These include retinopathies, renal failure, peripheral and autonomic neuropathy, occlusive peripheral vascular diseases and coronary artery diseases (Wilson & Reeves, 1986).

1.1.2 CLASSIFICATION OF DIABETES MELLITUS

Although the initial discovery, purification and commercial availability of insulin had an enormous impact on the treatment of diabetes and resulted in the saving of countless lives, it became clear to clinicians such as Himsworth (1949) that diabetes is a heterogeneous disease with more aetiologies than just insulin deficiency. He pointed out that the disease could be divided into 'insulin-sensitive' and 'insulin-insensitive' types, and postulated that the latter disease was not related to insulin deficiency (Himsworth, 1949). This hypothesis was later confirmed by measurements of plasma insulin concentrations by the radioimmunoassay of Yalow & Berson (1960): it was found that plasma from maturity-onset, non-ketotic diabetics contained insulin at concentrations which were, on average, greater than normal, suggesting that a resistance to insulin rather than its deficiency was responsible for this form of diabetes.

Although it is a heterogeneous disorder, diabetes mellitus can be classified into two major subtypes based on these initial observations (National Diabetes Data Group, 1979):-

A Insulin-Dependent Diabetes Mellitus (IDDM)

Also known as Type I diabetes, this disease is characterized by the acute onset of hypoinsulinaemia, due to pancreatic β-cell damage, and an abrupt onset of symptoms such as a susceptibility to ketoacidosis. Sufferers are therefore dependent on the administration of exogenous insulin to prevent ketoacidosis and sustain life. It is also known as 'juvenile-onset' diabetes as the onset of symptoms usually occurs when patients are in their teens or early twenties. Approximately ten percent of all diabetics suffer from IDDM (National Diabetes Data Group, 1979).

In this disease, insulin resistance is not a major contributing factor to the complications of the disease: although it is present in newly diagnosed diabetics, it is readily alleviated once insulin treatment has begun (Kruszynska & Home, 1987). Any insulin resistance that does occur has most likely arisen from metabolic derangements of the diabetic state rather than any intrinsic target tissue defect in insulin action (Kruszynska & Home, 1987).

B Non-Insulin-Dependent Diabetes Mellitus (NIDDM)

Sufferers of Type II or non-insulin-dependent diabetes mellitus comprise approximately ninety percent of the diabetic population (National Diabetes Data Group, 1979). This form of diabetes is usually diagnosed in adulthood, hence the alternative term 'maturity-onset' diabetes. Unlike Type I diabetics, patients are not prone to ketosis and do not require the daily administration of insulin to sustain life, although in some cases insulin may be administered to treat persistent hyperglycaemia which does not respond to dietary control or anti-hyperglycaemic drugs (Olefsky *et al.*, 1988).

Hyperinsulinaemia and target tissue resistance to insulin are characteristic features of NIDDM. Furthermore, greater than half the patients with NIDDM exhibit obesity which is also associated with hyperinsulinaemia and insulin resistance (Olefsky *et al.*, 1988).

As this thesis is concerned with defects associated with Type II diabetes, the rest of this section will be devoted to the discussion of the development and potential mechanisms of insulin resistance associated with this state. However, to discuss systematically mechanisms underlying insulin resistance, we need to have some concept of the normal mechanism by which insulin exerts its intracellular effects.

1.2 THE MECHANISM OF INSULIN ACTION

1.2.1 INTRODUCTION

Insulin exerts a variety of anabolic effects in its three principal target tissues which are liver, skeletal muscle and fat (Denton *et al.*, 1981). Rapid effects of insulin include the stimulation of glucose uptake in skeletal muscle and fat, as well as the activation (e.g., glycogen synthase and pyruvate dehydrogenase) and inhibition (e.g., hormone-sensitive lipase) of specific regulatory enzymes (Denton *et al.*, 1981). Slower responses to insulin include those involved in regulating cell growth, such as the induction of c-*fos* and c-*myc*, which may take many hours to be expressed (Mottola & Czech, 1984; Stumpo *et al.*, 1988; Banskota *et al.*, 1989).

All these events are initiated by the binding of insulin to its receptor in the plasma membrane.

1.2.2 STRUCTURE & FUNCTION OF THE HUMAN INSULIN RECEPTOR

The structure of this receptor was initially determined by chemically cross-linking [^{125}I]insulin to its receptor, as well as by using biosynthetic and cell surface labelling techniques (Czech, 1985). It is an integral membrane glycoprotein with an approximate M_r=450 kDa, composed of two α-subunits (M_r=130 kDa) and two β-subunits (M_r=95 kDa) which are linked by disulphide bonds (Figure 1.1) (Czech, 1985). Affinity-labelling experiments using insulin derivatives predominantly

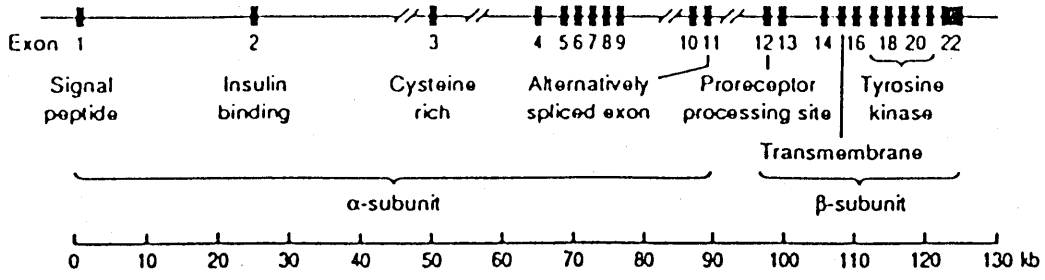
Figure 1.1:

Diagrammatical Representation Of The Human Insulin Receptor

The top figure depicts the intron/exon structure of the human insulin receptor. The 22 exons, their intronic boundaries and the corresponding coding regions of the receptor are shown.

The bottom figure gives depicts a simple structural model of the human insulin receptor in the plasma membrane. The blocks represent known functional domains, some of which are encoded by specific exons. Important tyrosine residues, either autophosphorylation sites (1146, 1150, 1151) or a presumed site of interactions with other proteins (960) are indicated as 'Y'. Two possible signal transduction mechanisms are shown; a non-covalent interaction with a G-protein which then couples to a second messenger-generating enzyme (phospholipase) resulting in diacylglycerol (DAG) and POS formation from the hydrolysis of a phosphatidylinositolglycan (PIglycan), and the tyrosyl phosphorylation of endogenous insulin receptor substrates (S) is also shown.

The figures are taken from Olefsky (1990).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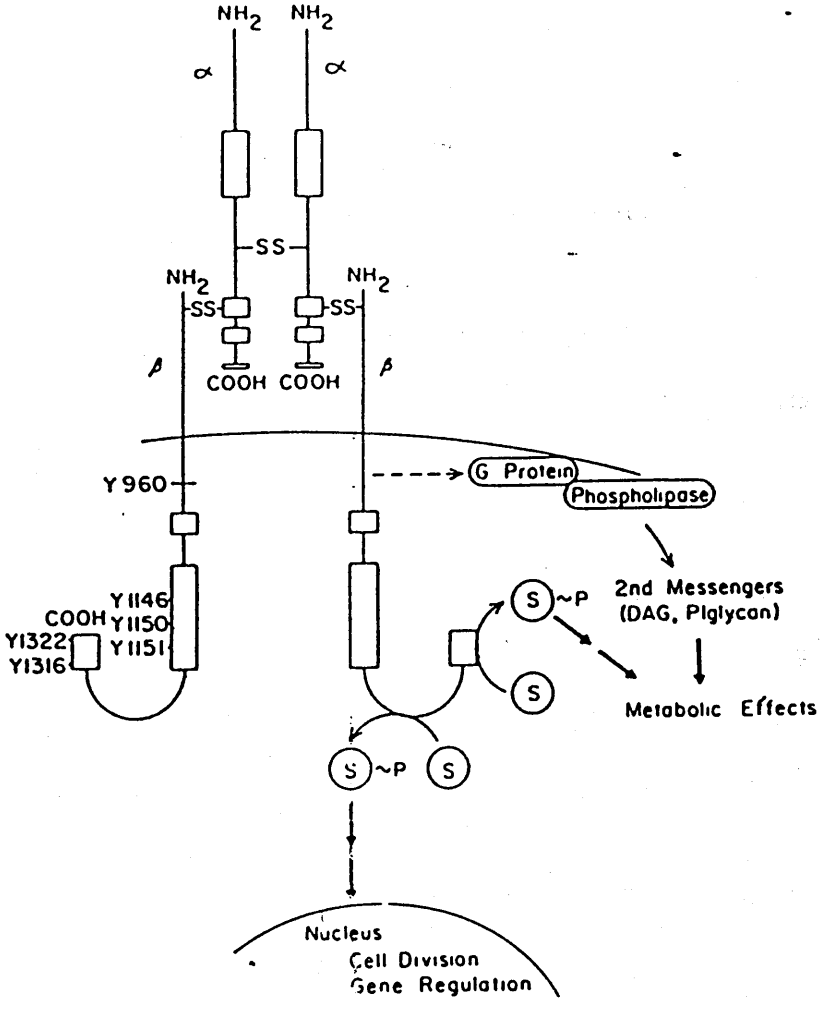
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G Protein
Phospholipase
2nd Messengers
(DAG, PIglycan)
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Y1322
Y1316
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Metabolic Effects
Nucleus
Cell Division
Gene Regulation

affect the α-subunit, suggesting that this subunit is responsible for insulin binding (Massagué *et al.*, 1981). However, even though two α-subunits are present per receptor molecule, the binding capacity of purified human insulin receptor is between 1 and 1.5 mol of insulin/mol of receptor (Petruzzelli *et al.*, 1982): the reasons for this discrepancy are unknown.

cDNA clones encoding the human insulin receptor were initially isolated from a human placental cDNA library using synthetic oligonucleotide probes designed from N-terminal amino acid sequences obtained from the α- and β-subunits of purified human placental insulin receptor (Ullrich *et al.*, 1985b; Ebina *et al.*, 1985). More recently it has been shown that this cDNA is derived from a single insulin receptor gene of greater than 120 Kbases in size, composed of 22 exons and which is located on the short arm of chromosome 19 (Seino *et al.*, 1989). As has been demonstrated for the low-density lipoprotein (LDL), platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) receptors, the insulin receptor is a protein consisting of several specific functional domains, some of which are encoded by distinct exons within its gene (Gilbert, 1985; Williams, 1989; Olefsky, 1990). Such a striking correlation between intron/exon structure and functionality suggests that such multidomain proteins have arisen by a series of exon shuffling and gene duplication events. Therefore, the insulin receptor can be seen as the product of a gene which may have evolved by the assembly of existing DNA sequences producing a novel gene. During the course of evolution, both gene rearrangements and degeneration of function at the protein level could have led to the overlap of functions across intron/exon boundaries (Olefsky, 1990).

The insulin receptor is synthesized as a single chain precursor containing either 1343 or 1355 amino acids preceded by a 27 residue signal sequence (Ullrich *et al.*, 1985b; Seino & Bell, 1989). During processing and transport from the endoplasmic reticulum to the plasma membrane the signal sequence is removed, the sequence of basic amino acids joining the α- and β-chains is cleaved,

oligosaccharides are added at specific side-chains and acylation occurs (Czech, 1985; Hedo *et al.*, 1987): these events lead to the formation of an ($\alpha\beta$) structure, the chains being linked by disulphide bonds. Two such processed monomers can associate with each other and form disulphide-linked dimers, $(\alpha\beta)_2$, which is the form of the mature receptor (Czech, 1985).

A Insulin Binding

Within each receptor molecule, the two α-subunits are identical and completely extracellular. They contain either 719 or 731 residues depending on the presence of a 12 amino acid insert which arises due to alternate splicing of exon 11 within the receptor mRNA: such splicing appears to occur in a tissue-specific manner (Seino & Bell, 1989).

Within each α-subunit is a Cys-rich region which is critical for ligand binding (Ullrich *et al.*, 1985b). A similar region has been identified within the ligand binding pockets of the EGF, PDGF and IGF-I receptors (Ullrich & Schlessinger, 1990). Interaction of the insulin molecule with other regions of the α-subunit has also been claimed, implying that these regions form a three-dimensional structure with the Cys-rich region to form the high-affinity binding site. The alternate splicing event within exon 11 has been shown to modify insulin binding affinity: inclusion of these residues produces a molecule with a 2- to 3-fold lower binding affinity for insulin (M^{c}Clain *et al.*, 1989b).

B Insulin-sensitive Tyrosyl Kinase Activity

The β-subunit of the human insulin receptor is a membrane-spanning protein containing 620 amino acids of which 403 residues are located in the cytoplasm and which are responsible for initiating insulin's effects in target tissues (Ullrich *et al.*, 1985b; Ebina *et al.*, 1985). The most important finding made in recent years has been the identification of this portion of the insulin receptor as an

insulin-sensitive tyrosyl kinase (Kasuga *et al.*, 1982). Such a property has been identified in every cell-type that responds to insulin, and can also be demonstrated using solubilised receptors (Gammeltoft & Van Obberghen, 1986; Olefsky, 1990). This is a property shared by several growth factor receptors, including EGF, PDGF, TGFα, and IGF-I, as well as some oncogene products, such as *src*, *ros* and *erb*-B (Ullrich & Schlessinger, 1990). Therefore the insulin receptor has been assigned as a member of the *src* family of protein kinases (Ullrich *et al.*, 1985b). In support of this hypothesis, analysis of the deduced amino acid sequence from the insulin receptor cDNA reveals the presence of an ATP-binding site (confirming biochemical studies by Shia & Pilch (1983)) as well as several Tyr residues which are capable of being autophosphorylated (Ullrich *et al.*, 1985b; Ebina *et al.*, 1985; Tavaré *et al.*, 1988).

Binding of insulin to the α-subunit induces a conformational change which relieves its inhibitory effect on the tyrosyl kinase activity of the β-subunit, and initiates a rapid cascade of autophosphorylation of the receptor on specific Tyr residues (White *et al.*, 1988). Three Tyr residues (residues 1146, 1150 and 1151 from the cDNA sequence of the original human receptor cDNA, which comprise "autophosphorylation domain A") are phosphorylated very rapidly after insulin binding and are the most important sites associated with an active receptor kinase (White *et al.*, 1988; Tornqvist *et al.*, 1987). Indeed, substitution of these Tyr residues for other amino acids causes a reduction in receptor kinase activity and compromises biological signalling with respect to glucose uptake (Ellis *et al.*, 1986a) but not with respect to insulin's mitogenic effects (Olefsky, 1990). However, mutation of the presumed ATP-binding site at Lys 1018 to Ala abolishes all cellular responses to insulin mediated by this receptor when it is transfected into rat fibroblasts (M^{c}Clain *et al.*, 1987). Two other Tyr residues in the C-terminal region of the molecule (Tyr 1316 & 1322) are also thought to be important in mediating signal transduction by the receptor as they are also autophosphorylated after exposure of the receptor to insulin, although they are not phosphorylated as

rapidly in the intact cell as domain A (Tavaré *et al.*, 1988).

Other studies on receptor constructs with either deletions or truncations which cause a loss in kinase activity also exhibit a loss of biological activity, further emphasizing the importance of the integrity of this property for signal transduction by the insulin receptor (Ellis *et al.*, 1986b; Ellis *et al.*, 1987). Experiments carried out on Rat-1 fibroblasts transfected with the ATP-binding site-defective human receptor (Lys 1018 substituted with Ala) have shown that although this receptor does not signal any of insulin's metabolic effects, the functioning of the native rat receptors is inhibited (McClain *et al.*, 1987; Chou *et al.*, 1987). The mechanisms by which this occurs are unclear: it is possible that both inactive rat/human receptor hybrids and mutant human receptors compete with the endogenous receptors for the next step in the signalling process (McClain *et al.*, 1987; Chou *et al.*, 1987).

C Post-Receptor Signal Transduction Mechanisms

As previously described, the insulin receptor can be thought of as a multidomain protein. Presumably some of these domains will be involved in mediating the interaction of the receptor with other cellular proteins. For example, one such domain may contain binding sites for substrates of the tyrosyl kinase activity of the receptor: these phosphorylated, activated proteins would then be able to participate in downstream signalling as direct effector units or coupling molecules. Several cellular substrates of the insulin receptor kinase have been identified in intact cells, yet they have only been identified by virtue of their molecular weights, for example pp185. Their function and potential importance remain uncertain (Kahn & White, 1988).

The unambiguous identification of an insulin receptor substrate, or any protein capable of interacting productively with the insulin receptor, as a serine/threonine kinase or phosphatase which participates in the propagation of the observed phosphorylation cascade would greatly enhance our understanding of the

mechanism of insulin action. Several kinases, such as microtubule-associated protein 2 kinase (MAP 2 kinase), *raf* and ribosomal subunit S6 kinase are all substrates of receptor tyrosyl kinases, with the MAP 2 kinase being of particular interest (Czech *et al.*, 1988; Boulton *et al.*, 1990b).

The activity of the MAP 2 kinase is stimulated by insulin and it is also capable of being activated by phosphorylation on Tyr residues *in vitro* (Ray & Sturgill, 1988). The fact that phosphorylation on Ser and Thr, as well as Tyr residues, is required for activation of MAP 2 kinase suggests that MAP 2 kinase activity is highly regulated, a property shared by other kinases intimately involved in phosphorylation cascades (*raf*, cdc^{2+}) (Anderson *et al.*, 1990). The cloning of the MAP 2 kinase from a Rat-1 fibroblast cDNA library showed this kinase to be highly homologous to two yeast Ser kinases (KSS 1 and FUS 3) which regulate the yeast cell cycle response to mating factors (Boulton *et al.*, 1990a).

Other signalling molecules have been proposed to interact directly with the insulin receptor. These include specific G-proteins (Heyworth *et al.*, 1985; Rothenberg & Kahn, 1988; Korn *et al.*, 1987) which may either be phosphorylated on Tyr (O'Brien *et al.*, 1987a & 1987b; Krupinski *et al.*, 1988) or may interact directly with the receptor without being covalently modified (Rothenberg & Kahn, 1988; Luttrell *et al.*, 1990). Such a G-protein may be capable of coupling to a phosphatidylinositol glycan-specific phospholipase C whose activity has been proposed to respond to insulin, leading to the release of a phospho-oligosaccharide (POS) 'mediator' and the accumulation of a specific diacylglycerol species (Saltiel, 1990; Standaert *et al.*, 1988) which may activate a specific isoform of protein kinase C (Saltiel *et al.*, 1987; Cooper *et al.*, 1987; Heidenreich *et al.*, 1990). Some evidence has suggested that the POS mediator can mimick the alterations in the Ser and Thr phosphorylation/dephosphorylation pattern observed on exposure of rat adipocytes to insulin (Alemany *et al.*, 1987), although the exact loci of action of the mediator have not yet been fully

characterized.

D Assignment Of Domains Of The Insulin Receptor With Function

The assignment of specific domains within the β-subunit responsible for interactions with specific signalling molecules or events has been attempted using molecular biological approaches (Olefsky, 1990; McClain, 1990): these findings are summarized in Figure 1.1. Two regions which have yielded particularly interesting information are the "Tyr 960 domain" and the C-terminal region of the β-subunit.

i Tyr 960 Domain

Unlike Tyr's 1146, 1150 and 1151, Tyr's 953, 960 and 972 are not autophosphorylated in the intact cell after exposure to insulin (Tavaré *et al.*, 1988) although Tyr 960 can be phosphorylated in truncated receptor constructs (White *et al.*, 1988). However, mutation of Tyr 960 to a Phe residue can block the insulin-stimulated sensitization of glucose uptake when the mutant receptor is transfected into Chinese Hamster ovary (CHO) cells without affecting autophosphorylation or tyrosyl kinase activity towards exogenous substrates (White *et al.*, 1988). Whilst the region flanking Tyr 960 is conserved between the insulin and IGF-I receptors, similar sequences are not present in either the EGF receptor or v-*ros*, perhaps implying that this domain may specify interaction with a signalling mechanism which is common to insulin and IGF-I.

ii C-Terminal Domain

The most divergent cytoplasmic region amongst tyrosyl kinases is their hydrophilic C-terminal region: this is partially deleted in the oncogenic forms of v-*erb*-B and v-*fms* compared with their proto-oncogenic sequences, suggesting a potential rôle for this region in mediating specific responses (Ullrich *et al.*, 1984; Ullrich *et al.*, 1985a and b; Ullrich *et al.*, 1986; Coussens *et al.*, 1986). To this end, mutant human insulin receptors in which the C-terminal 43 amino acids are

deleted (termed ΔChIR) have been studied after transfection into Rat-1 fibroblasts (McClain, 1990). The truncated receptor is normally synthesized and processed (McClain *et al.*, 1988) and undergoes insulin-stimulated autophosphorylation in a normal fashion (Maegawa *et al.*, 1988). Similarly, the truncated receptor is still capable of tyrosyl phosphorylating various exogenous and endogenous substrates (Maegawa *et al.*, 1988). However, the ability of insulin to sensitize intracellular glucose uptake is severely attenuated, presumably because the necessary structural information required for this process has been deleted (Maegawa *et al.*, 1988). This contrasts with an enhanced ability of insulin to mediate its mitogenic effects (measured by [^{3}H]thymidine uptake) in cells transfected with the ΔChIR constructs (Thies *et al.*, 1989). Hence, whilst the C-terminal domain may contain information critical for signalling the metabolic effects of insulin, absence of this domain also appears to relieve inhibitory constraints on insulin-stimulated mitogenesis (Thies *et al.*, 1989).

A concept of domain-specific insulin receptor signalling is supported by other mutations which can induce both selective insulin resistance (Wilden *et al.*, 1990) and a differential regulation of various actions of insulin (Marshall, 1990). For example, one study demonstrated that mutation of Tyr 1146 to a Phe residue in the primary autophosphorylation site had no effect on metabolic signalling yet mitogenic signalling was attenuated (Wilden *et al.*, 1990). However, more exhaustive studies are required to assign specific domains with putative effectors or substrates before any meaningful picture of the mechanism of insulin signal transduction can be drawn.

1.3 INSULIN-RESISTANT STATES

1.3.1 INTRODUCTION

Target tissue resistance to insulin is a characteristic feature of the Type II

diabetic state. It can be defined as the metabolic state in which a physiologically relevant concentration of insulin produces a subnormal response (Olefsky *et al.*, 1988). Such resistance to insulin can involve any of its multiple metabolic effects, but the hormone's effects on glucose metabolism are the most frequently studied and this is most often monitored in humans by the oral glucose tolerance test (OGTT) which measures the clearance of glucose from the blood after ingestion of a concentrated glucose solution (DeFronzo, 1988)

The previous section's discussion of the mechanism of insulin action in the normal state demonstrates that insulin resistance can potentially result from a defect in any point within the mechanism of signal transduction by the insulin receptor, from the insulin binding event to the transport of glucose into muscle and fat cells. The development of an insulin-resistant state and the potential mechanisms by which this phenomenon occur will be discussed as they apply to humans. This will be followed by a description of the murine models of obesity used in this thesis and their relevance to the Type II diabetic state as it occurs in man.

1.3.2 DEVELOPMENT OF AN INSULIN-RESISTANT STATE

The maintenance of glucose homeostasis depends on three processes which must occur in a co-ordinated fashion: ingestion of glucose must stimulate insulin secretion by the pancreas which must result in peripheral glucose uptake by muscle and fat, and the suppression of glucose production by the liver. Hence, defects at the level of either the pancreatic β-cell, muscle (the tissue which accounts for the majority of glucose cleared from the blood) or liver can cause diabetes. DeFronzo (1988) has proposed that there are two primary defects responsible for the development of NIDDM: in some patients the primary defect is at the level of insulin secretion, whilst in others it lies at the level of tissue insensitivity to insulin. Whichever defect initiates the diabetic syndrome, the second abnormality will eventually develop as both defects must be present simultaneously for full

expression of glucose intolerance (DeFronzo, 1988).

The major difficulty in attempting to determine the initial steps in the pathogenesis of NIDDM is that when patients are presented to a doctor, the defects in insulin secretion and insulin resistance are already present (DeFronzo *et al.*, 1983). However, two models which attempt to explain the development of NIDDM are outlined below:-

A "Failing Pancreas" Theory

In these NIDDM patients, the first phase of the insulin secretory response to hyperglycaemia is inadequate (Ward *et al.*, 1984; DeFronzo *et al.*, 1983). As a result, glucose concentrations remain elevated after glucose administration (Steiner *et al.*, 1982) and this presents a continual stimulus to the β-cell such that high basal insulin secretion, and a resulting hyperinsulinaemia, is necessary to return glucose levels to normal. As the β-cell defect becomes progressively worse, and the diabetes more pronounced, the basal plasma insulin response, although elevated compared with normal subjects, becomes insufficient to return plasma glucose concentrations to normal, resulting in the development of fasting hyperglycaemia. This causes a constitutive elevation in basal insulin secretion and fasting hyperinsulinaemia ensues (DeFronzo *et al.*, 1983; Faber & Damsgaard, 1984). Such a persistent hyperinsulinaemic state initiates a down-regulation of insulin receptors and the development of post-binding insulin resistance (Insel *et al.*, 1980; Garvey *et al.*, 1986; Marangou *et al.*, 1986).

Eventually, either as a result of the initial defect or β-cell exhaustion, the insulin response becomes absolutely deficient and fasting hyperglycaemia will persist due to excessive hepatic glucose output from the liver (Olefsky *et al.*, 1985) and a further reduction in glucose clearance from the blood by skeletal muscle and fat tissues: this is the final clinical picture of a Type II diabetic patient.

B "Resistant Receptor" Theory

In both diabetic and non-diabetic obesity, the plasma insulin response to oral and intravenous glucose is greater than for normal individuals (Felig *et al.*, 1974). It is thought that the disturbance in some of these patients is initiated by a cellular defect in insulin action. Initially, the insulin resistance is not sufficient to either impair glucose uptake by skeletal muscle or to increase hepatic glucose output. However, as the insulin resistance becomes more pronounced, peripheral glucose uptake becomes sufficiently impaired to induce hyperglycaemia, although hepatic glucose output remains normal due to the hyperinsulinaemia. The development of persistent hyperglycaemia exacerbates the hyperinsulinaemia which in turn causes down-regulation of insulin receptor number and function, thereby compounding the insulin resistance. In many patients the continual stimulation of β-cells due to hyperglycaemia causes a demise in β-cell function (DeFronzo, 1988). Such a sequence of events has been validated in several studies (Sims *et al.*, 1973; Bevilacqua *et al.*, 1985).

1.3.3 MECHANISMS OF INDUCTION OF DIABETES

There are several loci at which insulin resistance could potentially arise:-

A Secretion Of An Abnormal Insulin Molecule

This is a very rare syndrome. Only one such individual has been reported in the literature (Tager *et al.*, 1979).

B Secretion Of An Incompletely Processed Insulin Molecule

This disease is termed familial hyperproinsulinaemia. Again, this is a very rare disease with only two families reported to exhibit this defect (Gabbay *et al.*, 1976; Kanazawa *et al.*, 1978).

C Circulating Insulin "Antagonists"

These may take several forms. For example, excessive secretion of other hormones in disease states can antagonize insulin's metabolic effects. Examples include Cushing's syndrome (excess cortisol secretion), acromegaly (growth hormone secretion) and pheochromocytoma (catecholamine secretion). Of particular interest is the increase in glucagon secretion observed in Type I diabetes which may contribute to the form of insulin resistance present in newly-diagnosed IDDM patients (Kruszynska & Home, 1987).

Anti-insulin or anti-insulin receptor antibodies can theoretically antagonize the binding of insulin to the α-subunits of the receptor, although these syndromes are very rare (Kurz & Nabarro, 1980; Kahn *et al.*, 1981).

D Reduced Insulin Binding

Since the development of insulin resistance is initially associated with a hyperinsulinaemic state, one would expect a down-regulation of cell-surface insulin binding sites and a reduction in binding affinity to be induced. This has been observed in several cell types including adipocytes (Insel *et al.*, 1980). However, since many target cells possess "spare receptors" for most of insulin's biological effects, the relation between receptor number and physiological effect is not simple. For example, in rat adipocytes, maximal insulin stimulation of glucose transport occurs when only 10% of the insulin receptors are occupied (Kono & Barham, 1971; Olefsky, 1976), implying that 90% of the receptors are "spare". Hence, a successive reduction in the number of receptors is likely to shift the dose-response curve for insulin stimulation of glucose transport to the right, *i.e.* the sensitivity to insulin will be compromised but not the maximal response: only when greater than 90% of the receptors are lost will there be a reduction in the maximal response (Olefsky, 1976). Such a situation contrasts with liver, where only 10% of the total complement of insulin receptors are spare when insulin binding is compared with

activation of glycogen synthesis (Gammeltoft *et al.*, 1978; Gammeltoft, 1984). It has been suggested that these tissue-specific binding kinetics are a result of adaptation to different concentrations of insulin in the portal and extraportal circulations (Gammeltoft & Van Obberghen, 1986).

E Post-Binding Defects

In NIDDM, only part of the insulin resistance is accounted for by a reduction in insulin binding to its receptor: the major component has been attributed to a post-binding defect(s) (Kolterman *et al.*, 1981; Bolinder *et al.*, 1982). Two insulin-stimulated events which have been intensively studied with respect to potential defects in NIDDM are the tyrosyl kinase activity of the receptor (both autophosphorylation and modification of exogenous substrates) and glucose transport in skeletal muscle and fat (Olefsky *et al.*, 1988).

i Genetic Defects In Insulin Receptor Function

The types of patients most studied with respect to insulin receptor kinase functioning are those diabetics whose symptoms are severe and which arise due to genetic defects (Reddy & Kahn, 1988; Taylor *et al.*, 1990). Diseases arising from such defects include type A insulin resistance (Kahn *et al.*, 1976), lipoatrophic diabetes (Rossini & Cahill, 1979) and leprechaunism (Rosenberg *et al.*, 1980). Numerous studies on insulin receptors in different tissues from patients with these syndromes have shown there to be multiple insulin receptor aberations within each disease, including reduced binding associated with reduced expression of the receptor as well as autophosphorylation defects which vary in severity between patients (reviewed by Reddy & Kahn (1988)).

A variety of insulin-resistant patients have been studied by Taylor *et al.* (1990) and shown to exhibit a heterogeneous range of defects. Mutations in the insulin receptors isolated from these patients can produce any alteration from a reduced binding capacity, impaired processing leading to reduced functional

receptor number and reduced expression, as well as impaired tyrosyl kinase activity: the same alteration in function can arise from different patients exhibiting mutations in different parts of the insulin receptor (Taylor *et al.*, 1990). However, whilst such studies are illuminating from the point of elucidating putative functions of various domains of the insulin receptor, they have limited relevance to the most common form of insulin resistance associated with NIDDM and obesity.

ii Regulatory Defects In Insulin Receptor Function

Insulin resistance associated with NIDDM and obesity is distinct from the defects in insulin receptor function described above. Firstly, the degree of severity of the insulin-resistant state varies widely between different individuals, unlike the above syndromes which are all severe. Secondly, the common forms of insulin resistance are reversible to some degree, as the resistance may be alleviated by fasting and weight loss (Bar *et al.*, 1976; Freidenberg *et al.*, 1988), suggesting the existence of a readily-reversible regulatory defect.

The insulin resistance of NIDDM patients is associated with a reduced insulin-stimulated tyrosyl kinase activity of the receptor, with respect to autophosphorylation and tyrosyl phosphorylation of cellular substrates, in adipocytes (Freidenberg *et al.*, 1987; Sinha *et al.*, 1987; Thies *et al.*, 1990) and hepatocytes (Caro *et al.*, 1986) compared with non-diabetic counterparts. Studies on skeletal muscle have been less clear, with autophosphorylation in lean, obese and obese-diabetic groups being similar, yet kinase activity using exogenous substrates *in vitro* was reduced in the NIDDM patients (Caro *et al.*, 1987; Arner *et al.* 1987). Studies on adipocyte insulin receptors from NIDDM patients have shown that the reduced autophosphorylation observed is probably due to a decrease in the proportion of receptors capable of undergoing autophosphorylation (Olefsky *et al.*, 1988). The mechanism by which this occurs is unknown, although several possibilities have been proposed.

It has been suggested, on the basis of both direct (Karasik *et al.*, 1990) and indirect evidence (Houslay, 1989; van der Werve & Massillon, 1990) from studies on rodent models of obesity and insulin resistance, that there may be altered regulation of insulin receptor function by protein kinase C (PKC). Protein kinases A and C are capable of phosphorylating the insulin receptor, resulting in a dimunition of insulin binding and insulin-stimulated autophosphorylation (Stadtmauer & Rosen, 1986; Takayama *et al.*, 1988). Phorbol esters can also regulate insulin receptor internalization in some cell types (Hayachi *et al.*, 1987). The regulation of insulin receptor function has received particular attention by virtue of the findings from studies investigating the regulation of hepatic insulin receptor functioning from 72 hour-starved insulin-resistant rats (Karasik *et al.*, 1990). A drastic reduction in insulin-stimulated autophosphorylation of the receptor was noted, and was found to be associated with an increased PKC activity in both membrane and cytosolic fractions in livers from starved animals (Karasik *et al.*, 1990). It was suggested that an increase in the Ser/Thr phosphorylation by an enhanced PKC activity attenuated insulin receptor functioning (Karasik *et al.*, 1990).

In contrast, it has been noted in myocytes and hepatocytes from genetically obese Zucker rats that there is a reduced ability of hormone- or phorbol ester-stimulated PKC to inactivate glycogen synthase activity compared with their lean littermates (van der Werve *et al.*, 1987; van der Werve & Massillon, 1990). This contrasts with the conclusions of Bushfield *et al.* (1990b) who observed a phosphorylation and inactivation of the inhibitory regulatory protein of adenylate cyclase, $G_i\alpha$-2, in obese Zucker rat hepatocytes and ascribed this phenomenon to a constitutively elevated PKC activity. It is possible that the processes studied by these two groups of investigators are regulated by distinct PKC isoforms which are differentially regulated by the insulin-resistant state. In relation to this, it has been noted that both liver and skeletal muscle from genetically obese Zucker rats contain

elevated levels of *sn*-1,2-diacylglycerol compared with lean controls, which may result in the selective down-regulation or persistent activation of certain PKC isoforms (Turinsky *et al.*, 1990).

1.3.4 INSULIN-STIMULATED GLUCOSE TRANSPORT

Facilitative diffusion of glucose across the plasma membrane is characteristic of virtually all animal cells. Diffusion is mediated by a family of facilitative glucose transporter glycoproteins which display considerable homology with one another in their primary sequences, but exhibit distinct tissue-specific patterns of expression. The family consists of five different gene products, designated GLUT 1 to GLUT 5 based on the chronological order of publication of the cDNA sequences (Bell *et al.*, 1990). The basic characteristics and tissue distribution of these isoforms are detailed in Table 1.1. The transporters share a conserved presumed membrane topography of twelve transmembrane-spanning α-helices (Gould & Bell, 1990).

Insulin stimulates glucose uptake from the blood by enhancing the facilitative transport of glucose into skeletal muscle, heart and fat tissues (Gould & Bell, 1990). This is achieved by the translocation of specific transporter species from an intracellular vesicular pool to the plasma membrane (Cushman & Wardzala, 1980; Suzuki & Kono, 1980). In isolated rat adipocytes, exposure to insulin results in a 20- to 30-fold increase in the rate of glucose transport across the plasma membrane (Gibbs *et al.*, 1988). This is achieved by the translocation of GLUT 1 and GLUT 4 to the plasma membrane, with GLUT 4 accounting for the vast majority of the insulin-stimulated transport in both fat (Zorzano *et al.*, 1989) and skeletal muscle (Kern *et al.*, 1990).

As previously mentioned, peripheral insulin resistance is one of the major pathophysiological disorders of the Type II diabetic state. As this is associated with a reduced insulin stimulation of glucose utilization in skeletal muscle, the glucose transport system is a potential locus for this insulin resistance as glucose uptake

Table 1.1:

Family Of Human Facilitative Glucose Transporters

The table shows a list of the facilitative glucose transporters cloned thus far from the human genome, their size, tissue distribution and chromosomal locations.

The table is adapted from Gould & Bell (1990).

Common Name	No. Of Amino Acids	Major Sites Of: Expression	Chromosomal Location
GLUT 1	492	Fetal tissues, brain, kidney & colon	1
GLUT 2	524	Liver, β-cell, kidney & small intestine	3
GLUT 3	496	Many tissues	12
GLUT 4	509	Skeletal muscle, heart & adipocytes	17
GLUT 5	501	Small intestine	1

appears to be the rate-limiting step for glucose utilization in muscle (Morgan *et al.*, 1961). Any defect in insulin-stimulated glucose transport could potentially arise from perturbations in three processes: insulin receptor signal transduction (previously discussed), translocation of GLUT 1 and/or GLUT 4 to the plasma membrane or a reduced transport capacity of GLUT 1 or GLUT 4. Recent studies have demonstrated that induction of the Type I diabetic state in rats is associated with a drastic reduction in expression of GLUT 4, but not GLUT 1, in adipocytes (Berger *et al.*, 1989; Sivitz *et al.*, 1989) and skeletal muscle (Garvey *et al.*, 1989) with expression of the GLUT 4 transporter in adipocytes being regulated by the hypoinsulinaemia but not the hyperglycaemia (Sivitz *et al.*, 1990). In contrast, studies on GLUT 4 expression in various tissues from genetically Type II diabetic (*db/db*) mice showed that reduced insulin-stimulated glucose transport in skeletal muscle (Chan & Tatoyan, 1984) was not associated with a reduced expression of GLUT 4 in diabetic animals compared with lean controls (Koranyi *et al.*, 1990).

Numerous studies on insulin-stimulated glucose transport in adipocytes from Type II diabetic humans have been carried out by Olefsky and co-workers (Olefsky *et al.*, 1988). NIDDM patients exhibit drastically reduced levels of low density microsomal- and plasma membrane-associated glucose transporters as measured by D-glucose-inhibitable cytochalasin B binding measurements (Olefsky *et al.*, 1988). There also appears to be an impairment in the translocation of transporters to the plasma membrane as the rate at which maximal activation of glucose transport is achieved is reduced in NIDDM patients compared with non-diabetic controls (Molina *et al.*, 1989). Another possible mechanism for reduced transport in NIDDM sufferers is modification of the glucose transporter proteins: this has been implicated by a reduced affinity of glucose transporters for cytochalasin B in adipocytes from diabetic patients (Olefsky *et al.*, 1988). However, it is important to emphasize that, compared with skeletal muscle, adipose tissue is an inconsequential depot for circulating glucose and that GLUT 4 may be

differentially regulated in muscle in these insulin-resistant states (Ferrannini *et al.*, 1985; Mueckler, 1990).

Considering the rapid increase in the understanding of the proteins involved in glucose transport over the last three years, it is now necessary to determine the contribution of each of the glucose transporter subtypes (GLUT 1 or GLUT 4) in determining the reduced transport capacity in diabetic patients and by what mechanisms these changes occur.

Genetic studies are also underway in several labs in an attempt to determine a possible linkage between altered *GLUT* genes and the development of NIDDM: two obvious candidates are *GLUT 2* (liver and β-cell transporter) and *GLUT 4* (fat and muscle). Initial studies with *GLUT 1* (ubiquitous tissue distribution) have produced conflicting results, with one group suggesting a linkage between a genetic variation in *GLUT 1* and a susceptibility to NIDDM and others failing to observe such a relation in different populations (Bell *et al.*, 1990). Similar studies on *GLUT 4* have also shown there to be no association of a modified gene with susceptibility to NIDDM (Bell *et al.*, 1990). However as susceptibility to NIDDM is complex and polygenic in nature, any relation between altered glucose transporter genes and susceptibility to NIDDM will not be a simple one.

1.3.5 INDUCTION OF INSULIN RESISTANCE BY AMYLIN

Recently, it has been noted that islet amyloid polypeptide (IAPP or amylin), which is the major peptide component of pancreatic amyloid deposits found in diabetics, can induce insulin resistance *in vitro* and *in vivo* (Leighton & Cooper, 1990). Maximally effective concentrations of amylin, and the related calcitonin gene-related peptide (CGRP), can inhibit basal and insulin-stimulated glucose uptake into rat soleus muscle (Leighton & Cooper, 1988) and BC_3H1 myocytes but not rat adipocytes (Molina *et al.*, 1990). As the major glucose transporter isoform in BC_3H1 myocytes which translocates in response to insulin is GLUT 1 and not

Table 1.2:

Rodent Models Of Type II Diabetes Mellitus And Obesity

SINGLE GENE MUTANTS

Obese (*ob/ob*) mouse

Diabetic (*db/db*) mouse

Zucker (*fa/fa*) rat

Obese Yellow (A^{vy}) mouse

POLYGENIC INHERITANCE

Chinese hamster

NZO mouse

KK mouse

Djungarian hamster

South African hamster

Pbb/Ld mouse

CBA/Ca mouse

HYBRIDS

Wellesley hybrid mouse

ACQUIRED

High fat-fed rat

High fructose-fed rat

Spiny mouse (partial genetic susceptibility)

GLUT 4 (Calderhead *et al.*, 1990), amylin may be responsible for reducing basal glucose transport activity in skeletal muscle. It also remains to be determined what range of circulating physiological amylin concentrations is present in diabetics, and what relation this bears to the concentrations used in the studies carried out with the peptide thus far (Leighton & Cooper, 1990).

1.4 RODENT MODELS OF TYPE II DIABETES MELLITUS & INSULIN RESISTANCE

1.4.1 INTRODUCTION

Several different rodent models of Type II diabetes and insulin resistance associated with obesity have been developed to study the metabolic perturbations characteristic of insulin-resistant states: some of these are listed in Table 1.2. Only the two models of obesity used in this thesis will be described in detail.

1.4.2 THE GENETICALLY DIABETIC (*db/db*) MOUSE

"Diabetes" (*db*) is a single gene mutation on chromosome 4 initially observed in a C57BL/KsJ mouse strain at the Jackson Laboratory, Maine, U.S.A. (Hummel *et al.*, 1966). On this background, which is characteristic of the mice used in this thesis, the gene produces marked hyperglycaemia, hyperphagia, obesity and atrophy of pancreatic β-cells. Inheritance of the diabetes syndrome is autosomal recessive with complete penetrance.

Hyperinsulinaemia in these animals is detectable from as early as 10 days of age and after 4 weeks the animals are profoundly diabetic, although a transient hypoinsulinaemia is detectable before the hyperinsulinaemic state prevails (Coleman & Hummel, 1974). In this state the obese diabetic animals can rapidly gain weight so that after 6 weeks of age they weigh 50% more than pair-fed lean controls and substantially more if they are allowed *ad libitum* access to food (Cox & Powley, 1977). During the hyperinsulinaemic phase of their life, the β-cells degranulate and

secrete insulin at greatly enhanced rates compared to normal animals until the cells die and fail to secrete insulin in response to glucose. This initiates the final hypoinsulinaemic phase of the syndrome when the animals become ketotic and die.

The etiology of this syndrome has yet to be defined. Coleman & Hummel (1974) have suggested that a defect in insulin secretion initiates the the syndrome and that insulin resistance eventually follows in a manner similar to that described for the "Failing Pancreas" model of NIDDM development in humans. Insulin resistance has been noted in liver, where insulin binding is reduced (Soll *et al.*, 1975) and gluconeogenesis is non-suppressible (Chan *et al.*, 1975), and in skeletal muscle where insulin-stimulated glucose uptake is reduced (Chan & Tatoyan, 1984). The rôle of other hormones in contributing to the observed insulin resistance is unknown: glucagon secretion is elevated throughout the life of a diabetic mouse due to increased pancreatic A-cell number (Laube *et al.*, 1973) and the secretion of thyroid hormones is normal, although steroid metabolism is severely perturbed (Leiter, 1990).

The activities of several insulin-sensitive enzymes involved in hepatic and adipose tissue lipogenesis are increased in diabetic animals compared with controls (Chan & Exton, 1977; Coleman & Hummel, 1967). Furthermore, an impaired mobilization of fat in response to lipolytic hormones has been observed by several investigators (Steinmetz *et al.*, 1969; Yen *et al.*, 1970). Presumably these phenomena cause the accumulation of triglyceride and the associated obesity. These factors will be discussed in more detail in Chapter 4.

1.4.2 THE GENETICALLY DIABETIC CBA/CA MOUSE

The CBA/Ca mice used in this thesis were obtained from the University of Bristol Medical School, Bristol, U.K. They were derived from an inbred colony in which approximately 80% of males exhibit a maturity onset diabetic syndrome, consisting of hyperphagia, hyperinsulinaemia, hypertriglyceridaemia, obesity and

impaired glucose tolerance (Connelly & Taberner, 1989). Onset of the syndrome occurs between the ages of twelve and sixteen weeks, although the afflicted mice have a similar life expectancy to that of non-diabetic animals (Connelly & Taberner, 1989). The background of the CBA syndrome is unknown, but presumably susceptibility does not involve an environmental component as the obese male mice are kept under identical conditions as their lean littermates (Connelly & Taberner, 1989).

Since this colony of mice has been developed recently, only limited biochemical studies have been performed thus far. Insulin binding studies, performed on liver plasma membranes from lean and obese animals, have demonstrated that the numbers of high- and low-affinity insulin binding sites are reduced by some 40% in obese animals compared with their lean littermates (Taberner & Connelly, 1991). Considering the directly proportional relationship between insulin receptor number and insulin action in liver (Gammeltoft *et al.*, 1978; Gammeltoft, 1984), this may partially contribute to the insulin resistance these animals exhibit. Insulin resistance has also been noted with respect to the ability of insulin to exert anti-lipolytic effects in brown and white adipose tissues (Mercer *et al.*, 1991)

1.5 HISTORICAL PERSPECTIVES ON G-PROTEIN REGULATION OF SIGNAL TRANSDUCTION

In 1956, Earl Sutherland first recognized the importance of the heat-stable compound adenosine 3', 5'-cyclic monophosphate (cyclic AMP) in mediating the activation of glycogen phosphorylase by glucagon in rat liver (historical summary by Sutherland, 1972). This observation gave rise to the concept of an extracellular hormonal "first messenger" activating an effector enzyme which produces relatively large amounts of an intracellular "second messenger". Subsequent studies on the hormonal sensitivity of cyclic AMP formation demonstrated that occupation of a hormone receptor by a suitable agonist induces a conformational change in the catalytic unit of adenylate cyclase thereby activating it and increasing the rate of formation of cyclic AMP from ATP. Therefore hormone-dependent adenylate cyclase functions as an allosterically regulated enzyme, the receptor occupying the status of a regulatory site (Robison *et al.*, 1967).

The importance of a guanine nucleotide binding regulatory component, or G-protein, was first suggested by a requirement for GTP in the activation of adenylate cyclase by glucagon in rat liver plasma membranes (Rodbell *et al.*, 1971b). This was associated with an ability of GTP to reduce the specific binding of glucagon to its receptor (Rodbell *et al.*, 1971a). Further research demonstrated that this effect was specific for agonists and was a consequence of reduced affinity for receptor (Maguire *et al.*, 1976). The agonist dependence of the effect of guanine nucleotides on ligand binding to β-adrenergic receptors was shown by Cassel and Selinger to be related to their ability to stimulate a catecholamine-sensitive GTPase activity in turkey erythrocyte membranes (Cassel & Selinger, 1976).

From these early studies, the basic properties of G-protein-linked systems were determined. Thus G-protein-linked systems stimulate a specific protein by interacting with it such that bound GDP dissociates allowing GTP to bind and

activate the protein: hydrolysis of GTP to GDP initiates deactivation. The correlation of receptor occupancy by an agonist with GDP release leads to the negative heterotropic effect of guanine nucleotides on agonist binding.

Subsequent research over 15 years has shown G-proteins to be general intermediates in many hormone-sensitive signal transduction pathways. The number of receptors involved is vast and includes those for biogenic amines (catecholamines, muscarinic receptors for acetylcholine), autocoids (prostaglandins, leukotrienes), peptide hormones (glucagon), neurotransmitters (opioids) and the photoreceptor rhodopsin. However, relatively few effector enzymes are known to be regulated by G-proteins: adenylate cyclase and the retinal cyclic GMP-specific phosphodiesterase have been intensively studied, but other effectors include a phosphoinositide-specific phospholipase C, phospholipase A_2 and some ion channels (Birnbaumer *et al.*, 1990).

Recent research has also demonstrated that G-protein-linked signalling pathways are not restricted to mammalian systems. For example, in *Saccharomyces cerevisiae* G-proteins are involved in both the activation of adenylate cyclase and the "pheromone response", which results in yeast cells mating. Both of the proteins involved exhibit strong structural and functional homologies with their mammalian counterparts (Levitzki, 1988; Fields, 1990).

1.6 STUCTURES & FUNCTIONS OF G-PROTEIN SUBUNITS

1.6.1 INTRODUCTION

To describe adequately both structures and mechanisms of G-protein action it must be appreciated that G-proteins are heterotrimeric species with subunits designated α, β and γ in order of decreasing molecular weight. Each protein is distinguished by virtue of its α-subunit which confers the abilities to bind GTP and interact with receptor and effector. The β- and γ-subunits bind to the α-subunit but

can dissociate from it as a βγ complex after activation of the G-protein by the appropriate receptor under suitable conditions: β- and γ-subunits are only separated under conditions were they are denatured.

Certain details about G-protein structure have been gleaned from molecular biological approaches, which have resulted in the cloning of several G-protein subunits, and from X-ray crystallographic structures of the bacterial elongation factor EF-Tu (Jurnak, 1985) and more recently the crystal structure of c-Ha-*ras* p21 (deVos *et al.*, 1988; Pai *et al.*, 1990). Therefore we are at a stage where nearly all the G-protein α-subunits purified to date have been cloned, expressed and their primary structures determined. In the following section, the properties of the major G-protein subunits thus far cloned will be described. This will be followed by a discussion of the 'G_{avg}' α-subunit proposed by Bourne and co-workers (Masters *et al.*, 1986), and modified by Johnson and colleagues (Osawa *et al.*, 1990), which attempts to unify these studies to produce a 'predicted' secondary structure containing important structural features common to all members of the G-protein family.

1.6.2 THE MAJOR MAMMALIAN G-PROTEIN ALPHA-SUBUNITS

A The α-subunits Of G_s

The stimulatory guanine nucleotide-binding regulatory protein, G_s, was first identified by its ability to stimulate adenylate cyclase activity. The original tissue source used for the isolation of $G_s$was rabbit liver (Northup *et al.*, 1980) and on purification, two oligomeric proteins were obtained containing differing α-subunits (apparent M_r of 45 and 52 kDa) and indistinguishable β-subunits. A cDNA corresponding to $G_s\alpha$ was isolated initially from a bovine brain cDNA library using an oligonucleotide probe with a nucleotide sequence taken from a region conserved in $G_0\alpha$ and the α-subunit of transducin (Harris *et al.*, 1985). Anti-peptide antisera raised against deduced amino acid sequences taken from the isolated cDNA

nucleotide sequence recognised proteins of the appropriate molecular weight in Western blotting studies, and were identified as $G_s\alpha$-subunits by the failure to find both messenger RNA and protein in the $G_s\alpha$–deficient S49 *cyc*$^-$ murine lymphoma cell line (Harris *et al.*, 1985). Subsequent expression of the cDNA from bovine brain, as well as $G_s\alpha$ cDNA's isolated from bovine adrenals and rat brain, verified these observations (Robishaw *et al.*, 1986a and b; Itoh *et al.*, 1986). Northern and Western blotting studies have shown the protein to be ubiquitous in mammalian tissues.

The original cDNA clone for $G_s\alpha$ encoded a protein of 394 amino acids with a corresponding molecular weight of 46 kDa. However expression in either COS-m6 cells or in *E. coli* under the control of bacterial promoters produces the 52 kDa form, thus suggesting that the molecular weight of this subunit in SDS-PAGE gels is anomalous (Robishaw *et al.*, 1986a and b). The situation is further complicated by the studies of Bray *et al.* (1986) which revealed four different $G_s\alpha$ cDNA's in human brain, one of which corresponded to the original cDNA isolated by Harris *et al.* (1985): these cDNA's have been designated $G_s\alpha$-1 to 4. $G_s\alpha$-1 and $G_s\alpha$-3 are identical except that $G_s\alpha$-3 lacks a stretch of 45 nucleotides. $G_s\alpha$-2 and $G_s\alpha$-4 have 3 additional nucleotides (CAG) to $G_s\alpha$-1 and $G_s\alpha$-3 which are located at the 3' end of the 45 nucleotide stretch.

The isolation and characterisation of the human $G_s\alpha$ gene provided an explanation for the origin of the four messenger RNA species (Kosaza *et al.*, 1988). The human G_s gene is composed of 13 exons and 12 introns which extend approximately 20 kilobases and it maps to chromosome 20 in the human genome (Blatt *et al.*, 1988). Comparing the exon-intron organisation of the gene with the predicted functional domain structure of 'G_{avg}', various exons can be putatively assigned certain functions. Exons 1 and 2 may code for the GTPase activity, whilst exon 9 contains a conserved Asp 223 which may form a salt bridge with the Mg^{2+} ion bound to the β-phosphate of GDP. Therefore, exchange of GDP for GTP may

displace the surrounding residues, causing a GTP-dependent conformational change in the hydrophilic region of residues 230-238 in exon 9. A consensus sequence sequence Asn-Lys-X-Asp is found in exon 11: a similar sequence in EF-Tu was found by X-ray crystallographic analysis to be adjacent to the guanine ring of GDP (Jurnak, 1985).

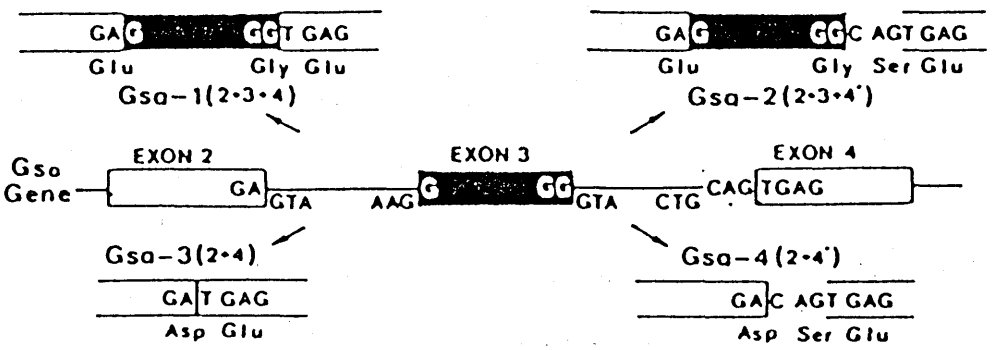
These regions are common amongst all G-protein α-subunits, but exons 4 and 5 encode a region which is heterogeneous amongst G-protein subunits, while exon 3 is unique to $G_s\alpha$. Figure 1.2 shows how the four different $G_s\alpha$ mRNA species are derived from the differential splicing of a single primary RNA transcript, utilizing GT-TG donor/acceptor splice sites as well as the more usual GT-AG sites. These splicing events allow for the potential expression of four $G_s\alpha$-subunits per cell. Two of these ($G_s\alpha$-1 and $G_s\alpha$-2) will contain an extra 14 amino acids from residues 72 to 85 (corresponding to the inclusion of exon 3 from the gene). Also $G_s\alpha$-2 and $G_s\alpha$-4 contain an extra Ser residue compared with $G_s\alpha$-1 and $G_s\alpha$-3 respectively due to splicing events causing the inclusion of the CAG nucleotide sequence in their mRNAs (Kozasa *et al.*, 1988).

In native membranes, relative amounts of the various isoforms of $G_s\alpha$ vary: while some tissues contain only 45 kDa or 52 kDa forms, multiple forms exist in most tissues (Mumby *et al.*, 1986). Studies on partially-purified preparations of $G_s\alpha$ demonstrated that both forms can mediate hormonal stimulation of adenylate cyclase (Sternweis *et al.*, 1981) although some preliminary studies have suggested that there may be quantitative differences in the extent to which both forms can activate the enzyme (Kaslow *et al.*, 1979; Larner & Ross, 1981). Kinetic measurements of GDP release and rates of GTP hydrolysis by recombinant 45 kDa and 52 kDa $G_s\alpha$-subunits purified from *E. coli* expression systems have shown that while both forms have similar k_{cat} (GTPase) values, the larger form releases GDP more rapidly. Hence, under basal conditions, a greater proportion of the larger form is GTP-bound and active with respect to stimulation of adenylate

Figure 1.2:

Origin Of Four $G_s\alpha$-Subunit mRNA Species

The $G_s\alpha$-subunit gene is shown in the centre. Exons 2 and 4 are shown by open boxes while exon 3 is depicted as a solid box. Nucleotide sequences of exon-intron boundaries are shown, as are the consequences of the alternate splicing events on the amino acid sequence. Figure is taken from Kozasa *et al.* (1988).


GA G GG T GAG
Glu Gly Glu
Gsα-1(2·3·4)
GA G GG C AGT GAG
Glu Gly Ser Glu
Gsα-2(2·3·4')
Gso Gene
EXON 2
GA
GTA
AAG
EXON 3
G GG
GTA
CTG
CAG
EXON 4
TGAG
Gsα-3(2·4)
GA T GAG
Asp Glu
Gsα-4(2·4')
GA C AGT GAG
Asp Ser Glu

cyclase in both S49 *cyc*⁻ cells and with the purified enzyme in detergent (Graziano *et al.*, 1989). The demonstration of a similarly increased hormone receptor-dependent rate of GDP dissociation of the larger form of $G_s\alpha$ would also be of considerable interest. However it must be appreciated that in these experiments recombinant $G_s\alpha$ had a much lower affinity (5- to 10-fold) for the adenylate cyclase catalytic unit compared with purified rabbit liver $G_s\alpha$ under the same conditions, and so results with recombinant molecules may not be extrapolated to the situation in the plasma membrane.

B The α-subunits Of G_i

The inhibitory guanine nucleotide-binding regulatory protein, G_i, was first identified as a 41 kDa substrate for ADP-ribosylation catalysed by the islet-activating protein (IAP) from the whooping cough bacterium *Bordetella pertussis*, or pertussis toxin. It was first purified from rabbit liver and human erythrocytes using a very similar protocol to that employed for purification of G_s (Bokoch *et al.*, 1983; Codina *et al.*, 1983). Like G_s, G_i was purified as an oligomer containing an α-subunit distinct from $G_s\alpha$, but apparently identical β-subunits (Manning & Gilman, 1983). The functional attributes of G_i were deduced by reconstituting the purified protein with S49 lymphoma and platelet membranes which resulted in the inhibition of adenylate cyclase activity (Katada *et al.*, 1984a, b and c).

However, the heterogeneity of pertussis toxin substrates observed in several tissues, including brain (Milligan & Klee, 1985) and fat (Rapiejko *et al.*, 1986), suggested that there may be several isoforms of this protein and that this inhibitory function may not be specific for one form of G_i. Indeed, the possibility that different G_i's control other pertussis toxin-sensitive signalling events, such as activation of phospholipases C and A_2, has produced an area of intense research but its discussion is beyond the scope of this thesis.

Nakada *et al.* (1986) isolated a G_i cDNA by purifying a 41 kDa pertussis

toxin substrate from bovine brain and using the sequences of the trypsin-digested peptides to generate oligonucleotide probes. The cDNA isolated contains nucleotide sequences which correspond to all the tryptic peptide sequences, and codes for a protein of 354 amino acids with a calculated molecular weight of 40.4 kDa. This protein has been designated $G_i\alpha$-1, as it was the first G_i protein to be cloned, and corresponding cDNA species have subsequently been isolated from rat C6 glioma cells and human cDNA libraries (Didsbury *et al.*, 1986; Itoh *et al.*, 1986). Immunological studies suggest that $G_i\alpha$-1 is most abundant in brain and less widely distributed in peripheral tissues (Brann *et al.*, 1987). The gene for $G_i\alpha$-1 maps to chromosome 7 of the human genome (Blatt *et al.*, 1988).

The screening of a rat C6 glioma cDNA library with an oligonucleotide probe containing a nucleotide sequence corresponding to an amino acid sequence of "purified" rat brain $G_i\alpha$ led to the isolation of 2 cDNA clones (Itoh *et al.*, 1986). One corresponded to rat $G_i\alpha$-1, which differs in sequence from bovine $G_i\alpha$-1 by 2 amino acid residues. The other clone encoded a 355 amino acid protein which differed significantly (11%) from the $G_i\alpha$-1 clone, and was termed $G_i\alpha$-2. This encodes for a protein of predicted M_r=40.1 kDa. Molecular biological studies showed that the $G_i\alpha$-2 gene maps to chromosome 3 in humans (Blatt *et al.*, 1988). Northern and Western blotting studies have also demonstrated the expression of this protein in several peripheral tissues, including liver, kidney, heart and white adipose tissues (Suki *et al.*, 1987).

cDNA clones for a $G_i\alpha$-3 protein were initially isolated by 2 groups from human HL-60 cell and human liver cDNA libraries (Didsbury & Snyderman, 1987; Suki *et al.*, 1987). $G_i\alpha$-3 cDNA has since been cloned from rat C6 glioma cell, rat olfactory and human T-lymphocyte cDNA libraries (Beals *et al.*, 1987; Jones & Reed, 1987; Itoh *et al.*, 1988). These species code for a protein with a deduced M_r=40.5 KDa. Subsequent Northern and Western blotting analyses have shown the mRNA for this species to be present in many peripheral tissues, including liver,

heart, kidney and white adipose tissues (Didsbury & Snyderman, 1987; Mitchell *et al.*, 1989). The human $G_i\alpha$-3 gene has been mapped to chromosome 1 (Blatt *et al.*, 1988).

Between each of $G_i\alpha$-1 , 2 and 3 there is a very strong conservation of overall structure. There is also a striking conservation of sequence between each G_i subtype (more than 98% homology) between rat, mouse, bovine and human proteins (Itoh *et al.*, 1988). Such a strong conservation of sequence may reflect evolutionary pressure to maintain the specific functioning of each G_i gene product. One interesting point is that the homologies between the three G_i species are higher than that between rod and cone transducins ($G_t\alpha$-1 and $G_t\alpha$-2) (Lerea *et al.*, 1986). Considering that $G_t\alpha$-1 and $G_t\alpha$-2 activate the same effector, whereas the $G_i\alpha$'s probably interact with several different ones depending on the cell-type, the degree of homology is quite remarkable.

As with $G_s\alpha$-subunits, different tissues express particular combinations of $G_i\alpha$-subunits, as determined by Western blotting analysis of various tissues using antisera specific for each of the three $G_i\alpha$-subunits. For example, while rat liver expresses only $G_i\alpha$-2 and $G_i\alpha$-3, white adipose tissue expresses all three $G_i\alpha$-subunits (Suki *et al.*, 1987; Mitchell *et al.*, 1989).

The isolation of genes for the three G_i species was achieved by Itoh *et al.* (1988), initially using a rat $G_i\alpha$-2 probe on a human genomic DNA library under conditions of low stringency. The resulting clones were used for restriction analysis and Southern hybridisation at high and low stringencies, and isolation of the three $G_i\alpha$ genes. Both $G_i\alpha$-2 and 3 consist of eight exons and seven introns. $G_i\alpha$-2 contains an additional exon in the 3' flanking region but this does not code for amino acids. The positions of the exon junctions in the cDNA sequences were found to be identical even though intron lengths were different between each gene.

Comparison between the exon-intron structure of the $G_i\alpha$ genes and the predicted domain structure reveals some interesting relationships. Whereas exons

1, 2, 5, 6 and 7 are fairly conserved between different Gα-subunits, exons 3, 4 and 8 show the greatest structural divergence. In particular, residues 80-130 in exons 3 and 4 show a very significant sequence diversity within the $G_i\alpha$-subunits, implying a role of this region in specifying effector interactions. The heterogeneous C-terminal domain encoded by exon 8, which contains the Cys residue ADP-ribosylated by pertussis toxin, is thought to be involved in mediating G-protein contact with the appropriate receptor (Masters *et al.*, 1986; see later).

C The α-subunits Of Transducin, G_t

G_t, or transducin, was first purified from bovine retina at about the same time as G_s was being purified. It is easily isolated, being the major component of retinal rod outer segment (ROS) disc membranes, and large amounts (mg quantities) can be obtained (Kuhn, 1980). This facilitated the manufacture of polyclonal antisera and determination of enough partial amino acid sequence data to generate probes, allowing isolation of $G_t\alpha$ cDNA seqences (Hurley *et al.*, 1984; Lochrie *et al.*, 1985; Yatsunami *et al.*, 1985). Two cDNA clones ($G_t\alpha$-1 and $G_t\alpha$-2) were isolated which differ by approximately 20% in their respective primary sequences. Whereas $G_t\alpha$-1 cDNA encodes for a protein of 350 residues, $G_t\alpha$-2 codes for 354 amino acids (Yatsunami *et al.*, 1985; Lochrie *et al.*, 1985). Both $G_t\alpha$'s contain Arg 174 and Cys 347 residues which can be ADP-ribosylated by cholera and pertussis toxins respectively (Van Dop *et al.*, 1984a and b). Anti-peptide antisera raised against sequences specific for each $G_t\alpha$ demonstrated that $G_t\alpha$-1 was located exclusively in rod outer cell segments whereas $G_t\alpha$-2 was expressed only in cone cells (Lerea *et al.*, 1986). The two $G_t\alpha$-subunits were found to be the products of two separate genes located on human chromosomes 3 ($G_t\alpha$-1) and 1 ($G_t\alpha$-2) (Blatt *et al.*, 1988). Both subtypes are presumed to activate the cyclic GMP-specific phosphodiesterase effector enzyme of visual signal transduction.

D Other G-Protein Subunits

i The α-subunit Of Go

G_o was first identified by Sternweis & Robishaw (1984) as the predominant pertussis toxin substrate in bovine brain: it was designated "o" for "other" to signify its distinction from G_i. Like G_i , G_s and G_t, it exists as a heterotrimer of α-, β- and γ-subunits. Several lines of biochemical evidence suggested the existence of two forms of $G_o\alpha$. These were confirmed by molecular biological studies leading to cDNA cloning and determination of deduced primary sequences. $G_o\alpha$-subunit cDNA's have been cloned from several mammalian tissues, including rat brain (Itoh *et al.*, 1988), heart (VanDongen *et al.*, 1988), neuroepithelium (Jones & Reed, 1987) and bovine retina (VanMeurs *et al.*, 1987). Tissue expression studies show that $G_o\alpha$-subunits are expressed at relatively high levels in brain and retina compared with heart, but are not expressed in liver (Brann *et al.*, 1987). Bovine $G_o\alpha$ ($G_o\alpha$-1) contains 354 residues, the same number as $G_o\alpha$-2. Their primary sequences are identical up to position 248: thereafter 26 amino acids are different. It has been proposed by Hsu *et al.* (1990) that $G_o\alpha$-1 and 2 arise due to the alternative splicing of a single $G_o\alpha$ transcript. Unlike other G-protein subunits, the gene for $G_o\alpha$ has not yet been mapped to a chromosome in the human genome.

The function of G_o is unknown although it has been proposed by different groups to control distinct processes in certain cell types. These include opiate-induced Ca^{2+} channel opening in neural cell lines (Hescheler *et al.*, 1987), and activation of phospholipase C in oocytes from *Xenopus laevis* (Moriarty *et al.*, 1990). One important finding made recently is that the activity of G_o is regulated by a GAP protein (designated 'GAP-43') which stimulates binding of GTPγS to $G_o\alpha$ (Strittmatter *et al.*, 1990). GAP-43 is intimately involved in neural axonal growth and it is capable of interacting with the N-terminal region of $G_o\alpha$, the region thought to be involved in mediating receptor/G-protein interaction

(Strittmatter *et al.*, 1990; Masters *et al.*, 1986). In particular, a consensus amino acid sequence containing two Cys and two basic residues is found in many G-protein-linked receptors as well as GAP-43 and the wasp venom mastoparan (Strittmatter *et al.*, 1990).

ii The α-subunit Of Golf

Considering the ubiquity of receptor/G-protein/effector mechanisms for mediating intracellular responses from extracellular ligands, it is not surprising to find that many oderants initiate their effects by binding to specific receptors on specialized neuronal olfactory membranes which couple to G-proteins and subsequent second messenger generation (Pace *et al.*, 1986; Sklar *et al.*, 1986). In particular, cyclic AMP is capable of opening the ion channels which initiate the oderant-stimulated nerve impulse in olfactory neurones, suggesting a model where oderant-induced elevation of intracellular cyclic AMP levels precedes the cyclic AMP-dependent depolarization of the sensory neurone (Nakamura & Gold, 1987).

However, as most of the $G_s\alpha$-subunit mRNA in olfactory tissue is derived from non-neuronal cells (Jones *et al.*, 1988), it seemed likely that a distinct G-protein was responsible for the activation of adenylate cyclase in olfactory neurones. Such a protein was identified by the screening of a rat olfactory tissue cDNA library with a degenerate oligonucleotide probe directed towards a conserved region of the G-protein GTP-binding domain (Jones & Reed, 1989). Amongst the clones isolated were some which hybridized weakly with a $G_s\alpha$ probe: the protein encoded by the open reading frame of these clones was termed $G_{olf}\alpha$ (Jones & Reed, 1989). Although the deduced amino acid sequence of the clones consisted of 381 amino acids which shared a significant identity (88%) with $G_s\alpha$, one important difference was the absence of the 45-base exon whose alternative splicing results in the different molecular weight forms of $G_s\alpha$ observed in many tissues (Bray *et al.*, 1986). Northern blot analysis showed that $G_{olf}\alpha$-subunit mRNA species of 3.5

and 2.7 kbases in size were expressed exclusively in olfactory epithelia, specifically in sensory neurones as determined by immunocytochemical experiments using a polyclonal antibody raised against a unique $G_{olf}\alpha$-subunit sequence (Jones & Reed, 1989). Expression of the $G_{olf}\alpha$-subunit clone in S49 *cyc⁻ kin⁻* cells demonstrated that activation of adenylate cyclase could be achieved by expression of the cloned protein (Jones & Reed, 1989).

iii The α-subunit Of Gz

A cDNA clone for the α-subunit of G_z was initially isolated by screening a bovine retinal cDNA library with bovine rod $G_t\alpha$ cDNA under conditions of low stringency (Fong *et al.*, 1988). An identical clone was isolated from a human genomic library using cDNA clones obtained from a rat C6 glioma cell cDNA library. Subsequent screening of a rat brain cDNA library with the human $G_z\alpha$ genomic clone isolated a rat $G_z\alpha$ clone of similar sequence (Matsuoka *et al.*, 1988). The deduced amino acid sequence consisted of 355 amino acids (M_r=40.8 kDa) exhibiting a particularly strong homology with the $G_i\alpha$ proteins (66-67% identity in sequence with $G_i\alpha$–1, $G_i\alpha$–2 and $G_i\alpha$–3) although not strong enough to be part of that separate subfamily ($G_i\alpha$-subunits exhibit 85–94% identity between each other). Northern and Western blotting analyses have shown that $G_z\alpha$ is expressed in retina, brain, adrenal gland, kidney and liver, but not testis, lung or spleen (Fong *et al.*, 1988). The gene for $G_z\alpha$ maps to human chromosome 22 (Blatt *et al.*, 1988).

A particularly interesting deviation from classical $G_i\alpha$ characteristics is the absence of a Cys residue 4 residues from the C-terminus which is the site for ADP-ribosylation by pertussis toxin on $G_i\alpha$- and $G_o\alpha$-subunits.

The purification of several 40 kDa pertussis toxin-insensitive G-protein α-subunits from bovine brain (Pang & Sternweis, 1990) and the molecular cloning of additional G-protein subunits using PCR (Strathmann *et al.*, 1990) suggests that a family of such proteins exist which may either be responsible for pertussis toxin-

insensitive signalling events or which may have other functions.

1.6.3 ASSIGNMENT OF FUNCTIONAL DOMAINS ON G-PROTEIN ALPHA SUBUNITS

The absence of a crystal structure for any of the mammalian Gα-subunits prompted Bourne and co-workers to predict the secondary structure of a composite G-protein α-subunit ("α_{avg}") from the deduced amino acid sequences of $G_i\alpha$-1, $G_t\alpha$-1, $G_t\alpha$-2, $G_o\alpha$ and $G_s\alpha$ as well as the crystal structure of bacterial elongation factor EF-Tu (Masters *et al.*, 1986). The model defines three putative functional domains surrounding a hydrophobic guanine nucleotide-binding domain (Figure 1.3). The evidence assigning these domains with specific functions will now be discussed.

A Guanine Nucleotide-Binding Domain

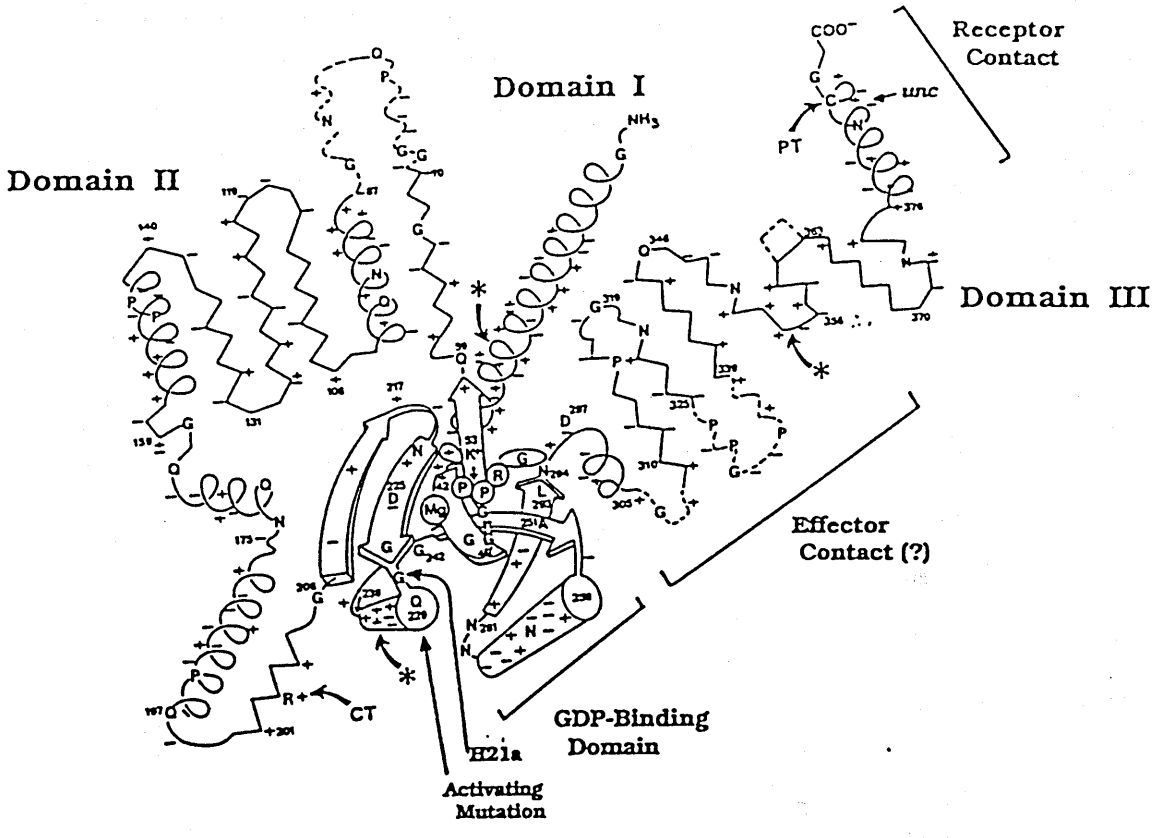
Four short stretches of amino acid sequence which are conserved amongst all G-protein α-subunits, EF-Tu and $p21^{ras}$ are presumed to form the guanine nucleotide-binding domain. The crystal structures of EF-Tu (Jurnak, 1985) and GTP-bound $p21^{ras}$ (Pai *et al.*, 1990) show these sequences to be located at turns between an α-helix and a strand of β-pleated sheet and have been designated A, C, E and G, after the assignments of $p21^{ras}$ and EF-Tu by Halliday (1984).

Region A, corresponding to residues 47-53 of α_{avg}, contains a Gly-4X-Lys consensus sequence. In EF-Tu, these residues comprise a loop which is adjacent to the α- and β-phosphoryl groups of bound GDP (Jurnak, 1985). The importance of this region in guanine nucleotide binding is further suggested by two observations. Firstly, mutation of Gly 12 in $p21^{ras}$ (corresponding to Gly 49 in α_{avg}) to any other amino acid produces a molecule with reduced GTPase activity and an

Figure 1.3:

Diagrammatical Representation Of A G-Protein Alpha-Subunit ('α_{avg}')

This figure is taken from Bourne *et al.* (1988) and represents a composite G-protein α-subunit, α_{avg}. The GDP-binding domain is adapted from that of EF-Tu (Jurnak, 1985) and the GDP molecule (G=guanine ring, R=ribose, P=phosphoryl) nestles in a pocket formed by turns joining β-pleated sheets and α-helices. The rest of the molecule depicts a predicted secondary structure which is described in the text. Residues conserved amongst different α-subunits are represented by the corresponding single letter amino acid code, and numbers refer to their positions in α_{avg}. Asterisks represent locations of tryptic cleavage sites. Sites of α_s mutations and ADP-ribosylation sites are also indicated.


Domain II
Domain I
Domain III
Receptor Contact
Effector Contact (?)
GDP-Binding Domain
Activating Mutation
H21a
CT
PT
unc

enhanced transforming capacity (Bishop & Varmus, 1985). Secondly, replacement of the Lys residue in $p21^{ras}$ reduces the protein's affinity for GTP and GDP without altering the specificity of nucleotide binding (Sigal *et al.*, 1986).

Region C, corresponding to residues 225-228 in α_{avg}, has the signature sequence Asp-2X-Gly. In EF-Tu, the Gly begins a turn away from the bound GDP whilst the Asp group forms a salt bridge with the Mg^{2+} ion co-ordinated with the β-phosphoryl group of GDP (Jurnak, 1985). Presumably replacement of GDP with GTP causes a conformational change which may result in the displacement of C and the predicted adjacent α-helix (residues 229-238 in α_{avg}) on the surface of the molecule.

Region E is a hydrophobic region which forms a partial pocket for the guanine ring of GDP in EF-Tu, and which is highly conserved within G-protein α-subunits.

Region G forms a turn at the end of a β-strand which fixes the position of the guanine ring of GDP in EF-Tu. Asn 294 in α_{avg} may interact with the keto substituent of the ring while the next Asp residue (Asp 297 in α_{avg}) binds the 2-amino group (Jurnak, 1985).

B Domain I (residues 1-41)

The hydrophilic portion of α_{avg} has the predicted structure of an amphipathic α-helix, and several lines of evidence implicate its involvement in controlling binding to βγ-subunits. Tryptic cleavage of the amino-terminal 18 residues of $G_t\alpha$-1 (corresponding to residues 1-25 of α_{avg}) abolishes its ability to bind to photolysed rhodopsin and βγ-subunits (Fung & Nash, 1983). Subsequent work has shown this effect to be due exclusively to the reduced ability of $G_t\alpha$-1 to bind to βγ, which results in the abolition of the ability of pertussis toxin to catalyse the ADP-ribosylation of Cys 346 near the C-terminus of the molecule (Navon & Fung, 1987). Similar results were obtained by Neer *et al.* (1988) using 2 pertussis

toxin substrates purified from bovine brain, α_{41} and α_{39} ($G_i\alpha$-1 and $G_o\alpha$). In addition, they showed that trypsin treatment had no effect on the intrinsic GTPase activity of the α-subunits. Construction of $G_i\alpha$-2/$G_s\alpha$-subunit chimeras containing N-terminal regions from $G_i\alpha$-2 attached to a $G_s\alpha$-subunit has also demonstrated that the N-terminal region is involved in $\beta\gamma$ interactions and GDP dissociation independently from GTPase and effector activation (Osawa *et al.*, 1990).

Presumably the charged portion of the amphipathic helix in this region controls $\beta\gamma$ binding, and so we might expect a large degree of homology between the G-protein α-subunits: that this is not the case is therefore surprising. However, it is important to note that EF-Tu and $p21^{ras}$ do not contain residues corresponding to amino acids 1-41 of α_{avg} and neither binds $\beta\gamma$.

Another potentially important function of this domain may be to attach the relatively hydrophilic α-subunit to the plasma membrane after dissociation from $\beta\gamma$. In particular, $G_i\alpha$-1 to 3, $G_o\alpha$, $G_z\alpha$, $G_t\alpha$ but not $G_s\alpha$ have been shown to be N-myristylated on the Gly residue at their amino termini in a human astrocytoma cell line and when transiently expressed in COS-7 cells (Buss *et al.*, 1987; Mumby *et al.*, 1990): site-directed mutagenesis of these α-subunits or substitution of myristate with a less hydrophobic analogue affects α-subunit subcellular distribution within the cell. Successful reconstitution of the myristylated protein into phospholipid vesicles has an absolute requirement for $\beta\gamma$-subunits, so this modification may promote the attachment of α-subunit to $\beta\gamma$, consistent with the predicted function of this domain (Jones *et al.*, 1990).

C Domain II (residues 60-208)

Different α-subunits can interact with $\beta\gamma$-complexes from various G-protein sources, but their interactions with effector systems are rather more specific. Therefore any domain in α_{avg} involved in contact with an effector enzyme should be of variable sequence and should be involved in contact which is regulated in a guanine nucleotide-dependent manner. For α_{avg}, the amphipathic β-pleated sheets

and α-helices between regions A and C of the guanine nucleotide binding site may fit these criteria. Between EF-Tu, $p21^{ras}$ and the G-protein α-subunits, this region varies considerably in length, being as small as 18 amino acids ($p21^{ras}$) up to the 108 amino acids calculated for α_{avg}. In EF-Tu this region participates in the GTP-dependent binding of amino acyl tRNA, while in $p21^{ras}$ this region has been identified as the binding site of "GTPase activating protein" (GAP) which regulates the ability of $p21^{ras}$ to hydrolyse GTP and which may be its effector (McCormick, 1989).

Within this domain, residues 128-156 of α_{avg} exhibit the greatest variability and may conceivably account for the specificity of α-subunit/effector interactions, although the basic secondary structure is probably conserved. This secondary structure is most likely to consist of amphipathic helices located on the surface of the protein with effector specificity probably depending on unique distributions of charges within the helices (Masters *et al.*, 1986).

Residues 200-220 of α_{avg} comprise a region of β-pleated sheets whose sequence is highly conserved amongst the α-subunits. Such conservation, and the likelihood of GTP-dependent changes in conformation in this region, has raised the possibility that this region may be an alternative βγ contact site to domain I, although this hypothesis lacks any direct evidence. This region also contains the Arg residue (Arg 203 in α_{avg}) which is ADP-ribosylated by cholera toxin in $G_s\alpha$, $G_t\alpha$-1 and 2 and, under suitable conditions, the $G_i\alpha$-subunits (Owens *et al.*, 1985). This modification results in the dissociation of GTP-bound α-subunit from βγ and the persistent activation of the α-subunit due to the abolition of its GTPase activity.

1.3.4. **Domain III (residues 298-396)**

Domain III lies between region G of the guanine nucleotide-binding site and the C-terminus of α_{avg}, and has a predicted secondary structure consisting largely

of β-pleated sheets attached to a C-terminal α-helix. Several lines of evidence have suggested an involvement of this region in coupling the G-protein to its receptor. Firstly, ADP-ribosylation of $G_t\alpha$-1, $G_t\alpha$-2, and the $G_i\alpha$-subunits by pertussis toxin on a Cys residue which is 4 amino acids away from the C-terminus (Cys 392 in α_{avg}) prevents interaction with photolysed rhodopsin and inhibitory receptors respectively (Van Dop *et al.*, 1984a; Ui *et al.*, 1984). One possible explanation for this effect is that the incorporated ADP-ribose group sterically hinders interaction of this domain with the receptor. Other evidence has come from molecular biological studies on mutant $G_s\alpha$-subunits expressed in S49 lymphoma cells. Adenylate cyclase activity in S49 *unc* mutant cells responds normally to ligands acting on $G_s\alpha$ directly (Gpp[NH]p or AlF_4^- for example) but is incapable of responding to stimulatory receptor agonists such as those acting at β-adrenergic and prostaglandin E_1 receptors. This is associated with the abolition of the ability of guanine nucleotides to reduce agonist binding to β-adrenergic receptors (Haga *et al.*, 1977; Bourne *et al.*, 1982). Preparation of cDNA libraries from wild type and *unc* mutant S49 cells, and the use of a restriction fragment from murine $G_s\alpha$ cDNA as a probe, led to the isolation of cDNA sequences encoding approximately 70% of the $G_s\alpha$ open reading frames from both cell types. While the wild type nucleotide sequences gave a deduced amino acid sequence identical to that of normal murine $G_s\alpha$, $G_s\alpha$ cDNA from the *unc* cells had a single base mutation resulting in the replacement of an Arg residue with a Pro at residue 372, 6 residues from the C-terminus. Construction of a complete recombinant $G_s\alpha_{unc}$ cDNA containing the Pro mutation and expression of the mutant in S49 *cyc*$^-$ cells reproduced all the characteristics of the *unc* phenotype with respect to its regulation of adenylate cyclase (Sullivan *et al.*, 1987). Presumably substitution of Arg 372 for a Pro residue in the predicted C-terminal α-helix will introduce a kink in the helix as well as alter the distribution of charge in this region, hence preventing interaction with receptor.

Another study by the same group examined the properties of a $G_s\alpha/G_i\alpha$-2 chimeric α-subunit expressed in S49 *cyc*$^-$ cells (Masters *et al.*, 1988). This chimera contained the N-terminal 212 amino acids of $G_i\alpha$-2 (*i.e.* domains I and II) attached to the 160 C-terminal residues of murine $G_s\alpha$ (*i.e.* domain III). One surprising but important finding from this study was that the chimeric G-protein was capable of stimulating adenylate cyclase even though it contained the presumed effector binding site of $G_i\alpha$-2. Therefore it is possible that structural features of domain III may be involved in regulating effector interactions, although which particular sequences are important within the 160 amino acids of $G_s\alpha$ in the chimera are unknown. However it must be noted that swapping domain III of $G_i\alpha$-2 for the corresponding region of $G_s\alpha$ could impair the functioning of the rest of the molecule and lead to erroneous assignment of function to a given domain. Another important finding from this work was that stimulation of adenylate cyclase activity by this chimera was similar to that mediated by wild type $G_s\alpha$ and it was concluded that the ability of $G_s\alpha$ to interact with receptor resided within the C-terminal 160 amino acids of $G_s\alpha$ in the chimera.

Indeed the abilities of the β-adrenergic receptor and rhodopsin to discriminate between different G-protein α-subunits *in vitro* also seems related to differences in the latter's C-termini. Activated rhodopsin is capable of stimulating the GTPase activity of $G_t\alpha-1$ and 2 as well as $G_o\alpha$ and $G_i\alpha$ but not $G_s\alpha$. Similarly, the β-adrenergic receptor can stimulate the GTPase activity of $G_s\alpha$ more efficiently than any other G-protein, consistent with the C-terminal region of $G_s\alpha$ being distinct from those of $G_i\alpha$, $G_o\alpha$ and $G_t\alpha$ (Cerione *et al.*, 1985; Asano *et al.*, 1984). Also in support of the assignment of this domain in mediating interaction with receptors has been the abilities of specific antisera raised against C-terminal peptides of G-protein α-subunits to block hormone-stimulated events. For example, an antiserum raised against the C-terminal decapeptide sequence of $G_s\alpha$ reduces isoproterenol stimulation of adenylate cyclase activity in turkey erythrocytes without disrupting the ability of the antiserum to immunoprecipitate $G_s\alpha$.C

complexes (Simonds *et al.*, 1989a). Similarly, antibodies raised against the C-terminal decapeptide of $G_i\alpha$-2 attenuate receptor-dependent inhibition of adenylate cyclase activity in human platelet membranes (Simonds *et al.*, 1989b).

However, it is important to stress that there may be at least one more site of receptor/G-protein contact other than the C-terminus. This has been demonstrated by studies on purified α_2-adrenergic receptors reconstituted with $G_i\alpha$, $G_o\alpha$ and $G_t\alpha$, where agonist-stimulated GTP hydrolysis was not observed for $G_t\alpha$ even though the C-terminal regions of these three proteins are virtually identical (Cerione *et al.*, 1986).

1.6.4 BETA-SUBUNITS

On purification of G_s, G_i and G_t, similar 35 kDa proteins were found to be associated with the more distinctive α-subunits. Peptide maps of these β-subunits from G_s, G_i and G_t yielded identical patterns, and the use of several anti-transducin antisera in Western blotting studies could not discriminate between β-subunits of different G-proteins (Manning & Gilman, 1983; Hildebrandt *et al.*, 1985; Gierschik *et al.*, 1985). They are associated with smaller γ-subunits *in vivo* and these $\beta\gamma$-complexes appear to be functionally interchangeable; for example, $\beta\gamma$-subunits from G_i can interact with G_t to reconstitute rhodopsin-stimulated GTPase activity (Kanaho *et al.*, 1984). However, evidence is now accumulating which suggests that β-subunits are structurally heterogeneous proteins. Although the β-subunit of G_t migrates as a single 36 kDa band on SDS-PAGE gels, the subunits for other G-proteins run as a 36/35 kDa (β_1/β_2) doublet under suitable electrophoretic conditions (Sternweis & Robishaw, 1984). Moreover, antisera raised against a synthetic peptide with an amino acid sequence corresponding to a region of $G_t\beta$ recognizes only β_1 (Evans *et al.*, 1986). A cDNA clone for $G_t\beta$ was isolated from a bovine retinal cDNA library by Sugimoto *et al.* (1985) and was found to code for a protein of 340 amino acids (M_r=37.4 kDa). Northern

hybridization experiments with the isolated cDNA recognized 2 bands of 1.8 and 3.3 kbases in RNA from brain, liver and retinal tissues, suggesting multiple forms (Tagahashi *et al.*, 1985). Another cDNA was subsequently isolated from bovine brain, bovine adrenal and human myeloid leukemia (HL-60) cell cDNA libraries (Fong *et al.*, 1987). This cDNA also codes for a protein of 340 residues (M_r=37,329) which is 90% identical in predicted amino acid sequence with the protein encoded by human and bovine β_1 cDNAs. The issue has been further complicated by the cloning of a β_3 cDNA sequence from a retinal cDNA library by Levine *et al.* (1990). Screening of bovine and human retinal cDNA libraries with a probe corresponding to residues 61-75 of the amino acid sequence of bovine β_1 resulted in the isolation of the distinct β_3 cDNA. As for β_1 and β_2, the β_3 cDNA codes for a protein of of 340 amino acids (M_r=37.2 kDa) whose size on electrophoretic gels is unknown as the cDNA has not yet been expressed. Northern blotting of human retinal RNA with a probe corresponding to a unique region of the 3' untranslated region of human β_3 cDNA recognised 2 bands, suggesting that there are two β_3 mRNA species arising from alternative splicing of a single gene product (Levine *et al.*, 1990). Data from several laboratories have shown that these subunits are derived from 3 non-allelic genes located on human chromosomes 1 (β_1), 7 (β_2) and 12 (β_3) (Blatt *et al.*, 1988; Levine *et al.*, 1990).

Deduced amino acid sequences of human β_1- and β_2-subunits differ only in 34 (10%) out of 340 residues, with these differences representing conservative changes in the N-terminal half of the molecule. In contrast, human β_3 differs from β_1 by 17% and from β_2 by 19%, with both conservative and divergent changes throughout the sequence (Levine *et al.*, 1990). The relationship between sequence divergence and functional interactions with α- and γ-subunits, as well as hormone receptors, has yet to be elucidated.

1.6.5 GAMMA-SUBUNITS

γ-subunits remain tightly associated with β-subunits throughout G-protein purification, and they can only be separated from one another by denaturation. The γ-subunit of G_t was recognized as a 5 kDa band on SDS-PAGE gels early in its purification (Fung *et al.*, 1981) whereas its association with G_i and G_s was delayed because of its poor staining (Hildebrandt *et al.*, 1984). The primary sequence of $G_t\gamma$'s have been deduced from both cDNA isolation and protein chemistry (Yatsunami *et al.*, 1985; Ovchinnikov *et al.*, 1985). $G_t\gamma$ has 74 amino acid residues (M_r=8.4 kDa) and is very hydrophobic although it contains a large proportion of acidic residues. Two-dimensional peptide mapping of bovine and frog $G_t\gamma$ produces similar patterns, but different from the pattern observed with human erythrocyte $G_s\gamma$ and bovine brain $G_i\gamma$, suggesting that $G_t\gamma$ is an isoform distinct from that of other G-proteins (Hildebrandt *et al.*, 1984). A γ-subunit cDNA has been cloned from a bovine adrenal cDNA library which codes for a 71 amino acid peptide of estimated M_r=7.85 kDa (Robishaw *et al.*, 1989). This was shown to be distinct from the γ-subunits present in brain and retina (Robishaw *et al.*, 1989). The nature of γ-subunit diversity has been further complicated by the recent isolation of cDNA's encoding several more distinct γ-subunits (Gautam *et al.*, 1990).

An important observation made recently is that the γ-subunit of G_t can alter the ability of the α-subunit to interact with activated rhodopsin. Fukada *et al.* (1990) were able to separate bovine retinal $G_t\beta\gamma$-subunits into $G_t\beta\gamma$-1 and $G_t\beta\gamma$-2 components. GDP-bound $G_t\alpha$-subunits have a higher affinity for the latter and, moreover, $G_t\beta\gamma$-2 is capable of enhancing by approximately 30-fold the metarhodopsin II-stimulated binding of GTP to $G_t\alpha$ compared with $G_t\beta\gamma$-1. This is associated with the presence of a farnesyl (polyisoprenyl) group and a methyl-ester at the C-terminal Cys residue of $G_t\gamma$-2; these post-translational modifications are absent from $G_t\gamma$-1 (Fukada *et al.*, 1990). Unlike the proposed function of the similar modification in $p21^{ras}$ proteins (Hancock *et al.*, 1989), the farnesylation

event does not seem to be involved in promoting membrane attachment as $G_t\beta\gamma$-2 is easily extracted in soluble form. Such a polyisoprenylation event has recently been shown to occur on the bovine adrenal G-protein γ-subunit after expression of the appropriate cDNA in COS cells and after translation *in vitro* with a rabbit reticulocyte lysate (Maltese & Robishaw, 1990).

The potential importance of these modifications on γ-subunits from other mammalian G-proteins has yet to be determined, although it has been observed that a similar polyisoprenylation event on STE18, the γ-subunit equivalent of a yeast G-protein involved in the pheromone-mating response (G_m), is essential for membrane association and proper functioning (Finegold *et al.*, 1990).

1.7 G-PROTEIN-LINKED RECEPTORS

1.7.1 INTRODUCTION

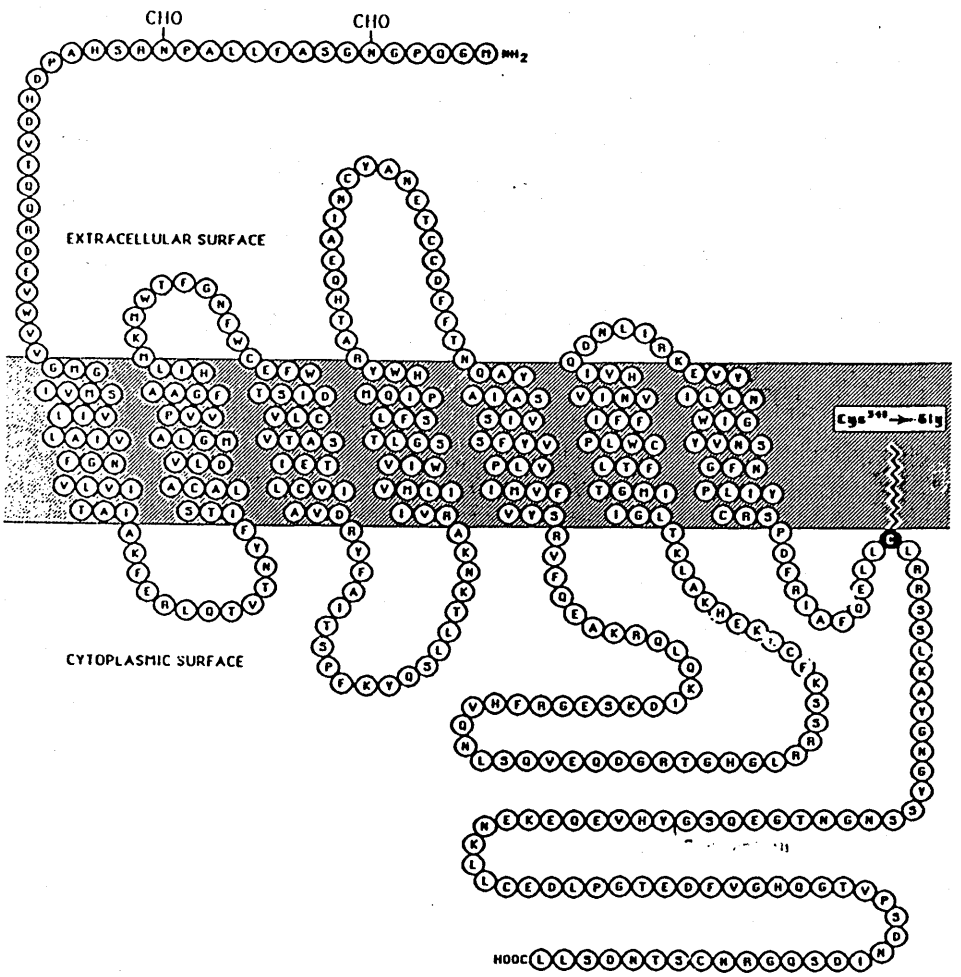
G-protein-linked receptors encompass the largest group of hormone and drug receptors known. This receptor family includes those for amines (catecholamines, acetylcholine, serotonins), small peptides (vasopressin, oxytocin, tachykinins), proteins (glucagon, luteinizing hormone) and the 'light receptor' rhodopsin. It is highly likely that this family will expand to include other sensory receptors.

Based on the purification to apparent homogeneity of the adrenergic receptor subtypes, limited protein sequences were obtained which allowed the synthesis of oligonucleotide probes for cloning studies. As a result, the last 5 years has seen the elucidation of the primary structures of several members of this receptor family by recombinant DNA technology (Figure 1.4). Among these are included some of the adrenergic (α_1, α_2, β_1, β_2 and β_3), muscarinic acetylcholine (m_1 to m_5), serotonin (5-HT) and tachykinin receptor subtypes. Deduced amino acid sequences have shown that these receptors are homologous proteins, exhibiting variability within a

Figure 1.4:

Diagrammatical Representation Of A G-Protein-Linked Receptor

The figure shows the amino acid sequence and predicted membrane-spanning topography of the human β_2-adrenergic receptor and is taken from O'Dowd *et al.* (1989). Potential sites of N-linked glycosylation are shown (CHO) as is Cys341, which may be palmitoylated *in vivo*.


CHO
CHO
EXTRACELLULAR SURFACE
CYTOPLASMIC SURFACE
Cys³⁴⁰ → Gly
HOOC

common structural framework (Lefkowitz *et al.*, 1989).

1.7.2 HORMONE-RECEPTOR TOPOGRAPHY &STRUCTURE

A Membrane Spanning Regions

All the G-protein-linked receptors thus far cloned share a conserved structure and presumed membrane topography. The major feature of their deduced amino acid sequences are the 7 hydrophobic segments predicted by hydropathy analysis, each containing 24-28 amino acids, which are long enough to span the plasma membrane as a series of α-helices. These presumed membrane-spanning regions are connected by a series of extracellular and cytoplasmic loops, of which the third cytoplasmic loop varies most between different receptor types (O'Dowd *et al.*, 1989). Between all the G-protein-linked receptors thus far cloned, conservation of sequence is greatest in the membrane spanning regions (40-50% between members of the adrenergic receptor subfamily; 20-30% between adrenergic and muscarinic receptors).

It is important to note, however, that the predicted membrane spanning topography has not yet been unequivocally proven for any of these receptors. Its existence rests on the 7-helical structure obtained from the high resolution electron diffraction pattern for the bacteriorhodopsin molecule in the purple membrane of *Halobacterium halobium* (Henderson & Unwin, 1975). A similar structure has been proposed for mammalian rhodopsin on the basis of circular dichroism, infra red circular dichroism and neutron diffraction studies (Applebury & Hargrave, 1987). A similar structure for the adrenergic receptors has also been proposed but on the basis of limited proteolysis studies (Dohlman *et al.*, 1987).

In bacteriorhodopsin, three of the helices seem to be tilted, suggesting a structural requirement for the interlocking of amino acid side chains from adjacent helices. Results with rhodopsin, which could be extrapolated to other hormone

receptors, have shown that there are Pro residues in some helices which could induce kinks of the sort observed for the bacteriorhodopsin molecule (Michel-Villaz *et al.*, 1979).

B Ligand Binding Sites

Comparing the transmembrane sequences of the muscarinic and adrenergic receptors, the majority of amino acids found exclusively in one or other of the receptor types are located on the side of the helices closest to the extracellular surface (67% for adrenergic receptors and 64% for muscarinic receptors) (O'Dowd *et al.*, 1989). It has been proposed that this may reflect the unique ligand binding characteristics of these receptor types.

Presumably the cluster of α-helices within the membrane form a pocket within which a ligand binding site exists, as is the case for rhodopsin where the retinal chromophore is buried and surrounded by the seven helices (Stryer, 1986). Mutagenesis studies have demonstrated that highly conserved Asp residues at positions 79, 113 and 130 of the human β-adrenergic receptor are essential for normal agonist binding (Strader *et al.*, 1988). In particular, residues 113 and 130 may be important in forming a negative counterion for the protonated amino group of the catecholamine moiety (Strader *et al.*, 1988).

Hydrophilic cytoplasmic and extracellular regions appear, in general, not to affect this function, although the second extracellular loop of the β-adrenergic receptor contains several highly conserved Cys residues which may be important in disulphide bridge formation. Site-directed mutagenesis studies have demonstrated that deletion of these residues causes the loss of ligand binding (Dixon *et al.*, 1987; Lefkowitz *et al*, 1989).

The construction of α_2/β_2 chimeric receptors has also provided information on the nature of ligand binding. If one constructs β_2-adrenergic receptor chimeras containing progressively larger proportions of α_2-adrenergic receptor spreading from the N-terminus and expresses the chimeras in *Xenopus* oocytes, or

transiently in COS-7 cells, progressive changes in agonist binding affinity occur. Binding patterns intermediate between α_2 and β_2 characteristics are observed, and binding affinities for all agonists are reduced, suggesting an inability to form a stable ligand binding site structure (Kobilka *et al.*, 1988). However, with respect to antagonist binding, one can construct a chimera which consists of the appropriate α_2-adrenergic receptor sequence attached to the fifth, sixth, and seventh membrane-spanning and C-terminal regions of the β_2-adrenergic receptor, and still retain β_2-adrenergic receptor antagonist binding characteristics. Conversion of the seventh membrane-spanning region to an α_2-adrenergic sequence restores α_2-antagonist binding properties, confirming the importance of this region in this process (Kobilka *et al.*, 1988).

Localization of specific residues within the membrane-spanning regions with probable importance in ligand binding agrees with data obtained from a biochemical approach using affinity and photoaffinity ligands (Dohlman *et al.*, 1988; Wong *et al.*, 1988).

C G-Protein-Coupling Region

The major function ascribed to the cytoplasmic domain of the adrenergic and related receptors is the agonist-dependent coupling with a specific G-protein. Specific cytoplasmic regions have been implicated by biochemical and site-directed mutagenesis studies as well as by expression of chimeric receptors in *Xenopus* oocytes or transient expression in COS-7 cells.

Whereas amino acid substitutions in the first and second cytoplasmic loops have no effect on coupling with effector (O'Dowd *et al.*, 1989), the third cytoplasmic loop and the C-terminus have been directly implicated for both rhodopsin and the human β_2-adrenergic receptor (Takemoto *et al.*, 1985; Strader *et al.*, 1987). Several studies have shown that either deletion or substitution of amino acids in the C-terminal tail causes a marked reduction in agonist-stimulated

adenylate cyclase activity (O'Dowd *et al.*, 1989). In particular, Strader and collegues demonstrated that deletions in either the amino or carboxy ends of of the third cytoplasmic loop uncouple the hamster β_2-adrenergic receptor from cyclase stimulation (Strader *et al.*, 1987). Residues in the amino terminal end of of the C-terminal tail are also important in this coupling process (O'Dowd *et al.*, 1989). The assignment of this domain in G-protein coupling has been challenged by Rubenstein *et al.* (1987) examining the interaction of G_s with partially digested turkey erythrocyte β_2-adrenergic receptor in phospholipid vesicles. Nevertheless, evidence accumulated from the construction and expression of chimeric receptors implicating the third cytoplasmic loop and the C-terminal tail is convincing. For example, a hybrid receptor containing the fifth and sixth transmembrane regions with the interconnecting 3rd cytoplasmic loop of the β_2-adrenergic receptor within an α_2-adrenergic receptor exhibits α_2 binding characteristics yet activates cyclase with classical α_2-adrenergic specificity. This implies that swapping of the third cytoplasmic loop connecting transmembrane helices 5 and 6 confers the ability to interact with G_s and stimulate cyclase (Kobilka *et al.*, 1988). This region is highly conserved amongst receptors which couple to the same G-protein, although more work is required to determine the generality of these sequences and the conformational requirements for G-protein interaction within them.

It has also been shown that the third cytoplasmic loop contains amino acid sequences containing Cys residues and basic residues which are conserved amongst many different receptor subtypes as well as mastoparan, a wasp venom which activates G-proteins (Strittmatter *et al.*, 1990; Higashijima *et al.*, 1988). One of these Cys residues must be palmitylated for proper functioning of the β-adrenergic receptor (O'Dowd *et al.*, 1989) and such a modification may be essential for the functioning of other receptors as well as GAP-43, which is a peripheral membrane protein capable of interacting with G_o (Strittmatter *et al.*, 1990). Indeed, a acylation/deacylation cycle could potentially be an important mechanism for

regulating receptor functioning.

1.8 THE CATALYTIC UNIT OF ADENYLATE CYCLASE

1.8.1 PURIFICATION

Of the five components involved in the dual regulation of hormone-stimulated generation of cyclic AMP at the plasma membrane, least is known about the catalytic unit. Biochemical evidence suggests the existence of several isoforms which have molecular weights between 150 and 180 kDa (Pfeuffer *et al.*, 1985; Mollner & Pfeuffer, 1988). Purification of adenylate cyclase to homogeneity from bovine brain using a forskolin-agarose affinity step produces a 120 kDa glycoprotein (Pfeuffer *et al.*, 1985). Polyclonal antisera raised against this glycoprotein recognized a protein of the appropriate molecular weight in solubilized brain membranes and was able to precipitate adenylate cyclase activity from detergent solutions containing the partially-purified enzyme (Mollner & Pfeuffer, 1988). This 120 kDa form of adenylate cyclase binds tightly to calmodulin, a property which has been used to further purify the protein after the forskolin-affinity stage (Minocherhomjee *et al.*, 1987). Indeed, the activity of this isoform of adenylate cyclase can be stimulated on binding Ca^{2+}-calmodulin (Katada *et al.*, 1987). $\beta\gamma$-subunits can competitively inhibit the enzyme by binding to the Ca^{2+}-calmodulin complex, thereby preventing its interaction with and activation of the catalytic unit (Katada *et al.*, 1987). The addition of purified, activated G_s further enhances activation by Ca^{2+}-calmodulin (Harrison *et al.*, 1989).

1.8.2 PRIMARY STRUCTURE

Isolation of cDNA sequences encoding the primary sequence of the calmodulin-sensitive form of adenylate cyclase was achieved by screening a bovine brain cDNA library using a probe constructed from the amino acid sequence of a peptide obtained after exhaustive tryptic digestion of adenylate cyclase purified from

bovine brain (Krupinski *et al.*, 1989). When one of the isolated clones was used as a probe in Northern blotting analysis of bovine brain mRNA at high stringency, a single band of 11.5 kbases in size was visualized. The deduced amino acid sequence of the isolated cDNA's encoded a molecule with an estimated molecular weight of 124 kDa containing several potential glycosylation sites. Hydropathy analysis showed the protein to consist of two large cytoplasmic domains attached to the plasma membrane by two sets of six transmembrane α-helices. The cytoplasmic domains contained regions of similarity with each other as well as with sequences from soluble and membrane-bound guanylate cyclases (Krupinski *et al.*, 1989). Moreover, the similarity between the proposed structure and topography of adenylate cyclase with other G-protein-linked effectors, such as dihydropyridine-sensitive Ca^{2+} channels and K^+ channels, suggested that such proteins have similar motifs within which limited variability produces different functions (Krupinski *et al.*, 1989).

Putative Ser residues have been identified which may be substrates for the observed PKC-catalysed phosphorylation and sensitization of adenylate cyclase observed in frog erythrocytes (Krupinski *et al.*, 1989; Yoshimasa *et al.*, 1987). The regions of the protein involved in the interactions with G_s, G_i, calmodulin and the region involved in forming the inhibitory P-site have yet to be assigned.

1.9 DUAL REGULATION OF ADENYLATE CYCLASE

1.9.1 INTRODUCTION

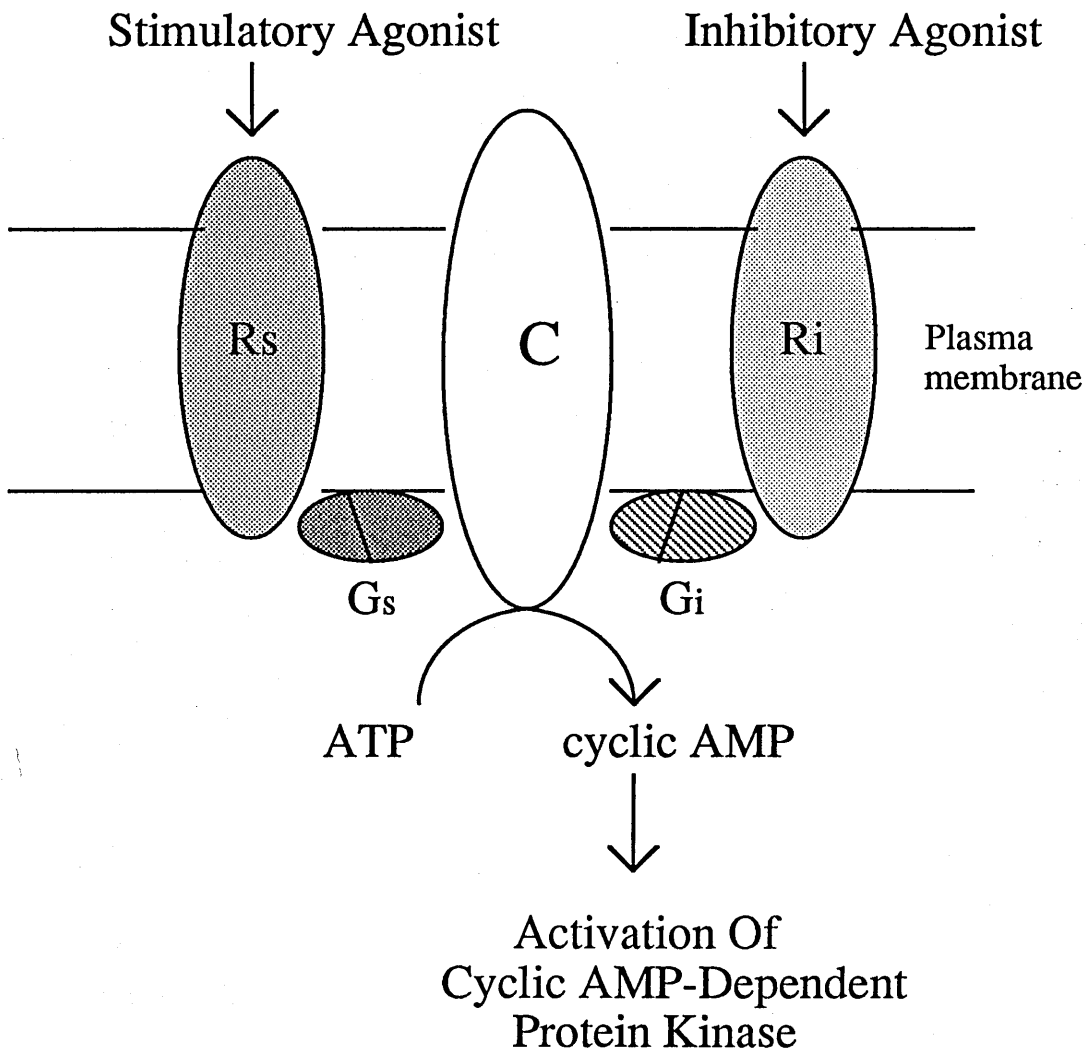
As previously described, hormone-sensitive adenylate cyclase is a multicomponent system consisting of five functional units (Figure 1.5):-

i The stimulatory receptor, of which the β-adrenergic receptors are the most thoroughly characterised. Other examples include those for glucagon,

Figure 1.5:

Dual Regulation Of Adenylate Cyclase Activity

Figure depicts how the activity of the adenylate cyclase catalytic unit is regulated by a stimulatory (G_s) and inhibitory (G_i) G-protein. The activities of G_s and G_i are regulated by their interaction with appropriate receptors; after binding of the appropriate agonists, R_s couples to G_s, and R_i couples to G_i.


Stimulatory Agonist
Inhibitory Agonist
Rs
C
Ri
Plasma membrane
Gs
Gi
ATP
cyclic AMP
Activation Of
Cyclic AMP-Dependent
Protein Kinase

adrenocorticotropic hormone (ACTH), thyroid-stimulating hormone (TSH) and secretin.

ii The stimulatory guanine nucleotide-binding regulatory protein, G_s .

iii The inhibitory receptor, homologous in structure to its stimulatory counterpart.

iv The inhibitory guanine nucleotide-binding regulatory protein, G_i. Unambiguous identification of the isoform(s) responsible for mediating inhibition of adenylate cyclase activity is still lacking.

v The catalytic unit (C) responsible for generating cyclic AMP from ATP.

It has been observed that the mechanisms of information transfer between receptors and G-proteins are highly conserved, presumably reflecting similar structural motifs present within the bewildering number (eighty to date) of different receptors coupled to various G-proteins and effectors (Birnbaumer *et al.*, 1990). The intensively studied β-adrenergic receptor/G_s and rhodopsin/transducin systems have provided the basis for understanding the interactions involved, although different cells appear to exploit the same basic mechanisms to their own advantage.

1.9.2 STIMULATION OF ADENYLATE CYCLASE ACTIVITY

A Hormone-Sensitive Activation/Deactivation Cycle Of G_s

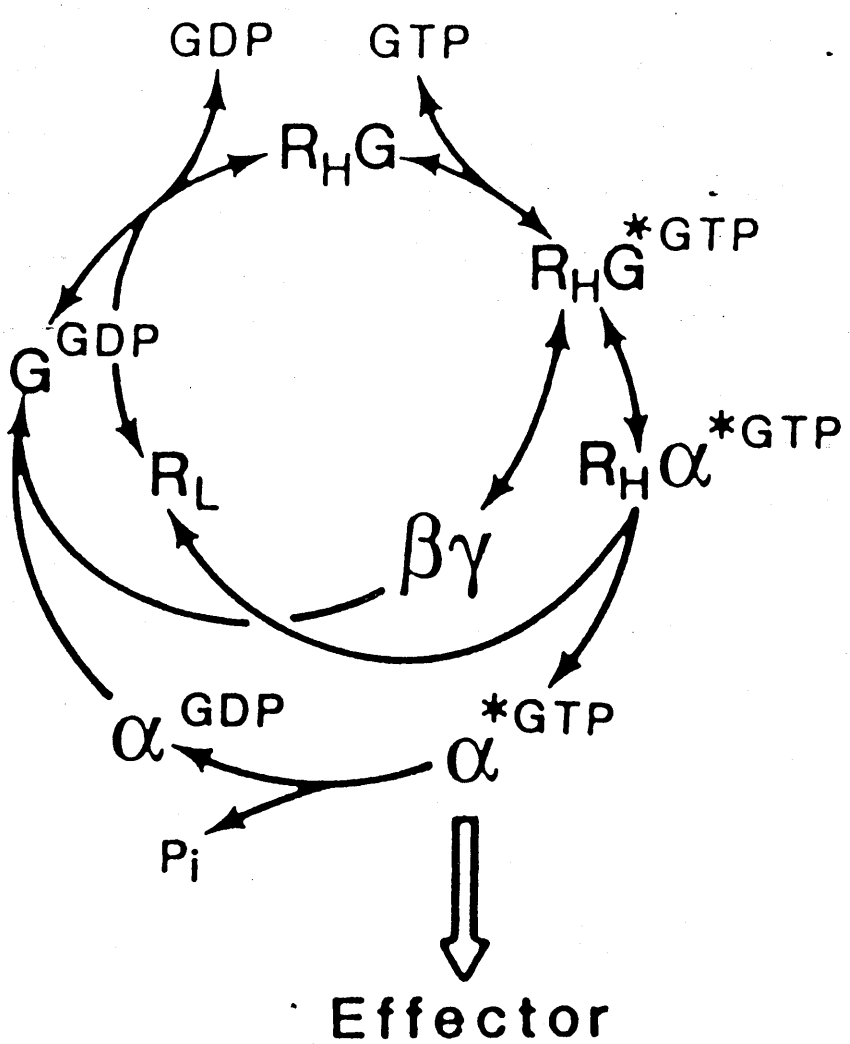
Studies on the interactions of G_s with β-adrenergic receptors in phospholipid vesicles (Pederson & Ross, 1982) and isolated membranes (Levitzki, 1986) have revealed the essential features of their interactions (Figure 1.6):-

Figure 1.6:

Regulatory Cycle Of G-Protein Activation/Deactivation

Figure depicts the mechanism by which a receptor catalyses the activation of a G-protein and the possible reaction sequences in which a receptor mediates the release of GDP from the $\alpha\beta\gamma$ heterotrimer, promotes the binding of GTP and triggers the activation of the heterotrimer before a possible dissociation of α*-GTP from $\beta\gamma$. GTP-bound α-subunits activate the appropriate effector enzyme, a process which is turned-off by the intrinsic GTPase activity of the α-subunit, allowing the reassociation of α-GDP with $\beta\gamma$ to initiate another cycle. R_h=receptor with high affinity for agonist; R_i=receptor with lower affinity for agonist; G*=activated heterotrimeric G-protein; α*=activated α-subunit; P_i=inorganic phosphate.

Figure is taken from Birnbaumer (1990).


GDP
GTP
R_HG
R_HG^{*GTP}
G GDP
R_L
$\beta\gamma$
$R_H\alpha^{*GTP}$
α GDP
α^{*GTP}
P_i
Effector

i Agonist-occupied receptors, in concert with intracellular Mg^{2+}, stimulate the rate of dissociation of GDP from the guanine nucleotide-binding site on the α-subunit of the αβγ heterotrimeric G-protein. In the absence of activated receptor, GDP only dissociates very slowly (1-5 minutes) (Brandt & Ross, 1985).

ii Agonist-occupied receptors stimulate GTP binding, even when the nucleotide binding site is empty. Therefore the life-span of the the "open" G-protein/receptor/agonist complex is short as physiological GTP concentrations (100 μM) are sufficient to saturate G_s (K_mGTP is approximately 1μM) and so the nucleotide readily binds to G_s (Birnbaumer *et al.*, 1985).

This nucleotide exchange on the α-subunit described in steps "i" and "ii" requires the presence of the βγ-complex: in its absence, there is little effect of receptors on the α-subunit (Weiss *et al.*, 1988; Florio & Sternweis, 1989)

iii Binding of GTP precedes a Mg^{2+}- and temperature-dependent activation of G_s such that the α-subunits become capable of complexing with and activating the adenylate cyclase catalytic unit, increasing the k_{cat} for MgATP by approximately 200-fold (Northup *et al*, 1983). The importance of Mg^{2+} ions in the G-protein activation process was determined by Iyengar & Birnbaumer (1982) who found that the addition of glucagon to rat liver plasma membranes increases the affinity of the system for Mg^{2+} by three orders of magnitude (from a millimolar to a physiologically relevant micromolar range) and enhances the maximal activation of adenylate cyclase by GTPγS.

iv The intrinsic GTPase activity of $G_s\alpha$ hydrolyses GTP to GDP, causing deactivation of cyclase. The resulting G_s-GDP complex is then free to interact with another agonist-occupied receptor to re-initiate the cycle. Thus hormones stimulate a GTPase activity not by increasing the k_{cat} for GTP hydrolysis, but by increasing the number of molecules with GTP bound.

These events explain the basic properties of hormone-stimulated adenylate cyclase. In particular, the importance of the GTPase reaction in turning off the cycle was suggested by three observations. Firstly, non-hydrolysable GTP analogues (Gpp(NH)p and GTPγS) generate a persistently active enzyme as they block the hydrolytic "turn-off" step. Secondly, cholera toxin-catalysed ADP-ribosylation of $G_s\alpha$ abolishes its GTPase activity and results in the persistent activation of adenylate cyclase. Thirdly, a hormone-stimulated GTPase activity has been demonstrated in several systems, including turkey erythrocyte (Cassel & Selinger, 1976; Cassel *et al.*, 1977) and human platelet (Houslay *et al.*, 1986) membranes as well as for purified β-adrenergic receptors and $G_s\alpha$ reconstituted into phospholipid vesicles (Cerione *et al.*, 1984).

B The Role Of Hormone-Bound Receptor

The first amplification step in hormonal signalling is at the receptor/G_s interaction because the hormone-bound receptor functions as an exchange catalyst to activate several G_s molecules. Like conventional enzymes, agonist-bound receptors exhibit a higher affinity for the transition state (nucleotide-free G_s) than for the product (GTP-bound G_s). The catalytic nature of this interaction was demonstrated most unequivocally for the β-adrenergic receptor in turkey erythrocyte membranes (Tolkovsky & Levitzki, 1978) and reconstituted in phospholipid vesicles with purified G_s (Pederson & Ross, 1982). Similar results were also obtained for the glucagon receptor in rat liver plasma membranes (Houslay *et al.*, 1980). It was found that a progressive decrease in the number of stimulatory receptors reduces the rate of adenylate cyclase activation without affecting the maximum specific activity attainable with agonist and Gpp(NH)p, *i.e.* the rate of cyclase activation by G_s (k_{on}) is linearly related to the concentration of agonist-bound receptor. These observations led to the formulation of the collision-

coupling theory of adenylate cyclase activation whereby a hormone-bound receptor makes a transient contact with a G_s.C complex (Tolkovsky &Levitzki 1978). Such a model accounts for the observed kinetics in membranes and reconstituted systems, which include the linear dependence of k_{on} with the concentration of hormone-bound receptor, a non co-operative dependence of k_{on} with hormone concentration, and first order activation of adenylate cyclase activity by Gpp(NH)p and hormone (Pederson & Ross, 1982; Tolkovsky & Levitzki, 1978). Inactivation of C in membranes treated with N-ethylmaleimide, or reconstitution of the β-adrenergic receptor with purified G_s in the absence of C, demonstrated that these kinetic properties were not dependent on its presence (Iyengar & Birnbaumer, 1982; Pederson & Ross, 1982).

D The Effects Of Guanine Nucleotides On Agonist Binding

The capacity of a hormone to activate adenylate cyclase in isolated membranes is absolutely dependent on the presence of guanine nucleotides and Mg^{2+}. This observation came not from studies of adenylate cyclase activation but from studies on the interaction of glucagon with its receptor in liver plasma membranes. It was found that guanine nucleotides (present as contaminants of the ATP used in the adenylate cyclase assays) cause a reduction in the affinity of the receptors for glucagon (Rodbell *et al.*, 1971a). The search for a functional consequence of these effects led to the discovery of guanine nucleotide-mediated regulation of adenylate cyclase (Rodbell *et al.*, 1971a and b; Birnbaumer & Yang, 1974). Subsequently the importance of this phenomenon in G-protein activation has been extensively studied for many receptor subtypes linked to adenylate cyclase activation, particularly the β-adrenergic receptor (Maguire *et al.*, 1976), as well as the rhodopsin/ transducin system of visual signal transduction (Pfister *et al.*, 1983).

Diffusion of an agonist to within a few molecules diameters of its receptor

induces their interaction. The resulting free energy of binding is used to alter receptor conformation to a form which has a high affinity for the heterotrimeric G-protein with an unoccupied guanine nucleotide binding site on its α-subunit (Wessling-Resnick *et al*, 1987). Thus, activated receptors not only transfer the energy of binding to promote GDP release, but also hold open the guanine nucleotide binding site to allow GTP binding (Birnbaumer *et al.*, 1980; May & Ross, 1988). Once bound, the affinity of the receptor for the G-protein decreases and the two species dissociate from one another, thereby reducing the high affinity binding of agonist to receptor to a level commensurate with the energy used to activate the G-protein. However, in the absence of guanine nucleotides one can measure the receptor/G-protein association quite easily.

As previously stated, agonist-occupied receptors behave as catalysts, and as such have a high affinity for their transition state (the nucleotide-free heterotrimer). Presumably antagonist-bound receptors cannot bind more tightly than unoccupied receptors to the transition state. By binding with equal efficacy to different G-protein conformations, antagonist-occupied receptors would not alter the equilibrium between these conformations, and antagonist binding therefore would become insensitive to guanine nucleotides (Maguire *et al.*, 1976; Lefkowitz *et al.*, 1976). Alternatively, it is possible that antagonist-bound receptors can bind more tightly to non-transition state forms of G_s which would be stabilised by antagonist binding. Such antagonists exhibit a negative intrinsic activity and inhibit basal activity (Costa & Herz, 1989). Partial agonists are capable of forming high affinity guanine nucleotide-sensitive complexes with R_s receptors in a manner which is positively and linearly correlated with their abilities to activate cyclase by increasing the k_{on} (Birnbaumer *et al.*, 1985)

E G-Protein Activation

Activation of a G-protein requires the induction of a conformational change which allows the α-subunit to interact with and modify the activity of its effector.

The structural changes underlying these steps are poorly understood but presumably the effects of guanine nucleotides on receptor/G-protein interactions reflect these conformational changes. As agonist/receptor complexes bind tightly to the αβγ (nucleotide-free) transition state but not to the substrate (αβγ-GDP) or product (αβγ-GTP), the high affinity binding of R_s to G_s, or the linked function of high affinity binding of agonist to receptor, are disrupted by GDP, GTP or their analogues. However, whereas GTP promotes activation of G_s, GDP prevents their intreraction. Therefore, if GDP binds to $G_s\alpha$, the αβγ-GDP complex will leave the receptor exactly as it arrived whereas if GTP binds, the G-protein will alter its conformation such that it can now activate adenylate cyclase and can only interact with the receptor again after GTP hydrolysis (Higashijima *et al.*, 1987).

It has been suggested (Bourne *et al.*, 1991) that the ability of the receptor to preferentially bind the αβγ heterotrimer (Fung & Nash, 1983; Florio & Sternweis, 1989), coupled with the preference of βγ to bind GDP-bound rather than GTP-bound α-subunits, means that hormone receptors catalyse the replacement of GDP with GTP instead of the other way round. This is because the dissociation of βγ from GTP-bound α-subunits prevents further interaction with the receptor thereby avoiding receptor-mediated release of GTP.

Molecular biological and functional studies of a mutant H21a $G_s\alpha$ protein have attempted to analyse the mechanisms of G-protein activation (Bourne *et al.*, 1988). The mutant exhibits a single amino acid difference compared with wild type $G_s\alpha$, adjacent to the GTP-binding site, resulting in the divorce of guanine nucleotide-sensitive agonist binding from adenylate cyclase activation. Presumably this results from the mutation preventing the Mg^{2+}-dependent change in conformation which would allow the α-subunit to activate C. A similar separation of agonist binding and cyclase stimulatory effects of guanine nucleotides occurs in the absence of Mg^{2+}, supporting the hypothesis that a Mg^{2+}-dependent activation step is impeded in the H21a mutant (Higashijima *et al.*, 1987).

The nature of the conformational transitions which occur after GTP binding are controversial. When purified detergent-solubilised G-proteins bind GTPγS, dissociation into $G_s\alpha$-GTPγS and βγ complexes is promoted: GDP stabilizes the holomeric form (Gilman, 1984; Florio & Sterweis, 1989). These observations led to the proposal of a model whereby hormone-stimulated GTP binding to G_s leads to the dissociation of a $G_s\alpha$-GTP complex which then associates with and activates C. Upon GTP hydrolysis, the $G_s\alpha$-GDP complex dissociates from C and can preferentially reassociate with βγ since it has a higher affinity for the latter than $G_s\alpha$-GTP (Gilman, 1987). Hence βγ competes with C for $G_s\alpha$. Therefore one potential mechanism for cyclase inhibition is G_i dissociation producing elevated levels of βγ-subunits which can bind $G_s\alpha$ molecules, making them less accessible to C and thereby causing inhibition. In this respect, it is noteworthy that $G_s\alpha$-GTP binds more tightly to βγ than $G_i\alpha$-GTP (Pang & Sternweis, 1989).

However, Levitzki and co-workers have provided evidence which suggests that G_s remains tightly bound to C throughout the activation cycle and that it may not even dissociate into subunit complexes upon binding of GTP (Levitzki, 1990). Firstly, the mechanism proposed implies a "shuttling" of $G_s\alpha$ from holomeric G_s to C which is incompatible with the observed kinetics of cyclase activation in isolated membranes and reconstituted systems (Tolkovsky *et al.*, 1982). Secondly, very little dissociation of G-proteins has ever been observed in the presence of GTP (Huff & Neer, 1986). Finally, it is possible to extensively purify GDP-bound and Gpp(NH)p-preactivated C from turkey erythrocytes and bovine brain at low detergent/protein ratios such that β-subunits co-purify stoichiometrically with C and the associated $G_s\alpha$-subunit (Arad *et al.*, 1984; Marbach *et al.*, 1990). High salt washing of the forskolin affinity column to which this complex is bound results in a final forskolin-eluted product devoid of β-subunits, demonstrating the high salt sensitivity of the α/βγ interaction (Marbach *et al.*, 1990).

Despite the controversy and ignorance surrounding the conformational

changes occurring, it is accepted that the G-protein activation cycle provides a second amplification step after the R_s/G_s catalytic interaction. This is because the rate constant of GTP hydrolysis (approximately 10 per minute at 37°C) is 100-times less than the turnover number of the cyclase catalytic unit (approximately 1000 per minute). Therefore the events from agonist binding to activation of C result in a 10x100=1000-fold amplification factor (Levitzki, 1988).

1.9.3 INHIBITION OF ADENYLATE CYCLASE ACTIVITY

Proof that G_s and G_i were independent molecules under the separate control of GTP and hormone receptors came initially from studies which demonstrated that R_s receptor-mediated GTP hydrolysis was additive to the activity stimulated by R_i receptors (Bitonti *et al.*, 1980; Aktories & Jakobs, 1981). The subsequent purification and reconstitution of G_s and G_i with appropriate receptors confirmed these observations in isolated membranes (Kurose *et al.*, 1986).

Just as stimulation of adenylate cyclase activity by appropriate receptors appears to be ubiquitous, inhibition of adenylate cyclase activity also seems to be a universal phenomenon. Examples of inhibitory receptors which activate G_i to inhibit adenylate cyclase activity include α_2-adrenergic receptor subtypes and muscarinic acetylcholine receptor subtypes, as well as receptors for angiotensin II, dopamine (D_2 class), purinergics (P_{2y} class) and adenosine (A_1 class) to mention a few examples. The receptor-dependent activation/deactivation cycle of G_i is very similar to that of G_s but unlike R_s receptors, R_i receptors do not appear to enhance G_i activity by increasing its affinity for Mg^{2+} because under basal conditions, G_i already has an affinity for Mg^{2+} which is within the micromolar range (Hildebrandt & Birnbaumer, 1983). However like R_s receptors, agonist-bound R_i receptors are capable of catalysing nucleotide exchange at the α-subunit, as has been demonstrated in platelet and adipocyte membranes (Michel & Lefkowitz, 1982; Murayama & Ui, 1984). Similarly, GTP, GDP and their analogues exhibit virtually identical effects on the binding of agonists to R_i receptors as they do for R_s

receptor agonists (Birnbaumer *et al.*, 1985). One important difference is that pertussis toxin-catalysed ADP-ribosylation of $G_i\alpha$-subunits within holomeric G_i prevents formation of the high affinity nucleotide-free transition state, thereby inhibiting guanine nucleotide exchange and abolishing G_i function (Sunyer *et al.*, 1988).

A Proposed Inhibitory Mechanisms

As for G_s, studies on purified, ionic detergent-solubilised receptors and G-proteins led to the proposal of a subunit dissociation model to account for the observed non-competitive inhibitory effects of G_i on adenylate cyclase activity (Gilman, 1984). Such a model assumes that $G_s\alpha$ and $\beta\gamma$ are in continual equilibrium and that binding of GTP promotes a dissociated state whilst GDP stabilizes the holomeric form (Katada *et al.*, 1984a, b and c). Similarly, G_i molecules are presumed to dissociate into their constituent $G_i\alpha$ and $\beta\gamma$-subunits. Since $\beta\gamma$-subunits appear to be functionally interchangeable, and G_i is expressed in a 5- to 10-fold excess over G_s in most tissues, (Gilman, 1987) elevated levels of free $\beta\gamma$-subunits released by G_i dissociation will tend to increase the proportion of G_s molecules in the holomeric form by a Mass Action effect (Katada *et al.*, 1984a, b and c).

However, as a dissociation model cannot account for the observed kinetics of adenylate cyclase stimulation, Levitzki and co-workers have proposed a modification of this hypothesis which assumes the permanent association of $G_s\alpha$ and C that was used to account for G_s-mediated activation of C described in the previous section (Marbach *et al.*, 1990; Levitzki, 1990). The finding that $\beta\gamma$-subunits remain tightly associated with Gpp(NH)p-preactivated C places doubts on the ability of $\beta\gamma$ to inhibit cyclase by binding to 'free' $G_s\alpha$ (Marbach *et al.*, 1990), and leaves $G_i\alpha$ as the dominant inhibitory species. Whereas $G_i\alpha$.C interactions are not readily observable with purified components in phospholipid vesicles, they

must occur in S49 *cyc*$^-$ cells to account for the observed inhibitory effects of somatostatin and guanine nucleotides on forskolin-stimulated activity as there is no G_s present (Hildebrandt & Birnbaumer, 1983; Hildebrandt & Kohnken, 1990). The fact that G_i function, but not G_s function, may be preferentially uncoupled in certain systems using heparin (Willuweit & Aktories, 1987) suggests that its interaction with C involves weaker forces than those involved in G_s.C interaction: hence the lack of an inhibitory effect of $G_i\alpha$ on C in reconstituted systems even though R_i receptor-mediated activation of G_i can be observed (Smigel, 1986). Nevertheless, interaction between G_i and G_s.C has been detected in S49 lymphoma membranes by measuring β-adrenergic receptor binding affinity after treatment of membranes with pertussis toxin (Marbach *et al.*, 1988). Such experiments demonstrated that pertussis toxin pre-treatment reduced receptor affinity by approximately five-fold compared with untreated membranes, an effect not observed in S49 *cyc*$^-$ cells (no $G_s\alpha$ present) or turkey erythrocyte membranes (no G_i present). This suggested to the authors that G_i can interact more efficiently with C when C is complexed to G_s. The nature of these interactions is unknown (Marbach *et al.*, 1988).

One argument against a role of βγ-subunits in mediating inhibition is that activation of any G-protein will result in cyclase inhibition. However, preferential inhibition by βγ of basal and forskolin-stimulated activity rather than hormone-stimulated activity in S49 *cyc*$^-$ cells suggests that effects of these subunits on cyclase activity may not be artefactual or trivial, and a model has been proposed which tries to account for their inhibitory effects (Hildebrandt & Kohnken, 1990). These workers have proposed that a preferential inhibition of basal activty by βγ-subunits arises due to their ability to bind to GDP-bound $G_s\alpha$-subunits, thereby preventing exchange of GDP for GTP and subsequent activation. This assumes that a 'free' GDP-bound $G_s\alpha$-subunit can exist, and that the rate of nucleotide exchange of this species is greater than for holomeric G_i. Hence, the main function

of βγ-subunits would be to suppress basal activity thereby allowing any hormone working through any G-protein to inhibit basal adenylate cyclase activity, but only hormones working through G_i could inhibit hormone-stimulated cyclic AMP production (Hildebrandt & Kohnken, 1990). If this is the case, the importance of this basal inhibition of adenylate cyclase activity in different tissues depends on the amounts of free βγ-subunits in excess of $G_s\alpha$, perhaps accounting for the variation in potency of exogenously added βγ in causing inhibition of adenylate cyclase in different systems (Hildebrandt & Kohnken, 1990; Higashijima *et al.*, 1987).

2.9.3 EFFECTS OF ENVIRONMENT ON RESPONSIVENESS

Once a ligand has bound to its receptor, the activated complex must initiate a response. Since agonist-bound receptor complexes have a lifespan of only a few seconds, G-protein activation must be rapid so that the initial response is amplified by the activation of many G-proteins. Rapid activation of several G-proteins is achieved in several ways. Firstly, the dissociation of the G-protein from the receptor after the binding of GTP liberates the receptor so that it can activate another G-protein: this receptor recycling maximizes its catalytic efficiency. Secondly, receptors and G-proteins interact at the surface of a membrane and not free in solution, *i.e.* they are diluted in two rather than three dimensions, which increases the probability that they will interact with each other in a productive manner (Liebman *et al.*, 1987). This advantage will be counteracted to some degree by the relatively slow rate of lateral diffusion of receptors in the lipid bilayer compared to the situation in free solution. A comparison between vertebrate and invertebrate rhodopsin molecules also emphasizes the importance of membrane fluidity in regulating these interactions. Whereas vertebrate rhodopsin is highly mobile compared with most membrane proteins (Poo & Cone, 1974), squid rhodopsin is tightly embedded in microvilli (Foster, 1980). These differing environments may explain why a single photobleached vertebrate rhodopsin molecule can activate up to 500 transducins, whilst the invertebrate protein can only activate 8 transducins

(Stryer, 1986; Kirkwood *et al.*, 1989).

The rate of activation of an effector enzyme by a GTP-bound G-protein would be expected to depend more on cytoplasmic conditions than membrane conditions as none of the G-protein subunits are thought to be transmembrane species, and this has been shown to be the case for transducin-mediated activation of the retinal cyclic GMP-specific phosphodiesterase (Chabre, 1987). It has also been shown that the rate of β-adrenergic receptor- but not fluoride-mediated stimulation of adenylate cyclase activity is profoundly affected by alterations in membrane fluidity (Hanski *et al.*, 1979). The advantages of restricting interactions between signalling proteins to the membrane are not lost because G-proteins are membrane-bound, yet an additional benefit may be a faster transfer of information between G-protein and effector in the cytosol, which is rather less viscous than the plasma membrane. As yet, the assumption that the diffusion rates of transmembrane receptors and catalytic units within the membrane might limit the rates of activation or inhibition of adenylate cyclase activity has not been thoroughly tested experimentally.

1.10 REGULATION OF HORMONAL RESPONSIVENESS

1.10.1 STIMULATION OF ADENYLATE CYCLASE

The stimulation of second messenger generation by G-protein-linked receptors is under tight control from the regulatory mechanisms of the cell. This is manifested in the phenomenon of refractoriness or desensitization, which is defined as a continual temporal reduction in responsiveness observed despite the continuous presence of agonist at constant intensity. As intracellular cyclic AMP levels are the sum of three continuous processes (synthesis, degradation and export of cyclic AMP) it is possible that regulation at any of these points could account for the observed plateau in intracellular cyclic AMP levels observed after prolonged exposure to a stimulatory agonist. As both intracellular and extracellular levels of cyclic AMP plateau after exposure to a stimulatory agonist, cyclic AMP export cannot account for the observed desensitization (Shear *et al.*, 1976). Similarly, the observed cyclic AMP-induced increases in PDE activity at the level of increased gene transcription (Swinnen *et al.*, 1989) are not rapid enough to account for the observed plateau, and the effect also occurs in the presence of PDE inhibitors (Shear *et al.*, 1976). Instead, the bulk of the desensitization arises due to the attenuation of cyclic AMP synthesis

Desensitization of the β-adrenergic receptor has been most thoroughly characterised with respect to its activation of adenylate cyclase activity, and two processes are thought to be involved in its development: a rapid uncoupling of the receptor from the G-protein and receptor sequestration, followed by receptor down-regulation leading to a reduced receptor number (Collins *et al.*, 1990)

A Receptor/G-Protein Uncoupling & Receptor Sequestration

As previously mentioned in Section 1.7, the receptor/G-protein-linked systems are very homologous to the rhodopsin/transducin system. In the latter

case, a rhodopsin kinase is capable of specifically phosphorylating 'bleached' rhodopsin molecules, thereby uncoupling them from transducin and homologously desensitizing the response (Kuhn & Wilden, 1987). An analagous enzyme, the β-adrenergic receptor kinase (βARK) has been identified, purified to apparent homogeneity and cloned (Benovic *et al.*, 1987; Benovic *et al.*, 1989). The activity of this protein kinase is dependent on the presence of agonist-occupied β_2- and α_2-adrenergic receptors, and it is capable of phosphorylating the former to a stoichiometry of 8 mol phosphate per mole of receptor (Dohlman *et al.*, 1987). For the β_2-adrenergic receptor, most of the phosphorylated Ser and Thr residues are within the C-terminal tail of the receptor as carboxypeptidase Y treatment of reconstituted βARK-phosphorylated receptor removes most of the incorporated phosphate (Dohlman *et al.*, 1987).

Although phosphorylation by βARK reduces receptor function, an additional factor is required for the full expression of these effects (Benovic *et al.*, 1987). Again, this is analogous to the rhodopsin system where phosphorylation of at least one more Ser on the third cytoplasmic loop of rhodopsin is required before the binding of arrestin, a soluble 48 kDa protein which binds to phosphorylated rhodopsin and prevents its interaction with transducin (Stryer, 1986). Considering the ubiquity of G-protein-linked systems, it is not surprising that an analogous protein, termed βarrestin, has been shown to exist for the β-adrenergic receptor/G_s system and a family of such proteins may exist for other systems in which desensitization mechanisms have not yet been fully characterized (Benovic *et al.*, 1987; Lohse *et al.*, 1990b).

The cloning of βARK also resulted in the isolation of another homologous yet distinct clone, raising the possibility of the existence of βARK isoenzymes and perhaps a family of related molecules (Benovic *et al.*, 1989). The fact that muscarinic receptors have clusters of Ser and Thr residues at their C-termini might indicate that a similar kinase(s) are involved in an analogous homologous

desensitization mechanism (Peralta *et al.*, 1987). Indeed, this is given further credence by the finding that βARK is capable of phosphorylating muscarinic receptors in an agonist-dependent fashion (Kwatra *et al.*, 1989)

The β-adrenergic receptor also contains two consensus sequences for phosphorylation by cyclic AMP-dependent protein kinase (PKA): one site is within the third cytoplasmic loop, the other within the C-terminal tail (Blackshear *et al.*, 1988). Presumably these are the sites which are responsible for the heterologous desensitization elicited by R_S agonists in cells expressing several types of G_S-linked receptors (Hausdorff *et al.*, 1990). As with βARK, the rate of phosphorylation of the β_2-adrenergic receptor by PKA is increased by agonist occupancy of the receptor (Bouvier *et al.*, 1987).

Using specific inhibitors of βARK and PKA, Lohse *et al.*(1990a) have tried to assess the contribution of these kinases to the initial rapid uncoupling of the β-adrenergic receptor from adenylate cyclase after agonist exposure. At low agonist concentrations, approximately 90% of the desensitization response is due to equal contributions from PKA- and βARK-catalysed phosphorylation of the receptor. However, at higher doses of agonist, receptor sequestration becomes a significant component of the desensitization pathway. Therefore the contribution from each of these different mechanisms in the final desensitization response is intimately related to agonist concentration (Lohse *et al.*, 1990a).

A consensus sequence for protein kinase C also exists and has been shown to be phosphorylated in an agonist-independent manner, providing a potential means of 'cross-talk' between the inositol lipid and cyclic AMP signalling pathways, allowing one pathway to regulate the other (Bouvier *et al.*, 1987). Similarly, it is possible for PKA to phosphorylate the α_1-adrenergic receptor in an agonist-independent manner, thus regulating the function of the latter (Bouvier *et al.*, 1987).

Currently very little is known about the mechanisms of receptor dephosphorylation and resensitization of responsiveness, although latent

phosphatase 2A can catalyse the dephosphorylation of purified hamster β_2-adrenergic receptor which has been phosphorylated by βARK *in vitro* (Yang *et al.*, 1988). Also, the subcellular distribution of latent phosphatase 2A parallels that of β-adrenergic receptor phosphatase described in frog erythrocytes (Sibley *et al.*, 1986).

B Receptor Down-Regulation

Down-regulation leads to the irreversible reduction of receptor number and an associated reduction in agonist-stimulated adenylate cyclase activity (Lefkowitz & Caron, 1988). From studies with mutated β-adrenergic receptors which have been transfected and expressed in β-adrenergic receptor-deficient cells, as well as studies on transfected DDT_1 MF-2 smooth muscle cells, PKA appears to play an important rôle in the control of down-regulation (Collins *et al.*, 1990).

In cells expressing human β_2-adrenergic receptors with a single mutation (Ser to Ala) in either one or both of the PKA phosphorylation sequences, β-adrenergic receptor down-regulation proceeded with an initial delay, but after several hours the down-regulation rate paralleled that of cells transfected with the wild type receptor (Collins *et al.*, 1990). Hence, factors other than PKA-dependent phosphorylation of receptor are required for down-regulation to occur. Treatment of wild type and mutant receptor-transfected cells with forskolin or dibutyryl cyclic AMP causes identical reductions in the levels of β-adrenergic receptor mRNA. This reduction occurs in the absence of a promoter sequence, and must therefore result from a post-transcriptional event such as destabilisation of β-adrenergic receptor mRNA (Bouvier *et al.*, 1989; Hadcock *et al.*, 1989).

Recent evidence has also implicated two Tyr residues in the C-terminal tail of the β-adrenergic and other receptors in controlling down-regulation. Site-directed mutation of these Tyr residues to Ala's has no effect on receptor sequestration but drastically reduces down-regulation, providing more evidence that

these events are probably controlled by distinct mechanisms (Valiquette *et al.*, 1990).

1.10.2 GLUCAGON RECEPTOR DESENSITIZATION

Glucagon desensitization has been studied intensively in rat and chick hepatocytes: both systems exhibit distinct regulatory constraints on glucagon activation of adenylate cyclase activity. Unlike the β-adrenergic receptor system, a heterologous desensitization follows a homologous desensitization process (Premont & Iyengar, 1988; Premont & Iyengar, 1989). Whereas the homologous phase occurs over a relatively small time course (complete within 5 minutes), the heterologous phase, as monitored by fluoride-stimulated adenylate cyclase activity, takes longer (approximately 30 minutes) to reach completion (Heyworth & Houslay, 1983; Premont & Iyengar, 1988). Removal of glucagon within 30 minutes rapidly restores normal coupling of glucagon receptors to G_s.

In rat hepatocytes, angiotensin II, Arg-vasopressin and TPA can mimic the desensitization response, implying a role for PKC-catalysed phosphorylation of some component of the adenylate cyclase complex in mediating the desensitization response (Houslay, 1986). Indeed, it has been claimed that glucagon itself is also capable of eliciting a small stimulation of PIP_2 breakdown, resulting in the *sn*-1,2-diacylglycerol production which may initiate the PKC-mediated desensitization event (Wakelam *et al.*, 1986). This contrasts with the situation for chick hepatocytes where only cyclic AMP and its analogues can mimick the refractory response, a property not displayed by rat hepatocytes (Houslay, 1986).

1.10.3 PHOSPHORYLATION OF G-PROTEIN SUBUNITS

One obvious potential mechanism by which cells could rapidly adapt their hormonal responsiveness is by rapidly altering the functional status of a relevant G-protein by phosphorylation. This may result in either a heterologous desensitization

or supersensitization of a given second messenger event.

In vitro studies have shown that purified human insulin receptor is capable of phosphorylating on Tyr a mixture of bovine brain $G_i\alpha$ and $G_o\alpha$ when in their GDP-bound state (Krupinski *et al.*, 1988; O'Brien *et al.*, 1987a). However, insulin-stimulated phosphorylation of $G_i\alpha$ has not been observed in intact rat hepatocytes (Rothenberg & Kahn, 1988; Pyne *et al.*, 1989) and so the functional significance of these *in vitro* studies is unclear.

Ser/Thr phosphorylation of G-protein α-subunits has been noted by several investigators. In particular, Jakobs *et al.* (1985) initially claimed that α_2-adrenergic receptor-mediated inhibition of adenylate cyclase in platelets was attenuated by treatment with TPA which was thought to stimulate the PKC-catalysed phosphorylation of $G_i\alpha$. Since these initial observations, other workers have shown that $G_z\alpha$ may also be phosphorylated in platelets in response to thrombin, which stimulates inositol phospholipid metabolism (Carlson *et al.*, 1989).

In rat hepatocytes, $G_i\alpha$-2 can be specifically phosphorylated by hormones which elevate cyclic AMP and *sn*-1,2-diacylglycerol levels, resulting in the activation of PKA and PKC respectively (Bushfield *et al.*, 1990a). Whereas pure PKC can elicit a similar phosphorylation event on isolated hepatocyte membranes *in vitro*, pure PKA cannot, implying that PKA cannot phosphorylate $G_i\alpha$-2 directly but is required for the activation of a kinase which can. The inability of pure PKA to phosphorylate $G_i\alpha$ *in vitro* has been noted by others (Premont & Iyengar, 1989). In rat hepatocytes, these phosphorylation events abolish the ability of guanine nucleotides to inhibit adenylate cyclase activity: as it is possible to lose this effect while retaining receptor-dependent inhibition (Strassheim *et al.*, 1990), phosphorylation may enhance the sensitivity of adenylate cyclase to stimulation by stimulatory agonists such as glucagon.

1.10.4 HORMONAL INHIBITION OF ADENYLATE CYCLASE

Relatively few studies have investigated the mechanisms by which inhibitory regulation of adenylate cyclase is modified in response to persistent agonist exposure. Chronic (6 day) infusion of rats with the A_1 adenosine receptor agonist PIA causes a reduction in expression of $G_i\alpha$-1 and $G_i\alpha$-2 and an increased expression of $G_s\alpha$ without causing any alteration in the levels of $G_i\alpha$-3 in adipocyte membranes from treated animals compared with controls: these changes in expression are not accompanied by any alterations in the abundance of the mRNA species encoding these proteins (Longabaugh *et al.*, 1989). A 60% reduction in the number of adenosine receptors is also observed, with a greater proportion of the receptors existing in a low affinity state in the PIA-treated animals (Longabaugh *et al.*, 1989). With respect to the kinetics of adenylate cyclase regulation, PIA treatment leads to a progressive reduction in the ability of this ligand to inhibit adenylate cyclase activity while basal and forskolin-stimulated activities are increased compared with controls. Presumably the reduced expression of $G_i\alpha$-subunits and an increased expression of $G_s\alpha$-subunits produces elevated adenylate cyclase activity by relieving a tonic inhibitory input and potentiating a stimulatory input. Similar results have been obtained by treating isolated rat adipocytes in primary culture with PIA (Green, 1987).

Chronic treatment of S49 cells in culture with either isoproterenol or forskolin causes a time-dependent increase in expression of $G_i\alpha$-2 and a small reduction in expression of $G_s\alpha$ (Hadcock *et al.*, 1990). These changes arise due to an increased transcription of $G_i\alpha$-2 mRNA, and a reduced stability of $G_s\alpha$ even though the abundance of its mRNA is slightly increased. (Hadcock *et al.*, 1990). Presumably these alterations in G-protein subunit expression account for the increased inhibition of forskolin-stimulated adenylate cyclase activity by somatostatin in forskolin- and isoproterenol-treated cells. The alterations in expression of $G_i\alpha$-2 required the presence of a functional PKA activity as S49 *kin*$^-$

cells are unable to regulate expression of this protein when exposed to isoproterenol or forskolin under the same conditions as the wild type cell line (Hadcock *et al.*, 1990).

The mechanisms by which the mRNA's for different G-protein subunits are regulated are not fully understood at present, but the rapid increase in $G_i\alpha$-2 mRNA observed in treated S49 cells may be due to the presence of a cyclic AMP-responsive element in the 5' non-coding region of the $G_i\alpha$-2 gene which could be activated on exposure of the cells to stimulatory agents. This provides a means by which one arm of dually regulated adenylate cyclase can control the functioning of the other arm (Brann *et al.*, 1987; Hadcock *et al.*, 1990).

1.11 REGULATION OF ADENYLATE CYCLASE IN PATHOLOGICAL STATES

1.11.1 ALTERED THYROID STATUS

A Hypothyroidism

It has been recognized for many years that thyroid hormones can regulate catecholamine-stimulated lipolysis in adipocytes, with a hypothyroid state leading to triglyceride accumulation and obesity (Debons & Schwartz, 1961). This is achieved by regulating the number and function of β-adrenergic receptors coupled to the stimulation of adenylate cyclase (Stiles *et al.*, 1984) and by modulating the expression of specific G-protein subunits (Milligan *et al.*, 1987; Rapiejko *et al.*, 1989).

Fat cells from chemically-induced hypothyroid rats exhibit a drastically reduced response of adenylate cyclase to catecholamine stimulation (Malbon *et al.*, 1978) as well as an enhanced inhibitory response to inhibitory adenosine receptor agonists (Malbon *et al.*, 1985; Saggerson, 1986). These observed changes in

hormonal sensitivity are accompanied by an uncoupling of the β-adrenergic receptor from G_s without any change in expression of G_s (Milligan & Saggerson, 1990; Rapiejko *et al.*, 1989; Malbon *et al.*, 1984). However, the response of hypothyroid membranes to stimulation by either ACTH or glucagon is relatively unaffected (Saggerson, 1986). An increased abundance of $G_i\alpha$-1 and $G_i\alpha$-2 was observed in hypothyroid animals as determined by pertussis toxin-catalysed ADP-ribosylation *in vitro* (Malbon *et al.*, 1985) and immunoblotting (Milligan *et al.*, 1987; Rapiejko *et al.*, 1989), although $G_i\alpha$-1 and $G_i\alpha$-2 mRNA levels are unaltered between hypothyroid and euthyroid animals (Rapiejko *et al.*, 1989). An increased expression of $G_i\alpha$-3 has also been observed in adipocytes from hypothyroid animals by immunoblotting (Milligan & Saggerson, 1990). Immunoblotting studies have also shown there to be increased levels of β_1- and β_2-subunits in adipocyte membranes prepared from hypothyroid animals, which is associated with elevated amounts of their mRNA species (Rapiejko *et al.*, 1989). Human studies, although showing a general decreased effectiveness in lipolysis in hypothyroid patients, have shown that there are no alterations in G-protein expression. It is possible that the patients used exhibited enough genetic variability to mask any changes that occur in the wider population of hypothyroid individuals (Ohisalo & Milligan, 1989).

Preliminary studies have also shown that the hypothyroid state causes a similar potentiation of the inhibitory regulation of adenylate cyclase in synaptosomal membranes prepared from rat brain (Mazurkiewicz *et al.*, 1989). Whether this is due to the same alterations in G-protein expression as those occuring in fat cells is unknown.

B Hyperthyroidism

Hyperthyroidism is associated with a potentiated lipolytic response causing an elevated accumulation of cyclic AMP in response to catecholamines (Deykin & Vaughn, 1963; Malbon *et al.*, 1978). These changes may be explained by the

reduced levels of $G_i\alpha$-1, $G_i\alpha$-2 and β-subunits, as well as the slightly increased abundance of $G_s\alpha$-subunits seen in adipocytes from hyperthyroid animals compared with euthyroid controls (Ros *et al.*, 1988; Rapiejko *et al.*, 1989). Presumably these changes enhance cyclic AMP accumulation by reducing the tonic inhibitory effect of G_i on adenylate cyclase activity as well as slightly potentiating the stimulatory input from G_s. As with the hypothyroid animals, only β-subunits exhibited a co-ordinate reduction in the expression of their mRNA species (Rapiejko *et al.*, 1989), perhaps suggesting one point of permissive regulation of adenylate cyclase activity by thyroid hormones at the level of controlling β-subunit mRNA (Rapiejko *et al.*, 1989).

1.11.2 ALBRIGHT HEREDITARY OSTEODYSTROPHY (AHO)

AHO is a disorder inherited as an autosomal dominant trait (Van Dop *et al.*, 1984c), and is characterized by symptoms which include round facies, short stature, subcutaneous ossification and obesity (Levine *et al.*, 1988). Most AHO sufferers also exhibit pseudohypoparathyroidism type Ia (PHP-Ia), a state characterized by mental retardation, impaired olfaction and a resistance to parathyroid hormone and other ligands capable of stimulating adenylate cyclase activity in many tissues (Farfel & Friedman, 1986; Weinstock *et al.*, 1986; Levine *et al.*, 1983a). Presumably this is due to the drastically reduced functioning of $G_s\alpha$ detected in all tissues thus far examined from PHP-Ia sufferers (Downs *et al.*, 1983; Farfel *et al.*, 1982; Levine *et al.*, 1983b). AHO patients with normal hormone responsiveness are termed pseudo-pseudohypoparathyroidism sufferers (PPHP) (Albright *et al.*, 1952).

The autosomal dominant nature of the PHP-Ia characteristics suggested that the molecular lesion is at the level of one of the $G_s\alpha$ genes in the human genome. Fibroblasts and erythrocytes from all AHO patients examined, whether PHP-Ia or PPHP sufferers, have reduced levels of $G_s\alpha$ protein compared with normal

subjects and all but three of these also had correspondingly reduced levels of $G_s\alpha$ mRNA (Levine *et al.*, 1988). The lack of any differences in the genomic DNA restriction maps between normal and AHO patients rules out the possibility of large deletions, substitutions or re-arrangements within the $G_s\alpha$ gene being responsible for the observed reduced mRNA levels, but it does not exclude the possibility of small changes within the gene or its promoter region (Levine *et al.*, 1988). At present it is not known whether $G_s\alpha$ mRNA synthesis or processing is defective in these individuals.

The nature of the defect in the three PHP-Ia patients in the study by Levine *et al.*(1988) with normal $G_s\alpha$ mRNA levels but reduced $G_s\alpha$ protein is also unknown.

1.11.3 GROWTH HORMONE-SECRETING PITUITARY TUMOURS

Growth hormone releasing hormone (GHRH) is a peptide which interacts with receptors on pituitary somatotroph cells causing the elevation of intracellular cyclic AMP levels, resulting in uncontrolled somatotrophic cell proliferation and growth hormone (GH) secretion (Billestrup *et al.*, 1986). Measurement of adenylate cyclase activity in GH-secreting pituitary tumours from a subset of patients showed there to be a marked elevation of cyclic AMP levels which were not responsive to further stimulation by GHRH (Vallar *et al.*, 1987). Several lines of evidence implicated the existence of a constitutively active $G_s\alpha$ molecule in such individuals: these included a hyper-responsiveness of adenylate cyclase to activation by Mg^{2+}, elevated G_s activity as measured by complementation of $G_s\alpha$-deficient S49 *cyc*$^-$ cell membranes, and the inabilities of cholera toxin, Gpp(NH)p and NaF to elicit further activation of adenylate cyclase activity (Vallar *et al.*, 1987).

In four patients examined by Landis *et al.* (1989), two different mutations in $G_s\alpha$ were observed: three tumours replaced Arg 201 with a Cys or His residue, and one tumour had a Gln 227 to Arg substitution. Construction of $G_s\alpha$ molecules

with the appropriate mutations and expression in S49*cyc*$^-$ cells reproduced the same perturbed kinetics of adenylate cyclase stimulation as observed in the tumour cells (Landis *et al.*, 1989).

The Gln 227 mutation occurs in a region of $G_s\alpha$ within the GTP-binding domain (Masters *et al.*, 1986) and as for the corresponding residue in $p21^{ras}$ (residue 61), substitution to another amino acid results in the severe attenuation of the k_{cat} (GTPase) of the molecule (Landis *et al.*, 1989; Barbacid, 1987). Mutation of Arg 201 to a Cys or His residue produces a molecule with properties that are similar to those of wild type $G_s\alpha$ which has been ADP-ribosylated on Arg 201 by cholera toxin (Landis *et al.*, 1989). This Arg residue is within domain II of $G_s\alpha$ (Masters *et al.*, 1986), a domain that is lacking in both $p21^{ras}$ proteins and EF-Tu. Unlike G-proteins, the GTPase activity of these smaller molecules is regulated by their interaction with other proteins (GAP and a programmed ribosome respectively). Therefore it has been proposed that domain II acts as an 'intrinsic GAP' within $G_s\alpha$ thereby accounting for the higher intrinsic GTPase activity compared to the $p21^{ras}$ proteins (Landis *et al.*, 1989). Substitution of Arg 201 abolishes this GAP activity and causes the accumulation of GTP-bound $G_s\alpha$ (Graziano & Gilman, 1989; Masters *et al.*, 1989).

The existence of constitutively active $G_s\alpha$ molecules responsible for tumour promotion begs the question of their unambiguous identification as '*gsp* oncogene' products. They resemble dominantly acting oncogenes in several respects, including their abilities to mimic a growth factor response (GHRH in this case), the existence of oncogenic mutations exclusively in tumour DNA and the dominant expression of the *gsp* molecules with respect to wild type $G_s\alpha$ (Landis *et al.*, 1989). However, it is not yet known whether expression of these proteins in other cell types requiring cyclic AMP for growth will induce the same oncogenic phenotype.

One important implication from this work is that other mutant G-proteins may be responsible for tumour promotion in some cell types. Presumably similar

mutations in the corresponding Arg and Gln residues conserved within the other G-protein α-subunits in the appropriate cell types could also induce uncontrolled growth *via* constitutive activation of signalling pathways

1.11.4 ADRENALECTOMY

Steroid hormones can modulate the abilities of several tissues to respond to hormones which regulate adenylate cyclase activity (Davies & Lefkowitz, 1984), including liver and adipose tissues (Exton *et al.*, 1972; Allen & Beck, 1972). Hence, one might anticipate that adrenalectomy will perturb the permissive regulation of adenylate cyclase activity by steroid hormones in their target tissues. This is observed in adipocytes where adrenalectomy of rats impairs the catecholamine-stimulated lipolytic response in adipocytes (Ros *et al.*, 1989b). This study indicated that glucocorticoids regulate cyclic AMP production at the level of expression of $G_s\alpha$ and also the expression of β-subunits, since adrenalectomy causes a reduction in the cholera toxin-catalysed labelling of the two forms of $G_s\alpha$ and a decrease in the expression of β-subunit mRNA species present in adipocytes (Ros *et al.*, 1989b). A similar reduction in hormone-stimulated adenylate cyclase activity is also observed in reticulocytes and hepatocytes, although it is not known whether a similar mechanism is involved (Stiles *et al.*, 1981).

Consistent with adrenalectomy causing a reduction in expression of $G_s\alpha$, dexamethasone therapy of adrenalectomized animals enhances levels of $G_s\alpha$ in adipocytes and restores the catecholamine-stimulated lipolytic response to some extent, although the slightly reduced forskolin-stimulated adenylate cyclase activity observed might indicate a lower intrinsic activity of the catalytic unit (Malbon *et al.*, 1985). The fact that dexamethasone treatment of growth hormone-secreting cells causes increases in both cholera toxin-catalysed labelling of $G_s\alpha$ and forskolin-stimulated adenylate cyclase activity shows that the effects of steroids on the permissive regulation of adenylate cyclase activity are probably tissue-specific

(Chang & Bourne, 1987). Consistent with this idea is the finding that both $G_i\alpha$-2 and $G_s\alpha$ mRNA levels are regulated by glucocorticoids in brain, unlike adipocytes where expression of $G_i\alpha$-subunits appears to be unaffected (Saito *et al.*, 1989; Ros *et al.*, 1989b)

1.11.5 HEART FAILURE

Cyclic AMP is thought to play an important role in mediating the positive inotropic response of cardiac tissue (Bristow & Ginsberg, 1986). Therefore, one potential locus for a defect in certain heart diseases could be the adenylate cyclase signalling complex. A study by Neumann *et al.* (1988) demonstrated that heart membranes from three patients with idiopathic dilated cardiac myopathy displayed an enhanced pertussis toxin-catalysed labelling of $G_i\alpha$ compared with non-failing heart membranes. These changes are associated with a reduced response of cardiac adenylate cyclase activity to isoproterenol, which may be due in part to an increased expression of G_i enhancing the tonic inhibition of adenylate cyclase activity which is sufficient to reduce G_s-mediated stimulation (Neumann *et al.*, 1988).

Reduced number and function of β-adrenergic receptors also seems to be an important factor in the development of heart failure (Bristow *et al.*, 1985). However, as adenylate cyclase is activated by β-adrenergic receptors via a collision coupling mechanism which buffers the system against large decreases in receptor number (Tolkovsky & Levitzki, 1978), a defect in the coupling between the β-adrenergic receptor and G_s may be of greater significance.

1.11.6 DIABETES MELLITUS & INSULIN-RESISTANT STATES

A Type I Diabetes Mellitus (IDDM)

Type I diabetes mellitus (IDDM) arises due to the inability of the β-cells within the islets of Langerhans in the pancreas to secrete insulin: the consequences

of this state for whole body metabolism have been recently reviewed (Taylor & Agius, 1988) and are discussed briefly elsewhere in this Introduction.

i Liver Tissue

Evidence suggests that the hyperglucagonaemia associated with IDDM is responsible for the increased hepatic glycogenolysis, gluconeogenesis and ketogenesis in diabetic patients (Unger & Orci, 1981; Johnson *et al.*, 1982). In both genetic (BB/Wor rats) and chemically-induced (streptozotocin [stz] or alloxan) rat models of IDDM, these changes are associated with an increase in hepatic cyclic AMP concentrations (Pilkis *et al.*, 1974; Appel *et al.*, 1981). Several groups have attempted to ascertain whether this increase in cyclic AMP content is attributable to defects in the adenylate cyclase signalling complex, with variable results being obtained (Allgayer *et al.*, 1982; Dighe *et al.*, 1984; Gawler *et al.*, 1987; Lynch *et al.*, 1989).

Most groups have shown there to be an increase in the ability of glucagon to stimulate adenylate cyclase in plasma membranes prepared from either whole liver (Allgayer *et al.*, 1982; Lynch *et al.*, 1989) or hepatocytes (Gawler *et al.*, 1987). However the mechanisms by which this phenomenon occurs are still controversial. Lynch *et al.* (1989) claim that liver plasma membranes prepared from stz-induced diabetic animals and BB rats contain increased amounts of $G_s\alpha$-subunits compared with normal controls as assessed by immunoblotting and cholera toxin-catalysed ADP-ribosylation. This results in an enhanced activation of adenylate cyclase activity by cholera toxin, $[AlF_4]^-$ and GTPγS as well as glucagon (Lynch *et al.*, 1989). Also, inhibition of adenylate cyclase by angiotensin II in isolated membranes and intact hepatocytes was unchanged in stz-induced diabetic animals and slightly increased in BB rats compared with normal controls (Lynch *et al.*, 1989). These results contradict the findings of Gawler *et al.* (1987) who attributed the enhanced activation of adenylate cyclase activity by glucagon in hepatocyte plasma membranes to a loss of functional G_i activity relieving a tonic inhibitory

effect on stimulation, thereby sensitising the response to glucagon. It is thought that a 70% reduction in the expression of $G_i\alpha$-2 and $G_i\alpha$-3 associated with a phosphorylation of $G_i\alpha$-2 in stz-diabetic hepatocytes causes an abolition of the ability of Gpp(NH)p to inhibit forskolin-stimulated adenylate cyclase activity (Gawler *et al.*, 1987; Bushfield *et al.*, 1990c). However, whilst guanine nucleotide-mediated inhibition is abolished, P_{2y} purinergic receptor-mediated inhibition of cyclic AMP accumulation in stz-diabetic hepatocytes persists, albeit to a lesser extent commensurate with the reduction in expression of $G_i\alpha$-subunits (Bushfield *et al.*, 1990c).

ii Adipose Tissue

The reduction in plasma insulin concentrations associated with IDDM results in the mobilization of fatty acids from adipose tissue due to the concomitant reduction of insulin's anti-lipolytic effect and the enhanced action of lipolytic hormones (Williamson , 1989). The latter effect presumably accounts for the elevated cyclic AMP content of stz-diabetic rat adipocytes (Chiappe de Cingolani, 1983). As with hepatocytes, different groups have attempted to characterize possible defects in the regulation of adenylate cyclase which could explain the observed increase in lipolysis (Kissebah & Fraser, 1972; Zumstein *et al.*, 1980; Chiappe de Cingolini, 1986; Strassheim *et al.*, 1990).

Several investigators have noted either an increased sensitivity (La Casa *et al.*, 1983) or increased stimulation of adenylate cyclase in response to catecholamines (Chiappe de Cingolini, 1986; Strassheim *et al.*, 1990). Whereas Chiappe de Cingolini (1986) attributed the enhanced responsiveness to an increase in the number of β-adrenergic receptors, Strassheim *et al.* (1990) demonstrated that the tonic inhibitory effect of G_i is abolished in adipocyte membranes from stz-diabetic animals, which in this respect resemble hepatocyte membranes (Gawler *et al.*, 1987). Similarly, receptor-mediated inhibition of adenylate cyclase by PIA,

PGE_1 and nicotinic acid persists, and is even slightly enhanced in stz-diabetic animals (Strassheim *et al.*, 1990). These changes are also accompanied by a 2-fold increase in expression of $G_i\alpha$-3, although the authors claim that it is the phosphorylation and inactivation of $G_i\alpha$-2 which causes the abolition of guanine nucleotide-mediated inhibition (Strassheim *et al.*, 1990).

Human studies on IDDM patients failed to show any alteration in their ratios of expression of $G_i\alpha$-2 to $G_s\alpha$ compared with non-diabetic controls as determined by Western blotting (Ohisalo *et al.*, 1988) but functional studies have not been carried out to ascertain whether there is any alteration in adenylate cyclase regulation.

B Type II Diabetes Mellitus (NIDDM)

i Liver Tissue

The main characteristics of hepatic metabolism in the Type II diabetic state are increased lipogenesis, leading to triglyceride accumulation, and an enhanced hepatic glucose output which contributes to the observed hyperglycaemia (DeFronzo, 1988). As for IDDM, several animal models of NIDDM have been investigated to determine whether these effects can be explained by alterations in the regulation of adenylate cyclase activity. This possibility has been studied in several animal models of obesity and NIDDM including the genetically obese (*fa/fa*) Zucker rat (Houslay *et al.*, 1989), the genetically obese (*ob/ob*) mouse (Bégin-Heick & Welsh, 1988), and the genetically diabetic (*db/db*) mouse (present work).

In hepatocyte membranes prepared from obese Zucker rats, there seem to be two defects affecting adenylate cyclase regulation (Houslay *et al.*, 1989). Firstly, there is an abolition of the ability of Gpp(NH)p to inhibit forskolin-stimulated adenylate cyclase activity in membranes from obese animals, although the amounts of $G_i\alpha$-2 are similar in lean and obese membranes as determined by

immunoblotting and pertussis toxin-catalysed ADP-ribosylation studies (Houslay *et al.*, 1989). As with the stz-diabetic system, P_{2y} purinergic receptor-mediated inhibition of cyclic AMP accumulation persists in the obese state (Murphy & Houslay, unpublished data). There is also a defect in the ability of glucagon to stimulate adenylate cyclase activity, as hepatocyte membranes from obese animals exhibit a ten-fold higher EC_{50} value for glucagon activation of adenylate cyclase. However, the specific activities produced at maximal glucagon concentrations are the same in lean and obese rats (Houslay *et al.*, 1989). The reduced efficacy of hormonal activation was attributed to a 50% reduction in expression of the two forms of $G_s\alpha$ expressed in hepatocytes, as determined by cholera toxin-catalysed ADP-ribosylation (Houslay *et al.*, 1989).

Another study employing sucrose-purified liver plasma membranes from lean and genetically obese (*ob/ob*) mice gave similar but not identical findings to those in obese Zucker rats (Bégin-Heick & Welsh, 1988). G_i function was shown to be abolished but this was claimed on the basis that α_2-adrenergic receptor-mediated inhibition was lost unlike Zucker rat hepatocytes. This was associated with an enhanced ability of isoproterenol, but not glucagon, to stimulate adenylate cyclase activity in the presence of GTP (Bégin-Heick & Welsh, 1988). Whether these changes in regulation are related to the reduced cholera toxin-catalysed labelling of $G_s\alpha$ and increased pertussis toxin-catalysed labelling of $G_i\alpha$ observed in liver membranes from obese animals compared with leans is unknown (Bégin-Heick & Welsh, 1988).

ii Adipose Tissue

The most obvious characteristic of severe insulin-resistant states associated with NIDDM is obesity, arising from an increased adipocyte volume caused by triglyceride accumulation (Taylor & Agius, 1988). Many investigators have attempted to determine whether the triglyceride accumulation occurs due to a reduced lipolytic capacity of adipocytes in several animal models of obesity and

insulin resistance, including the Zucker rat (Vannucci *et al.*, 1990), the obese (*ob/ob*) mouse (Bégin-Heick & Heick, 1977) and the diabetic (*db/db*) mouse (Kupiecki & Adams, 1974). In these three systems a reduced lipolytic response is observed which can be attributed to a reduced ability of stimulatory hormones to activate adenylate cyclase activity in adipocyte membranes (Bégin-Heick, 1980; Vannucci *et al.*, 1990; Bégin-Heick & Coleman, 1988).

In adipocyte membranes prepared from obese (*ob/ob*) mice, stimulation by ACTH and the β-adrenergic receptor agonist isoproterenol are severely attenuated (Bégin-Heick, 1985; Bégin-Heick, 1986), even though there are no differences in the activity of the catalytic unit or the maximal stimulation by Gpp(NH)p between membranes prepared from lean and obese animals (Bégin-Heick, 1980; Bégin-Heick, 1985). Nevertheless, the EC_{50} value for stimulation of adenylate cyclase by Gpp(NH)p is ten-fold higher in membranes from obese animals compared with controls, perhaps suggesting an impairment in the G_s.C interaction (Bégin-Heick, 1980).

Adipocyte membranes from obese (*ob/ob*) animals also express a non-functional G_i activity, as determined by the inability of GTP and Gpp(NH)p to inhibit isoproterenol- and forskolin-stimulated adenylate cyclase activity respectively (Bégin-Heick, 1985; Bégin-Heick & Coleman, 1988). Another group demonstrated that receptor-mediated inhibition of adenylate cyclase activity remains intact (Greenberg *et al.*, 1987), although this has been disputed (Bégin-Heick, 1990). Such defects in the inhibitory regulation of adenylate cyclase activity in membranes from obese animals are associated with drastically reduced levels of $G_i\alpha$-1, with slightly reduced expression of the 45 kDa form of $G_s\alpha$ compared with lean controls (Bégin-Heick, 1990).

Virtually identical results have been found from studies on adipocyte membranes from lean and obese Zucker rats, where a non-functional G_i activity was found with respect to tonic inhibition, whilst receptor-dependent inhibition was

unaltered: such changes were associated with large reductions in the levels of $G_i\alpha$-1 and $G_i\alpha$-3 in membranes from obese rats compared with lean animals (Strassheim *et al.*, 1991b). There was also a reduced ability of stimulatory hormones (isoproterenol, glucagon, TSH and secretin) to activate adenylate cyclase activity, associated with an increase in the EC_{50} value for Gpp(NH)p activation of adenylate cyclase activity and a slight reduction in the expression of both forms of $G_s\alpha$ in obese membranes compared with leans (Strassheim *et al.*, 1991b). These results differ slightly from a study by Vannucci *et al.* (1990) who found that only the sensitivity of adenylate cyclase to activation by isoproterenol was altered. However, whilst their study used young (4 week old) rats, the animals used by Strassheim *et al.* (1991b) were more than 10 weeks old and in the plateau of their insulin-resistant phase (Bray, 1977).

CHAPTER 2

Materials & Methods

2.1 CHEMICALS

All reagents used were of the highest grade commercially available.

Supplier	Chemical/Enzyme
Sigma Chemical Co., Poole, Dorset, U.K.	BSA
	Cholera Toxin
	Theophylline
	PIA
	Nicotinic Acid
	Prostaglandin E_1
	Isoproterenol
	ATP
	Soya Bean Trypsin Inhibitor
	Non-ionic detergent NP 40
	Ortho-dianisidine
	Lubrol-PX
	Secretin (porcine)
	Alumina
	Dowex 50x4-400
	Mastoparan
Boehringer (U.K.), Lewes, East Sussex, U.K.	Creatine Kinase
	Creatine Phosphate
	Dithiothreitol
	Triethanolamine-HCl
	Tris
	GTP, Gpp(NH)p

F.S.A. Laboratory Supplies, Loughborough, Leics, U.K.	HEPES
May & Baker Ltd., Dagenham, Essex, U.K.	Hydrochloric Acid Ammonium Persulphate
Calbiochem, Cambridge, U.K.	Forskolin
National Diagnostics, Aylesbury, Bucks, U.K.	"Ecoscint" Scintillation Fluid
Porton Research Products Ltd. Maidenhead, Berks, U.K.	Pertussis Toxin
Whatman Biosystems Ltd., Maidstone, Kent.	DE-50 Cellulose
Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A.	Collagenase

Glucagon (porcine) was donated by Dr. W. W. Brommer, Eli Lilly & Co., IN, U.S.A.

ICI 118233 was a gift from I.C.I. Pharmaceuticals Ltd., Alderley Park, Macclesfield, Cheshire, U.K.

The following radiochemicals were from Amersham International plc, Amersham, Bucks, U.K.:-

[α-^{32}P] ATP in 50% (w/w) ethanol (Specific Activity 400 Ci/mmol)

[5', 8-^{3}H] cyclic AMP in 50% (w/w) ethanol (Specific Activity 40-60 Ci/mmol).

[α-^{32}P] NAD^+ (Specific Activity 800 Ci/mmol) was from New England Nuclear.

All other reagents were obtained from B.D.H. Ltd., Poole, Dorset, U.K.

2.2 ANIMALS

Male C57BL/KsJ heterozygous (*db*/+) lean and homozygous (*db*/*db*) obese diabetic animals aged between 6 and 15 weeks were obtained from Olac Ltd. Oxon., U.K. Male CBA/Ca lean and obese mice aged 16 weeks were obtained from the University of Bristol Medical School Breeding Unit, Bristol , U.K.

Animals were allowed *ad libitum* access to standard laboratory chow and water before use.

2.3 PREPARATION OF LIVER PLASMA MEMBRANES

Liver plasma membranes were prepared using a modification (Marchmont *et al.*, 1981) of the method of Pilkis *et al.* (1974) which is derived from the original method of Neville (1968).

Between 5 and 10 mice were stunned and killed by cervical dislocation before their livers were removed for washing and fine chopping with scissors in 1 mM potassium bicarbonate, pH 7.2. Using 3 volumes of liver to 1 volume 1 mM potassium bicarbonate, pH 7.2, the livers were homogenized with 6 up-and-down strokes of a Camlab motor-driven homogenizer at setting 3 with a teflon pestle in a 50 ml glass Potter-Elvejhem homogenizer. The resulting homogenate was then filtered through 2 layers of muslin and diluted to 300 ml with chilled 1 mM potassium bicarbonate, pH 7.2, before centrifugation at 2000 g_{max} for 10 minutes at 4°C in the 8x50 fixed angle rotor of a MSE 21 centrifuge. The resulting

supernatant was discarded and the loose pellets poured onto 72 g of sucrose in a plastic beaker before the volume was adjusted to 120 ml using chilled 1 mM potassium bicarbonate, pH 7.2, and the mixture stirred gently for 45 minutes to form a 60% (w/v) sucrose solution. 20 ml portions of the stirred solution were then pipetted into 40 ml Beckman Ultra-Clear centrifuge tubes. On top of this homogenate was layered 12 ml of ice-cold 48.2% (w/v) sucrose in 3 mM imidazole, pH 7.4, followed by 6.5 ml of ice-cold 42.5% (w/v) sucrose in 3 mM imidazole, pH 7.4. The gradients were centrifuged at 100,000 g_{max} for 3 hours at 4°C on the SW28 rotor of a Beckman L8 ultracentrifuge: acceleration and deceleration settings were slow (setting 7) to minimise disruption of the gradients. Plasma membranes were collected at the 48.2-42.5% sucrose interface using a Pasteur pipette, diluted 1:1 with chilled 1 mM potassium bicarbonate, pH 7.2, and centrifuged at 25,000 g_{max} at 4°C for 15 minutes in the MSE 21 8x50 rotor. The membrane pellet was resuspended in 5 ml of 1 mM potassium bicarbonate, pH 7.2, and stored in 100 µl aliquots at -80°C.

2.4 PREPARATION OF ISOLATED ADIPOCYTES

Adipocytes were prepared essentially as described by Rodbell (1964). Animals were killed by cervical dislocation before the white epididymal fat pads were removed and placed into pre-warmed (37°C) Krebs-Ringer-Henseleit buffer, pH 7.4, containing 3% (w/v) BSA (KRH/BSA). The KRH buffer had the following composition:- 25 mM HEPES, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5 mM KCl, 120 mM NaCl, 2 mM $CaCl_2$ and 20 mM glucose.

After fine chopping with scissors, the pieces of fat tissue were rinsed with KRH/BSA and placed into pre-warmed 100 ml glass conical flasks (pre-treated with dichlorosilane to prevent cell adhesion) containing 10 ml KRH/BSA supplemented with 2 mg/ml collagenase and 0.5 mg/ml soya bean trypsin inhibitor and placed in a shaking 37°C waterbath set at 120 cycles per minute. Each flask

was then sealed with a "Suba-seal" (Gallenkamp & Co., Widnes, Cheshire, U.K.) and gassed continuously for between 45 and 60 minutes in an atmosphere of O_2:CO_2 (95%:5%).

After digestion the contents of the flasks were filtered through a plastic teastrainer and washed into a pre-warmed glass beaker. The contents of the beaker were then poured into prewarmed 50 ml plastic centrifuge tubes and centrifuged at 600 r.p.m. for 5 seconds in an MSE Centaur 2 bench centrifuge to float the adipocytes. Adipocytes were removed from the top of the solution using a plastic Pasteur pipette and allowed to settle in a glass tube where the rest of the KRH/BSA was removed using a syringe with the attached needle placed below the floating cells. The cells were then added to 50 ml centrifuge tubes with 5 volumes of KRH/BSA and the procedure repeated twice more. Concentrated adipocytes from the last step were used for the subsequent membrane preparation .

2.5 PREPARATION OF A CRUDE MEMBRANE PELLET FROM ISOLATED ADIPOCYTES

Membranes were prepared according to the method of Strassheim *et al.* (1990). Cells were added to a pre-warmed (37°C) 50 ml measuring cylinder and pre-warmed buffered sucrose (0.25 M sucrose, 50 mM Tris-HCl, 3 mM ATP, pH 7.4) added such that the volume ratio of cells to buffer was 1:3. This mixture was transferred to a 50 ml glass Potter-Elvejhem homogenizer and hand-homogenized at room temperature by 6 up-and-down strokes of a teflon pestle. The homogenate was then poured into chilled (4°C) 50 ml plastic centrifuge tubes and centrifuged at 1500 g_{max} for 15 minutes at 4°C in the 8x50 rotor of a MSE 21 centrifuge. After removal of the fat cake from the top of the tube, the supernatant was re-centrifuged at 15,000g_{max} for 15 minutes in the same centrifuge at the same temperature. The resulting pellet was resuspended in chilled buffered sucrose by 5 up-and-down strokes of a teflon pestle in a 25 ml glass Potter-Elvejhem homogenizer at 4°C and

re-centrifuged at 15,000g_{max} at 4°C for 15 minutes. The membrane pellet was resuspended in 5 mM Tris-HCl, 1 mM EDTA, pH 7.4, to a concentration of between 1 and 2 mg/ml, and 100 µl aliquots rapidly frozen for storage at -80°C.

2.6 PREPARATION OF CYCLIC AMP BINDING PROTEIN FROM BOVINE HEART

The protocol used was based on that employed by Rubin *et al.* (1974).

2.6.1 Homogenization

After removal of pericardium and fat tissue, the heart was chopped into small (1 inch) cubes and minced using a Moulinex electric blender. The minced heart was then mixed with 4 l of 40 mM potassium phosphate, pH 6.1, containing 2 mM β-mercaptoethanol and homogenized in a Waring blender for 1 minute in small batches. After centrifugation at 10,000g_{max} for 10 minutes at 4°C, the supernatant was retained and filtered under vacuum through Whatman No. 54 filter paper using a Buchner funnel and flask. The remaining pellet from the centrifugation step was extracted twice more with 1 l of the same buffer and the filtered supernatants pooled for the next stage.

2.6.2 Ammonium Sulphate Precipitation

The pooled supernatants were made up to 55% saturation by the addition of solid ammonium sulphate to a concentration of 320 g/l, the pH being maintained between 7 and 8 units by the addition of ammonium hydroxide. Protein precipitation was allowed to occur for 3 hours and precipitated material was collected by centrifugation at 10,000g_{max} for 10 minutes at 4°C. The pellet was then dissolved in 500 ml 50 mM Tris-HCl, pH 7.6, containing 10 mM NaCl and 4 mM β-mercaptoethanol. This solution was dialysed overnight against 5 l 50 mM Tris-HCl, pH 7.6 containing 10 mM NaCl, overnight to remove the remaining ammonium sulphate.

2.6.3 Pretreatment Of DE-50 Cellulose

50 g DE-50 Cellulose was added to 2 l 0.5 M HCl and the mixture stirred for 30 minutes. The resin was then washed with distilled water until the pH rose to 4 units. The resin was stirred for 30 minutes with 2 l 0.5 M sodium hydroxide before washing with distilled water as before until the eluate was at neutral pH. The acid/alkali washing procedure was repeated once more and the resin finally equilibrated with 50 mM Tris-HCl, pH 7.6, containing 10 mM β-mercaptoethanol and 10 mM NaCl, such that the final slurry gave a wet settled volume:final volume ratio of 2:1.

2.6.4 Absorption Of Cyclic AMP Binding Protein By DE-50 Cellulose

The dialysed preparation was stirred for 1 hour with 800 ml equilibrated DE-50 Cellulose under conditions where cyclic AMP-dependent protein kinase activity should be absorbed. After filtration of the resin under vacuum with Whatman No. 54 filter paper on a Buchner funnel and flask, the resin was washed with 3 l 50 mM Tris-HCl, pH 7.6, containing 10 mM NaCl and 4 mM β-mercaptoethanol, for 45 minutes. The resin was collected by filtration and washed with the 50 mM Tris-HCl buffer, pH 7.6, containing 10 mM NaCl and 4 mM β-mercaptoethanol, using a Buchner flask and funnel.

2.6.5 Isolation Of The Cyclic AMP Binding Protein

The combined filtrates were brought to 35% saturation with 119 g/l solid ammonium sulphate and the pH maintained between 7 and 8 units with ammonium hydroxide solution. After stirring for 1 hour, the precipitate was collected by centrifugation at $10{,}000g_{max}$ for 10 minutes at 4°C and the resulting pellet discarded. The supernatant was brought to 75% saturation by adding 258 g/l solid ammonium sulphate and after stirring for 1 hour, was collected by centrifugation at

$10{,}000g_{max}$ as before. The pellet from this step was suspended in a minimal volume of 50 mM potassium phosphate buffer containing 4 mM β-mercaptoethanol, pH 7.0. This final suspension was dialysed against the same buffer overnight at 4°C. The purified protein kinase was stored in 250 µl aliquots at -80°C and under these conditions was stable for between 6 and 9 months. Aliquots were never refrozen but could be used for up to 48 hours after thawing if stored between 0 and 4°C.

2.7 ASSAY OF ADENYLATE CYCLASE ACTIVITY

This was achieved using either one of 2 methods:-

2.7.1 Determination Of Cyclic AMP Formation Using A Binding Protein Assay For Cyclic AMP

This method was based on that described by Heyworth & Houslay (1983). The first part of the procedure involves the assaying of cyclic AMP formed by the membranes under investigation. Incubations were carried out in a final volume of 100 µl containing 30 mM Tris-HCl and 0.75 mM EGTA, pH 7.4, with 10 mM theophylline (a non-selective cyclic AMP phosphodiesterase inhibitor), 22 mM creatine phosphate, 1.0 mM ATP, 2 mM $MgSO_4$ and 1 mg/ml creatine kinase. In experiments using inhibitory ligands in adipocyte membranes, 1 U/ml adenosine deaminase was included and theophylline was omitted from the reaction medium and replaced with 100 µM ICI 118233, a non-methylxanthine cyclic AMP phosphodiesterase inhibitor. These alterations enhance inhibition by reducing the effects of adenosine accumulation in the reaction medium. Other components of the reaction mixture were added at the final concentrations specified in figure legends and tables.

Reactions were initiated by the addition of either 10-15 µg of liver plasma membrane protein or 2-10 µg of adipocyte membrane protein, followed by

incubation for 15 minutes at 30°C (liver membranes) or 30 minutes at either 24°C or 30°C (adipocyte membranes) over which time periods cyclic AMP production was linear. Adipocyte membranes were subjected to longer incubation times because less protein was added per assay than for liver, in order to conserve adipocyte membrane samples as much as possible. Hence, in order to produce detectable levels of cyclic AMP with reduced amounts of membrane protein, reactions were carried out over a longer time period. Experiments involving the use of inhibitory ligands in adipocyte membranes were carried out at 24°C instead of 30°C as the former temperature allows slightly more potent inhibitory effects to be seen (Londos *et al.*, 1978).

Reactions were terminated by incubation in a boiling waterbath for 3 minutes. Samples were then centrifuged for 5 minutes at $14{,}000g_{av}$ in a Hettich Micro Rapide centrifuge at 4°C to sediment precipitated denatured protein. Aliquots were subsequently taken for assay of cyclic AMP using a competitive binding method.

Determination of amounts of cyclic AMP in the samples assayed for adenylate cyclase activity was based on the saturation binding assay of Brown *et al.* (1972) as modified by Tovey *et al.* (1974) and described by Whetton *et al.* (1983). This assay depends upon the specific binding of cyclic AMP to the cyclic AMP binding protein purified from bovine heart as described above. Aliquots from experimental samples are incubated with [^{3}H]cyclic AMP and a suitable concentration of binding protein until equilibrium has been reached. The labelled and unlabelled [^{3}H]cyclic AMP compete for a finite number of binding sites, thus under suitable conditions increased concentrations of unlabelled cyclic AMP will lead to reduced binding of the labelled nucleotide. The bound cyclic AMP is then separated from unbound cyclic AMP by the addition of a charcoal/BSA slurry, which absorbs free nucleotides, and centrifugation, thereby allowing estimation of the [^{3}H]cyclic AMP bound to the protein.

Total cyclic AMP binding to the protein is estimated by incubation of

[^{3}H]cyclic AMP in the absence of unlabelled cyclic AMP, while non-specific binding is estimated by incubating [^{3}H]cyclic AMP in the absence of both unlabelled cyclic AMP and binding protein. By using unlabelled cyclic AMP over a certain range of known concentrations, one can sequentially reduce the proportion of [^{3}H]cyclic AMP bound to the protein, allowing a standard displacement curve to be constructed and the estimation of cyclic AMP content.

Aliquots from the sample tubes were made up to a volume of 100 µl with binding buffer (50 mM Tris-HCl, 4 mM EDTA, pH 7.4) before 100 µl [^{3}H]cyclic AMP (stock solution of 5', 8 [^{3}H]cyclic AMP in 50% ethanol diluted in binding buffer to give 40-50,000 c.p.m. per 100 µl) and 100 µl of a suitable dilution of cyclic AMP binding protein in binding buffer were added. Also included in this procedure were duplicate tubes containing known amounts of cyclic AMP (0.0625, 0.125, 0.25, 0.5, 1, 2, 4, 8 and 16 pmol per tube) with [^{3}H]cyclic AMP and binding protein added to the same concentrations as the sample tubes. After incubation for at least 2 hours at 4°C, by which time binding of cyclic AMP to the binding protein had reached equilibrium, bound and free nucleotides were separated by the addition of 250 µl of a continuously stirred, chilled 2% (w/v) Norit GSX charcoal/1% (w/v) BSA slurry in binding buffer, and centrifugation at 14,000g_{av} at 4°C for 4 minutes. 300 µl of the supernatant was extracted and added to 4 ml Ecoscint before scintillation counting. The inclusion of tubes with known amounts of cyclic AMP allowed for a standard curve of c.p.m. versus pmol cyclic AMP to be derived, from which amounts of cyclic AMP formed in the sample tubes could be deduced. The samples were counted in the tritium channel of an LKB Rackbeta counter (Model 1219), which had a computer attachment allowing a curve of best-fit to be drawn for the standards used in each experiment, and which printed out pmol cyclic AMP present in each sample.

2.7.2 Determination Of Cyclic AMP Formation By Purification Using Sequential Column Chromatography With Dowex And Alumina

Reaction conditions employed were identical to those above , except that [α-^{32}P]ATP ($2x10^6$ c.p.m. per tube) was also present. Reactions were terminated by the addition of 100 µl of a "stopping solution" consisting of 1.4 mM cyclic AMP, 40 mM ATP and 0.2% (w/v) SDS, pH 7.5. Following the addition of 100 µl [^{3}H] cyclic AMP ($10x10^3$ c.p.m. per tube) and 750 µl distilled water, [^{32}P]cyclic AMP was partially purified by the double column method of Salomon *et al.* (1974). This procedure involves separating cyclic AMP from other nucleotides by sequential column chromatography with Dowex and alumina.

Dowex-H^+ 50 x 4-400 was mixed with distilled water to form a slurry (1:1 ratio, v/v). 2 ml of this was then added to each column and the water left to drain out: the columns were then ready for use. After each experiment, the Dowex was washed with 2 ml 1 M hydrochloric acid until just before the next set of samples were applied, when the columns were washed with 10 ml distilled water.

The alumina columns were prepared with 0.6 g of neutral alumina per column. These were washed with 8 ml 0.1 M imidazole, pH 7.4, before use and with 2 ml 0.1 M imidazole, pH 7.4, after each experiment. Before the next set of samples were loaded, the columns were washed with another 8 ml 0.1 M imidazole, pH 7.4.

Reaction tubes with added distilled water and [^{3}H]cyclic AMP were applied gently down the side of the Dowex columns using a Pasteur pipette. Once the columns had run dry, 1 ml distilled water was added slowly and the columns run dry once more. Another 1 ml distilled water was then added and the columns allowed to run dry again before being placed over the alumina columns. 3 ml distilled water were added to elute the Dowex columns and the system run until the alumina columns had run dry. Then the alumina columns were placed over

scintillation vials and eluted with 4 ml 0.1 M imidazole, pH 7.4.

The final eluate from the alumina columns was collected and added to 12 ml Ecoscint before counting on a dual label scintillation counting programme which automatically accounted for "spillover" from each channel. Recovery of cyclic AMP in these experiments was routinely between 70 and 90%. The amount of cyclic AMP per sample was calculated by a computer program which converted the d.p.m. per sample into pmol cyclic AMP by taking into account the specific activity of the [α-^{32}P]ATP used and the recovery of cyclic AMP from the column steps.

Unless otherwise stated, formation of cyclic AMP was linear over the incubation periods and initial rates were taken for subsequent analysis.

2.8 SODIUM DODECYLSULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

For these studies, a discontinuous SDS-PAGE system was used as originally described by Laemmli (1970), and modified by Milligan & Klee (1985). This involved concentrating samples in a 0.3% (w/v) acrylamide/0.08% (w/v) N, N'-methylenebisacrylamide stacking gel followed by resolution in a 10% (w/v) acrylamide/0.25% (w/v) N, N'-methylenebisacrylamide 12 cm x 14 cm resolving gel using a LKB Bromma electrophoresis system.

2.8.1 **Stock Solutions** (all solutions filtered and stored at 4°C)

a. Buffer 1:- 1.5 M Tris, 0.4% (w/v) SDS adjusted to pH 8.8 with HCl.

b. Buffer 2:- 0.5 M Tris, 0.4% (w/v) SDS adjusted to pH 6.8 with HCl.

c. Acrylamide solution:- 30% (w/v) acrylamide, 0.8% (w/v) N, N'-methylenebisacrylamide.

d. 50% (v/v) glycerol solution.

e. N, N, N', N'-Tetramethylenediamine (TEMED).

2.8.2 Gel Preparation

After assembly of the gel plates, a resolving gel solution was made consisting of 8.2 ml distilled water, 6 ml buffer 1, 8 ml acrylamide solution, 1.6 ml glycerol solution, 20 µl TEMED and 90 µl 10% (w/v) ammonium persulphate (prepared fresh). Once poured between the plates, 1 ml distilled water was added to cover the top of the solution thereby excluding air and enhancing polymerization. When set, the water was poured off and any unpolymerized acrylamide washed away with distilled water. A stacking gel mixture was then poured onto the resolving gel: this consisted of 9.75 ml distilled water, 3.75 ml buffer 2, 1.5 ml acrylamide solution, 20 µl TEMED and 150 µl 10% (w/v) ammonium persulphate. A gel comb was then inserted to facilitate loading of samples. Once set, the remainder of the kit was assembled and the samples loaded using a Hamilton Microsyringe. The anode and cathode were immersed in running buffer (25 mM Tris , 200 mM glycine and 0.1% (w/v) SDS) and samples run overnight at 12 mA, 60V.

2.8.3 Sample Preparation

The required amount of membrane protein (10-300 µg) was sedimented by centrifugation at 14,000g_{av} for 10 minutes at 4°C. After removal of the supernatant the membrane pellet was resuspended in 40 µl electrophoresis sample buffer (50 mM Tris, 4.5 M urea, 5% (w/v) SDS, 40 mM DTT and a grain of bromophenol blue dye). After solubilisation of samples by vortexing and incubation in a boiling water bath for 3 minutes, they were ready for electrophoresis.

2.8.4 Electrophoretic Separation Of G_i-alpha 1 And 2

Separation of the alpha-subunits of G_i1 and G_i2 was achieved by alkylating membrane samples before resolution of membrane proteins on a 12% (w/v)

acrylamide/0.06% (w/v) N, N'-methylenebisacrylamide resolving gel, as described by Mitchell *et al.* (1989).

Before alkylation, the required amount of membrane protein (10-300 µg) was sedimented by centrifugation at 14,000g_{av} at 4°C for 10 minutes. The supernatant was discarded and the pellet resuspended in 20 µl 10 mM Tris-HCl, 1 mM EDTA, pH 7.5. After the addition of 10 µl 5% (w/v) SDS in 50 mM DTT, the samples were boiled for 5-10 minutes before cooling on ice for 30 seconds (before the SDS precipitated out). 10 µl 100 mM N-ethylmaleimide (NEM) were added and the samples left at room temperature for at least 15 minutes to allow the alkylation reaction to occur. To stop the reaction, 20 µl electrophoresis sample buffer were added and the samples left overnight at 4°C.

The method for electrophoretically separating the alkylated samples is very similar to the protocol described in section 2.8.2 using the solutions described in section 2.8.1 apart from the following differences:-

a. A Bio-Rad Protean electrophoresis system was used to allow the use of a 20 cm x 20 cm resolving gel.
b. For the resolving gel, a different stock acrylamide solution was used, consisting of 30% (w/v) acrylamide, 0.15% (w/v) N, N'-methylene-bisacrylamide.
c. The resolving gel mixture consisted of the following:- 11.6 ml distilled water, 12 ml buffer 1, 20 ml acrylamide solution (described above), 4 ml glycerol solution, 160 µl 10% (w/v) ammonium persulphate and 40 µl TEMED.
d. The stacking gel mixture consisted of the following:- 14.6 ml distilled water, 5.6 ml buffer 2, 2.3 ml "normal" acrylamide solution, 225 µl ammonium persulphate and 12 µl TEMED.
e. Once loaded, samples were run at 45 mA, 100 V overnight in the

running buffer previously described.

2.8.5 Molecular Weight Markers

These were obtained from Gibco-BRL Research Products, Uxbridge, Middlesex, U.K. The biotinylated pre-stained proteins were 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).

2.8.6 Staining Of Gels For Protein

After electrophoresis, gels were stained for 1 hour in 200 ml of 0.1% (w/v) Coomassie Brilliant Blue R in 40% (v/v) methanol and 10% (v/v) acetic acid. Destaining was carried out overnight in the same solution minus the Coomassie Brilliant Blue R.

2.9 IMMUNOBLOTTING OF SDS PAGE GELS

2.9.1 Transfer Of Proteins To Nitrocellulose And Detection

This was achieved using a modification of the method of Mitchell *et al.* (1989) which is derived from the original method of Towbin *et al.* (1979).

After separation of proteins by SDS-PAGE the stacking gel was removed and the resolving gel allowed to soak in transfer buffer (25 mM Tris, 200 mM glycine in 20 (v/v) methanol) for a few minutes. After soaking, a blotting sandwich was constructed which allowed the gel to press tightly against a piece of nitrocellulose paper (Schleicher & Schull, 0.45 μm). These were kept firmly in place by pressing a piece of chromatography paper (Whatman) and foam rubber either side of this sandwich and keeping them together with plastic holders. This sandwich was placed into a LKB Transblot tank and completely immersed in transfer buffer. A LKB Transphor powerpack was connected such that the gel side of the sandwich was connected to the negative electrode and the proteins transferred

to the nitrocellulose paper for 2 hours at maximum voltage, producing a direct current of 1 A.

After transfer, the blotting sandwich was removed, dismantled and the nitrocellulose paper incubated for 3 hours at 30°C in a solution of 5% (w/v) gelatin in phosphate-buffered saline (PBS; 136 mM NaCl, 2.7 mM KCl, 80.5 mM Na_2HPO_4 and 14.7 mM KH_2PO_4) to block non-specific sites on the nitrocellulose which the subsequently-used antibodies might bind to. Following blocking, the nitrocellulose paper was washed free of excess gelatin with PBS before the primary antibody was added as a 1 in 200 dilution in PBS containing 1% (w/v) gelatin, 0.2% (v/v) non-ionic detergent NP-40 and 0.05% (w/v) thimerosal. The nitrocellulose paper was incubated overnight at 30°C in this solution, by which time the binding of antibody had reached equilibrium. The antibody solution was then poured off and the nitrocellulose washed with PBS. The paper was then washed twice for 10 minutes with shaking in PBS containing 0.2% (w/v) NP-40 before the second antibody (horse radish peroxidase-conjugated goat anti-rabbit IgG) was added as a 1 in 200 dilution in PBS containing 1% (w/v) gelatin, 0.2% (v/v) non-ionic detergent NP-40 and 0.05% (w/v) thimerosal .After incubation for 3 hours at 30°C, the antibody solution was poured off and the washing procedures outlined above were repeated. These were followed by a final wash for 10 minutes in PBS before this solution was removed and replaced by 40 ml 10 mM Tris-HCl, pH 7.5, and 1 ml of a 10% (w/v) orthodianisidine dihydrochloride solution dissolved in water. To develop the blot, 10 µl 30% (w/v) hydrogen peroxide solution were added and the mixture swirled over the nitrocellulose paper. Development was arrested by pouring off the developing solution and covering the blot with 50 ml 1% (w/v) sodium azide: quantification was achieved by scanning blots with an Abaton 300 scanner connected to a Macintosh P.C.(C-scan, v.1.0). When stored at 4°C before and after use, both the primary and secondary antisera could be stored as 1 in 200 dilutions in gelatin/PBS/NP-40 and used for up to five times.

2.9.2 Description Of Antisera

The polyclonal antisera used for these studies were donated by Dr. Graeme Milligan, Institute of Biochemistry, University of Glasgow, and have been described in several publications (Mitchell *et al.*, 1989; M^{c}Kenzie & Milligan, 1990; Milligan & Unson, 1989). Briefly, antisera were raised in New Zealand White rabbits against glutaraldehyde conjugates of keyhole-limpet haemocyanin and synthetic peptides which represent sections of the deduced amino acid sequences of various cloned G-protein subunits (Table 2.1).

Antiserum SG1 was raised against the C-terminal decapeptide sequence of $G_t\alpha$-1. This antiserum recognizes both $G_i\alpha$-1 and $G_i\alpha$-2 as well as $G_t\alpha$-1 and $G_t\alpha$-2, but as the expression of the latter two species is restricted to retinal tissue, SG1 can be used as a specific probe for $G_i\alpha$-1 and $G_i\alpha$-2 in other tissues. This antiserum does not recognize $G_i\alpha$-3 (Mitchell *et al.*, 1989). Antiserum I3B was raised against a decapeptide which corresponds to the C-terminal sequence of $G_i\alpha$-3. It does not cross-react with $G_i\alpha$-1 or $G_i\alpha$-2 but does exhibit weak cross-reactivity with $G_o\alpha$ (Mitchell *et al.*, 1989). However, the expression of $G_o\alpha$ in both liver (Lynch *et al.*, 1989; Huff *et al.*, 1985) and adipocytes (Mitchell *et al.*, 1989) is below current levels of detection. Antiserum CS1 was generated against a decapeptide corresponding to the C-terminal sequence of $G_s\alpha$ which is present in all four of the $G_s\alpha$ cDNA sequences (Bray *et al.*, 1987). Antiserum BN1 was raised against the N-terminal sequence of the β_1-subunit, which differs by one amino acid substitution from β_2 but by three residues from β_3 (Levine *et al.*, 1990). Hence BN1 could be used to probe for both β_1 and β_2 under suitable conditions (Evans *et al.*, 1986), although I was unable to resolve these proteins under any of the electrophoretic conditions described in this section.

Table 2.1:

Description Of Antisera Used For Immunoblotting Analyses

Table shows the antisera used in this thesis, the peptides with which they were raised and a list of the G-protein subunits which they recognize. For further details see Section 2.9.2.

Antiserum	Peptide Used	Recognizes:
SGI	KENLKDCGLF	$G_t\alpha$-1, $G_t\alpha$-2, $G_i\alpha$-1, $G_i\alpha$-2
I3B	KNNLKECGLY	$G_i\alpha$-3 ($G_o\alpha$ weakly)
CS1	RMHLRQYELL	$G_s\alpha$
BN1	MSELDQLRQE	β_1, β_2

2.10 CHOLERA TOXIN-CATALYSED ADP-RIBOSYLATION OF ISOLATED MEMBRANES

The method used was a modification of the protocol described by Heyworth *et al.* (1985) and consisted of 2 distinct stages.

2.10.1 Thiol Pre-activation Of Cholera Toxin

An aliquot of stock cholera toxin (1 mg/ml in 50 mM Tris, 0.2 M sodium chloride, 3 mM sodium nitride and 1 mM EDTA) was incubated with 25 mM DTT for 20 minutes at 30°C. This reduces disulphide links within the cholera toxin complex causing the release of the active A_1 subunit (Moss, 1987).

2.10.2 ADP-Ribosylation Of Membranes

Pre-activated cholera toxin was added to a ribosylation cocktail which included [α-^{32}P]NAD$^+$ and the necessary factors allowing ribosylation *in vitro*. To start the reaction, 50 µl of this cocktail was added to 50 µl of the required amount of membrane protein resuspended in 1.0% (v/v) Lubrol-PX such that the final reaction mix contained the following components: 20 µg/ml pre-activated cholera toxin, 5 µCi [α-^{32}P]NAD$^+$, 10 µM calcium chloride, 0.5 mM GTP, 15 mM thymidine, 5 mM DTT, 0.5% (v/v) Lubrol-PX and 5 mM magnesium chloride in 0.5 M potassium phosphate buffer, pH 7.0. This mix was incubated for 15 minutes at 30°C before the reaction was terminated by the addition of 500 µl ice-cold 10 mM Tris-HCl, 1 mM EDTA, pH 7.4. Membranes were collected by centrifugation at 14,000g_{av} for 10 minutes at 4°C and the resulting pellet resuspended in 30 µl electrophoresis sample buffer, boiled for 3 minutes and subjected to SDS-PAGE as described in Section 2.8. After electrophoresis, the gel was stained with Coomassie Brilliant Blue as previously described, and dried down under vacuum at 60°C for 90 minutes. The gel was then subjected to autoradiography with pre-flashed Kodak X17R-5 X-ray film and intensifying screen for 2-4 days and the film developed with a Kodak Xomat automatic

developer.

Quantitation of the relevant bands on autoradiographs was achieved by densitometric scanning using a Bio-Rad Video Densitometer connected to an Olivetti M21 computer driven by the Bio-Rad-ID analysis software package.

2.11 PERTUSSIS TOXIN-CATALYSED ADP-RIBOSYLATION OF ISOLATED MEMBRANES

This was carried out using a modification of the method of Heyworth *et al.* (1984).

2.11.1 Thiol Pre-Activation Of Pertussis Toxin

An aliquot of stock pertussis toxin (0.2 mg/ml in 50% glycerol-50% 0.05 M phosphate buffer, pH 7.2, containing 0.5 M sodium chloride) was incubated with 25 mM DTT at 30°C for 20 minutes. This activated the toxin in an analogous fashion to cholera toxin.

2.11.2 ADP-Ribosylation Of Membranes

The reaction conditions were very similar to those described for cholera toxin with the following exceptions:-

a. Final concentration of pertussis toxin used was 5 μg/ml.
b. Calcium chloride was not present in the reaction mix for pertussis toxin.
c. ATP was present at a final concentration of 1 mM.
d. The reaction was carried out for 45 minutes at 30°C.

Termination of the reaction, electrophoresis of the ribosylated samples, autoradiography and analysis of the results were exactly the same as described for cholera toxin.

2.12 TREATMENT OF ANIMALS WITH PERTUSSIS TOXIN

IN VIVO

Pertussis toxin injection of mice was carried out as described by Bégin-Heick (1985) which is a modification of the method of Garcia-Sainz (1981). Stock pertussis toxin was diluted in phosphate-buffered saline to a concentration of 10 μg/ml and each mouse given an i.p.injection of 100 μl using a 25 gauge needle. After 3 days, membranes were prepared as described in Section 2.3.

2.13 ESTIMATION OF PROTEIN CONCENTRATION

This was carried out according to the method of Peterson (1977) which is a modification of the method of Lowry *et al.* (1951). This procedure includes an initial solubilisation and precipitation step which prevents interference of membrane lipids with the reagents involved in the colorimetric step.

2.13.1 Reagents

a. Copper-Tartrate-Carbonate (CTC)-a solution of 20% (w/v) sodium carbonate added slowly to solution of 0.2% (w/v) copper sulphate in 0.4% (w/v) potassium tartrate to give final concentrations of 0.1% (w/v) copper sulphate, 0.2% (w/v) potassium tartrate and 10% (w/v) sodium carbonate.

b. 10% (w/v) SDS.

c. 0.8N sodium hydroxide.

d. 2 N Folin-Ciocalteau's reagent.

e. 0.15% (w/v) sodium deoxycholate.

f. 72% (w/v) trichloroacetic acid.

g. Reagent A-consists of equal volumes of CTC, sodium hydroxide, SDS and water: made up fresh before use.

h. Reagent B-1 volume of 2 N Folin-Ciocalteau's reagent mixed with 5 volumes of distilled water just before use.

2.13.2 Assay Protocol

The samples under investigation and BSA standards of known concentration (between 5 and 60 μg per tube) were brought up to a total volume of 1 ml with distilled water. Then 0.1 ml 0.15% (w/v) sodium deoxycholate was added and the samples left at room temperature for 10 minutes. 0.1 ml 72% (w/v) trichloroacetic acid were then added and the samples centrifuged for 30 minutes at 3000*g* in a MSE Centaur 2 bench centrifuge. After centrifugation, the supernatants were decanted off and the remaining liquid aspirated from the sides of the tubes. The remaining pellet was brought up to a volume of 1 ml with distilled water to which was added 1 ml reagent A. After standing at room temperature for 10 minutes to allow solubilisation of the pellet, 0.5 ml reagent B were added and the samples vigorously mixed. Absorbances of the solutions were read off within 2 hours after the addition of reagent B at a wavelength of 750 nm using a LKB Spectroplus spectrophotometer. A standard curve was constructed using the absorbances of the BSA standard tubes and the equation for a straight line of best-fit derived using the Cricketgraph programme of a Macintosh Plus P.C.

2.14 ANALYSIS OF DATA

For all adenylate cyclase assays shown in this thesis, values within a given experiment are presented as means ± S.D. of triplicate determinations. For immunoblotting and ADP-ribosylation studies, data are expressed as mean band intensity ± S.D. compared with control (set at 100%) for at least three experiments. When appropriate, statistical significance of data is assessed using Student's t-test (paired).

CHAPTER 3

The Regulation Of Adenylate Cyclase Activity In Liver Plasma Membranes From Lean And Genetically Diabetic (*db/db*) Mice

3.1 INTRODUCTION

The Type II diabetic state in human beings is associated with several alterations in carbohydrate, lipid and protein metabolism. For example, hepatic glucose output becomes elevated, contributing to the observed hyperglycaemia, and triglyceride accumulates in both liver and fat tissues (DeFronzo, 1988). Presumably these changes arise due to perturbed regulation of key enzymes involved in glycolysis and gluconeogenesis as well as glycogen and triglyceride turnover. As regulation of hepatic metabolism has consequences for whole body homeostasis, such key enzymes are regulated in a complex manner by hormones, in particular insulin and those hormones modulating levels of cyclic AMP and Ca^{2+} (Pilkis *et al.*, 1988; Nuttall *et al.*, 1988). As an example, consider the rôle of cyclic AMP in inhibiting hepatic glycolysis and stimulating gluconeogenesis *via* the activation of cyclic AMP-dependent protein kinase. The nucleotide can act at two levels: rapid alteration of the phosphorylation state of rate-limiting enzymes (pyruvate kinase and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase), and long-term regulation of gene transcription (phosphoenolpyruvate carboxykinase, pyruvate kinase and glucokinase). Insulin opposes the acute effects of cyclic AMP on enzyme phosphorylation presumably by activating cyclic AMP phosphodiesterases and lowering cyclic AMP levels as well as by altering gene expression *via* an undefined mechanism (Pilkis *et al.*, 1988).

Alterations in the hepatic metabolism of genetically diabetic (*db/db*) mice have been known for several years but the mechanisms by which they occur are unknown. Work by Chan *et al.*(1975) demonstrated that glucose production was elevated, glycogen was turned over more rapidly and that activities of both glycolytic and gluconeogenic enzymes were increased in diabetic (*db/db*) animals compared with lean controls. The excessive fat deposition observed in livers from diabetic (*db/db*) animals was associated with increased activities of several lipogenic enzymes, although whether this can completely account for the increased

triglyceride accumulation is uncertain (Coleman & Hummel, 1967).

Considering the alterations observed with respect to the hormonal regulation of adenylate cyclase activity in livers from genetically obese (*fa/fa*) Zucker rats (Houslay *et al.*, 1989) and genetically obese (*ob/ob*) mice (Bégin-Heick & Welsh, 1988), it was decided to examine the regulation of hepatic adenylate cyclase activity in genetically diabetic (*db/db*) mice. Such investigations were initiated for two reasons; firstly, it was important to determine whether any of the alterations observed in cyclase regulation in other models of obesity were also present in diabetic (*db/db*) animals. In this respect, it was of particular interest to see if inhibitory regulation of adenylate cyclase was intact in the diabetic state as it has been suggested that the inability of G_i to tonically inhibit adenylate cyclase activity in the presence of guanine nucleotides, but in the absence of inhibitory hormones, is characteristic of insulin-resistant states (Bushfield *et al.*, 1990d). Secondly, any changes in hormonal regulation of adenylate cyclase activity, in association with an insulin-resistant state, may potentially explain some of the long-term alterations in liver metabolism observed in diabetic animals.

Table 3.1:

Adenylate Cyclase Activity In Mouse Liver Plasma Membranes

Sucrose-purified liver plasma membranes prepared from lean and diabetic (*db/db*) animals were assayed for adenylate cyclase activity in the absence (basal) and in the presence of the ligands mentioned below for 15 minutes at 30°C as described in Section 2.7.1. The [^{32}P]cyclic AMP formed was measured by the double column method described in Section 2.7.2. Specific activities are in pmol/min/mg protein and data are expressed as MEAN ± S.D. for four experiments using different membrane preparations.

(Fold activations above basal activity are given in parentheses).

Ligand	Lean	Diabetic (*db/db*)
Basal	5.5 ± 1.8	4.6 ± 1.4
100 μM Forskolin	131.7 ± 14.1 (23.9)	130.8 ± 17.6 (23.4)
100 μM GTP	46.6 ±10.2 (8.5)	41.2 ± 12.4 (8.9)
100 μM Gpp(NH)p	48.4 ± 8.1 (8.8)	48.3 ± 7.3 (10.5)
10 mM NaF	101.7 ± 11.3 (18.5)	81.0 ± 12.0 (17.6)

Figure 3.1:

Dose-Response Curves For NaF Stimulation Of Adenylate Cyclase Activity In Liver Plasma Membranes From Lean And Diabetic (*db/db*) Animals

Sucrose-purified liver plasma membranes from lean (❑) and diabetic (*db/db*) (○) mice were assayed for adenylate cyclase activity in the presence of increasing concentrations of NaF for 15 minutes at 30°C as described in Section 2.7.1. The [^{32}P]cyclic AMP formed was measured by the double column method described in Section 2.7.2. The figure shows an experiment representative of three performed with different membrane preparations which gave quantitatively similar results.

Basal activities in this experiment were 6.0 ± 2.1 (lean) and 5.5 ± 1.8 (diabetic) pmol/min/mg.

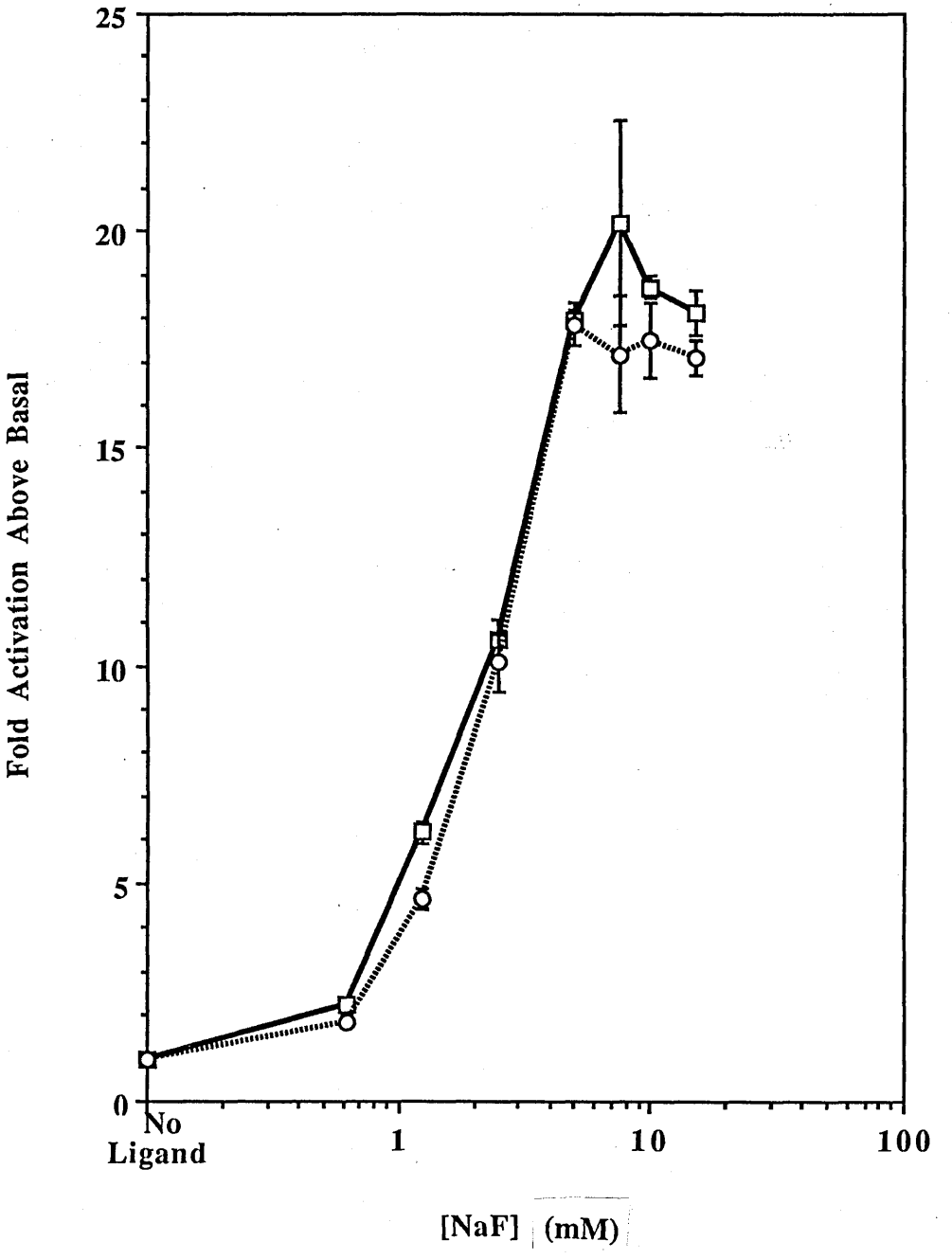

25
20
15
10
5
0
Fold Activation Above Basal
No Ligand
1
10
100
[NaF] (mM)

Figure 3.2:

Dose-Response Curves For Gpp(NH)p Stimulation Of Adenylate Cyclase Activity In Liver Plasma Membranes From Lean & Diabetic (*db/db*) Animals

Sucrose-purified liver plasma membranes from lean (❑) and diabetic (*db/db*) (O) animals were assayed for adenylate cyclase activity in the presence of increasing concentrations of Gpp(NH)p for 15 minutes at 30°C as described in Section 2.7.1. The [^{32}P]cyclic AMP formed was measured by the double column method described in Section 2.7.2. The figure shows an experiment representative of three performed on different membrane preparations which gave quantitatively similar results.

Basal activities in this experiment were 3.9 ± 1.0 (lean) and 4.8 ± 0.5 (diabetic) pmol/min/mg.

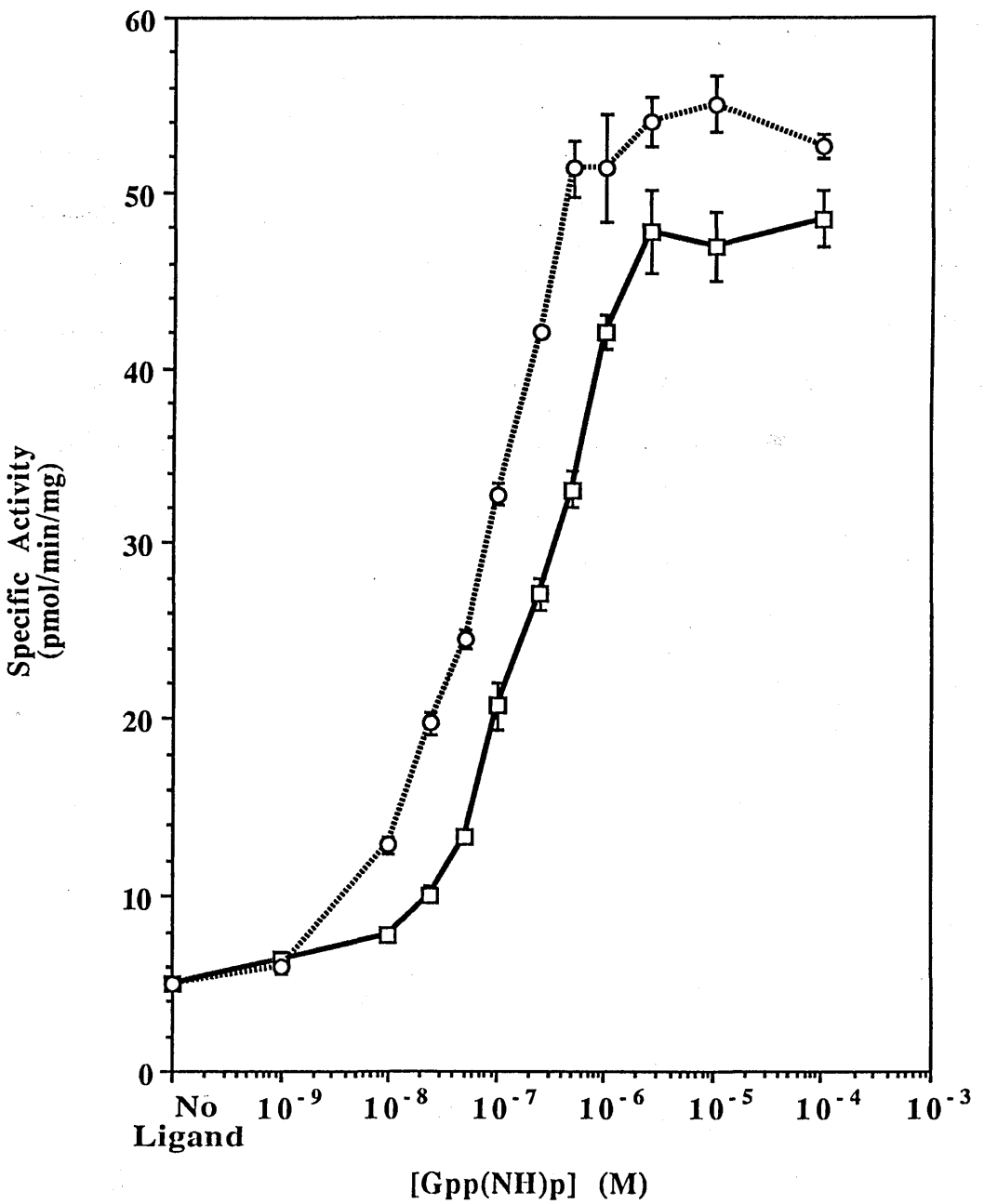

60
50
40
30
20
10
0
Specific Activity
(pmol/min/mg)
No
Ligand
10-9
10-8
10-7
10-6
10-5
10-4
10-3
[Gpp(NH)p] (M)

Figure 3.3:

Effect Of Pertussis Toxin Treatment On The Dose-Response Curve For Gpp(NH)p Stimulation Of Adenylate Cyclase Activity In Liver Plasma Membranes From Lean Animals

Sucrose-purified liver plasma membranes from lean (❑) and pertussis toxin-treated lean (■) animals were assayed for adenylate cyclase activity with increasing concentrations of Gpp(NH)p for 15 minutes at 30°C as described in Section 2.7.1. The [^{32}P]cyclic AMP formed was measured by the double column method described in Section 2.7.2. The figure shows an experiment representative of three performed on different membrane preparations which gave quantitatively similar results.

Basal activities in this experiment were 6.4 ± 2.2 (lean) and 5.0 ± 2.0 (PTx-treated lean) pmol/min/mg..

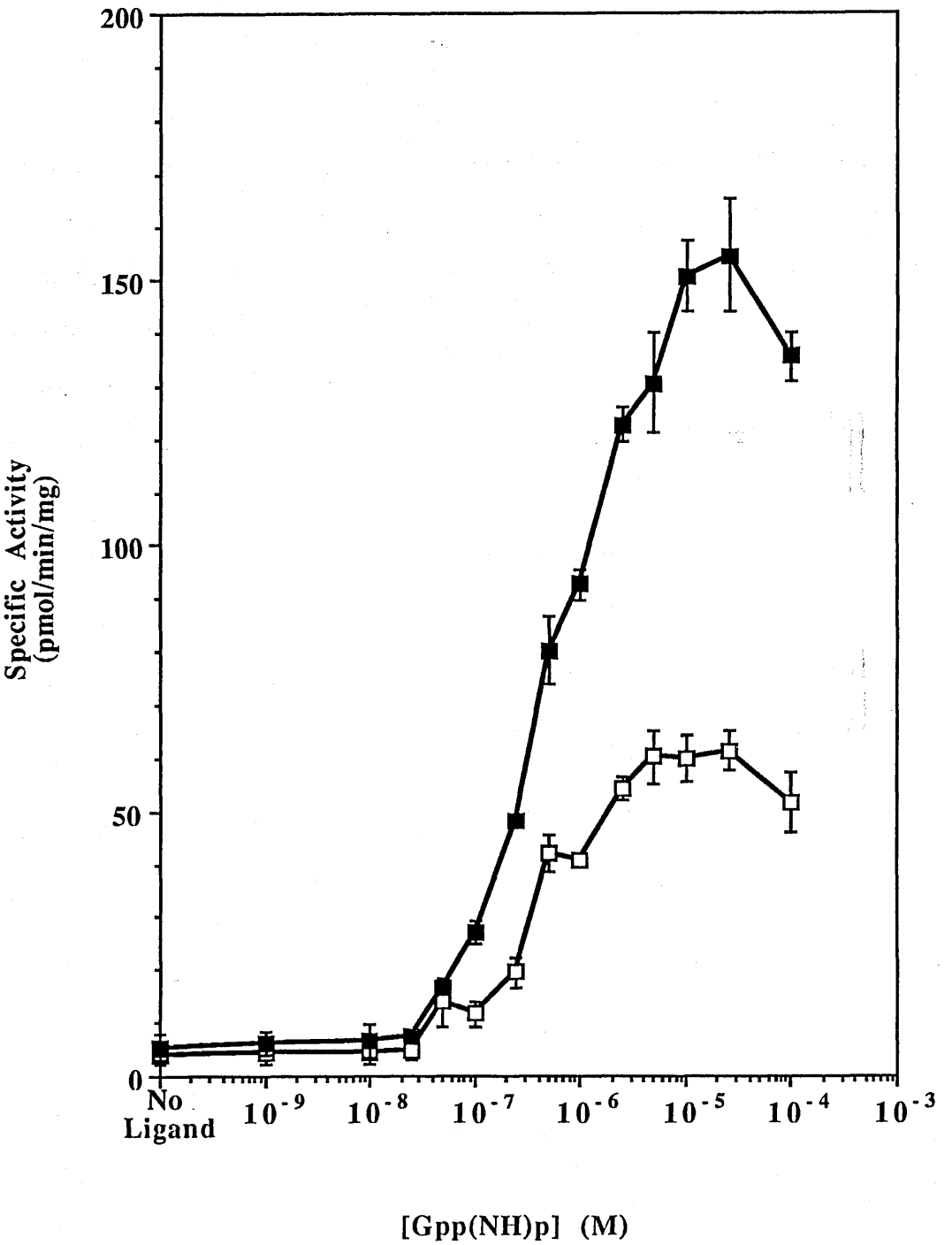

200
150
100
50
0
Specific Activity
(pmol/min/mg)
No
Ligand
10-9
10-8
10-7
10-6
10-5
10-4
10-3
[Gpp(NH)p] (M)

Figure 3.4:

Effect Of Pertussis Toxin Treatment On The Dose-Response Curve For Gpp(NH)p Stimulation Of Adenylate Cyclase Activity In Liver Plasma Membranes From Diabetic (*db/db*) Animals

Sucrose-purified liver plasma membranes from diabetic (*db/db*) (○) and pertussis toxin-treated diabetic (●) animals were assayed for adenylate cyclase activity in the presence of increasing concentrations of Gpp(NH)p for 15 minutes at 30°C as described in Section 2.7.1. The [^{32}P]cyclic AMP formed was measured by the double column method described in Section 2.7.2. The figure shows an experiment representative of three performed on different membrane preparations which gave quantitatively similar results.

Basal activities in this experiment were 5.0 ± 1.5 (diabetic) and 6.2 ± 1.9 (PTx-treated diabetic) pmol/min/mg.

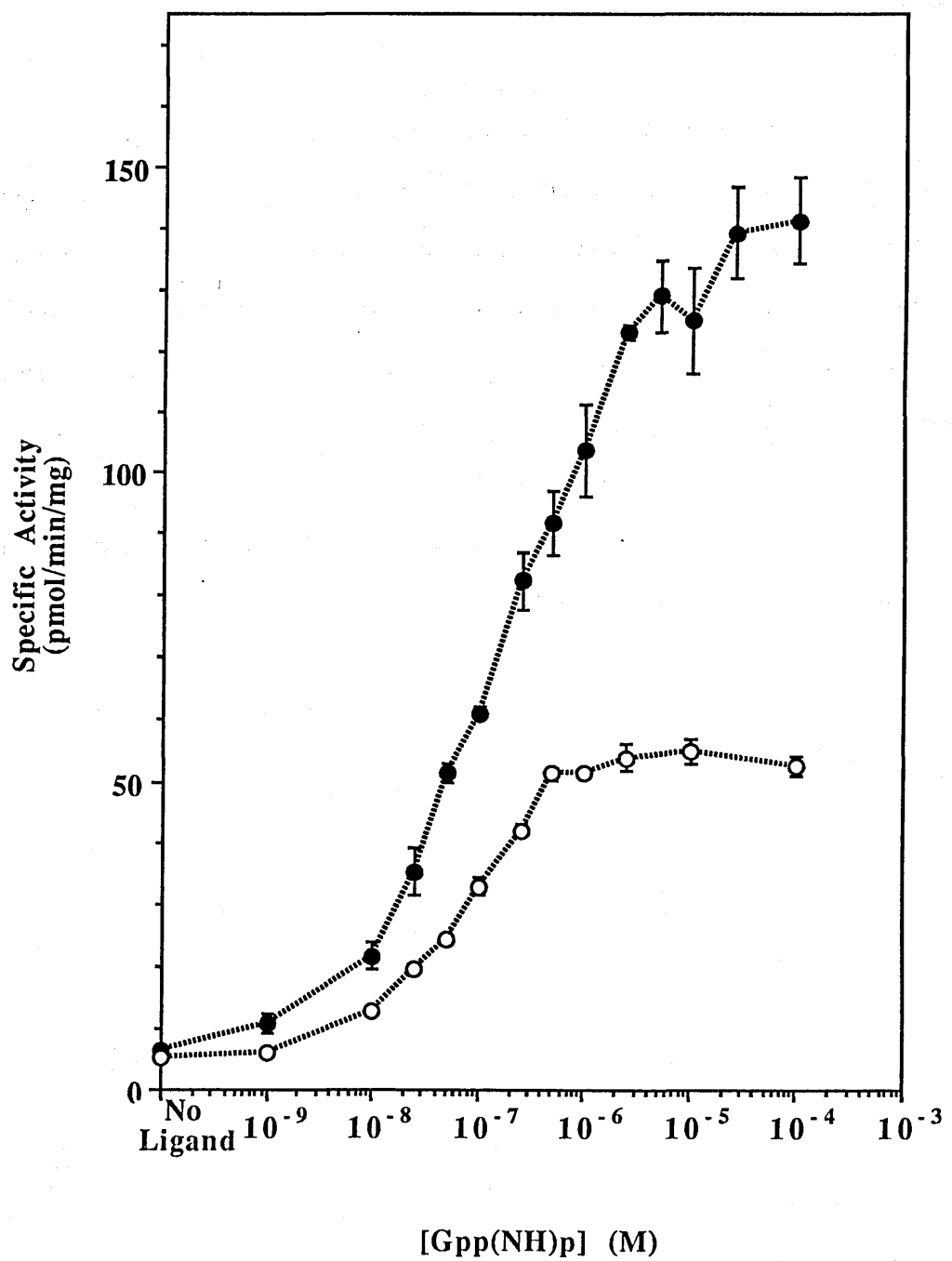

Specific Activity
(pmol/min/mg)
150
100
50
0
No
Ligand
10-9
10-8
10-7
10-6
10-5
10-4
10-3
[Gpp(NH)p] (M)

Figure 3.5:

Dose-Response Curves For Glucagon Stimulation Of Adenylate Cyclase Activity In Liver Plasma Membranes From Lean And Diabetic (*db/db*) Animals

Sucrose-purified liver plasma membranes from lean (❑) and diabetic (*db/db*) (○) animals were assayed for adenylate cyclase activity for 15 minutes at 30°C in the presence of 100 μM GTP and increasing concentrations of glucagon as described in Section 2.7.1. The [^{32}P]cyclic AMP formed was measured by the double column method described in Section 2.7.2. The figure shows results pooled from four experiments performed on different membrane preparations and values are presented as MEAN ± S.D.

The activities obtained with 100 μM GTP alone are given in Table 3.2.

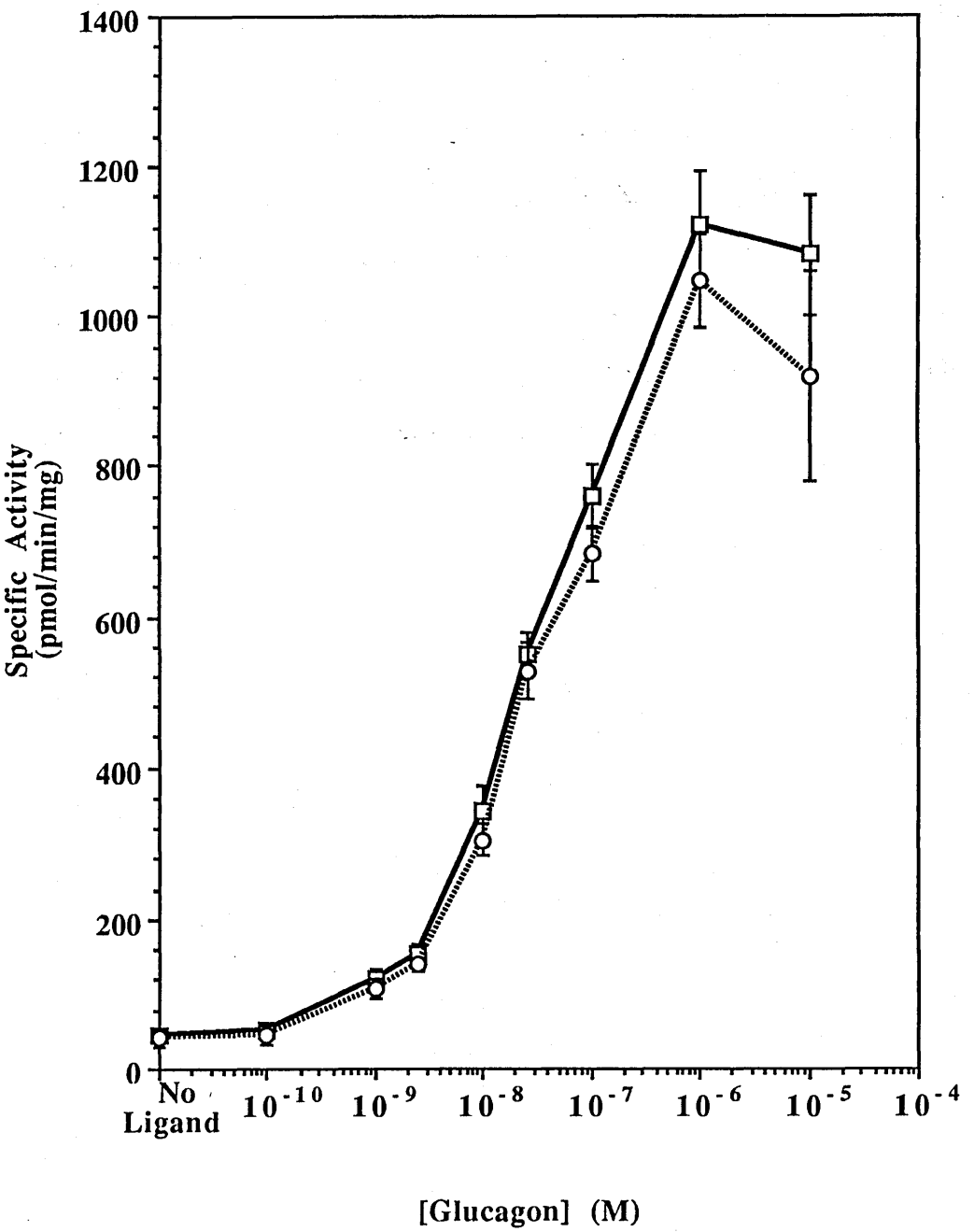

1400
1200
1000
800
600
400
200
0
Specific Activity
(pmol/min/mg)
No
Ligand
10-10
10-9
10-8
10-7
10-6
10-5
10-4
[Glucagon] (M)

Figure 3.6:

Effect Of Pertussis Toxin Treatment On The Dose-Response Curve For Glucagon Stimulation Of Adenylate Cyclase Activity In Liver Plasma Membranes From Lean Animals

Sucrose-purified liver plasma membranes from lean (❑) and pertussis toxin-treated lean (■) animals were assayed for adenylate cyclase activity in the presence of 100 μM GTP and increasing concentrations of glucagon for 15 minutes at 30°C as described in Section 2.7.1. The [^{32}P]cyclic AMP formed was measured by the double column method described in Section 2.7.2. The figure shows results pooled from four experiments performed on different membrane preparations and values are presented as MEAN ± S.D.

The activities obtained with 100 μM GTP alone are given in Table 3.2.

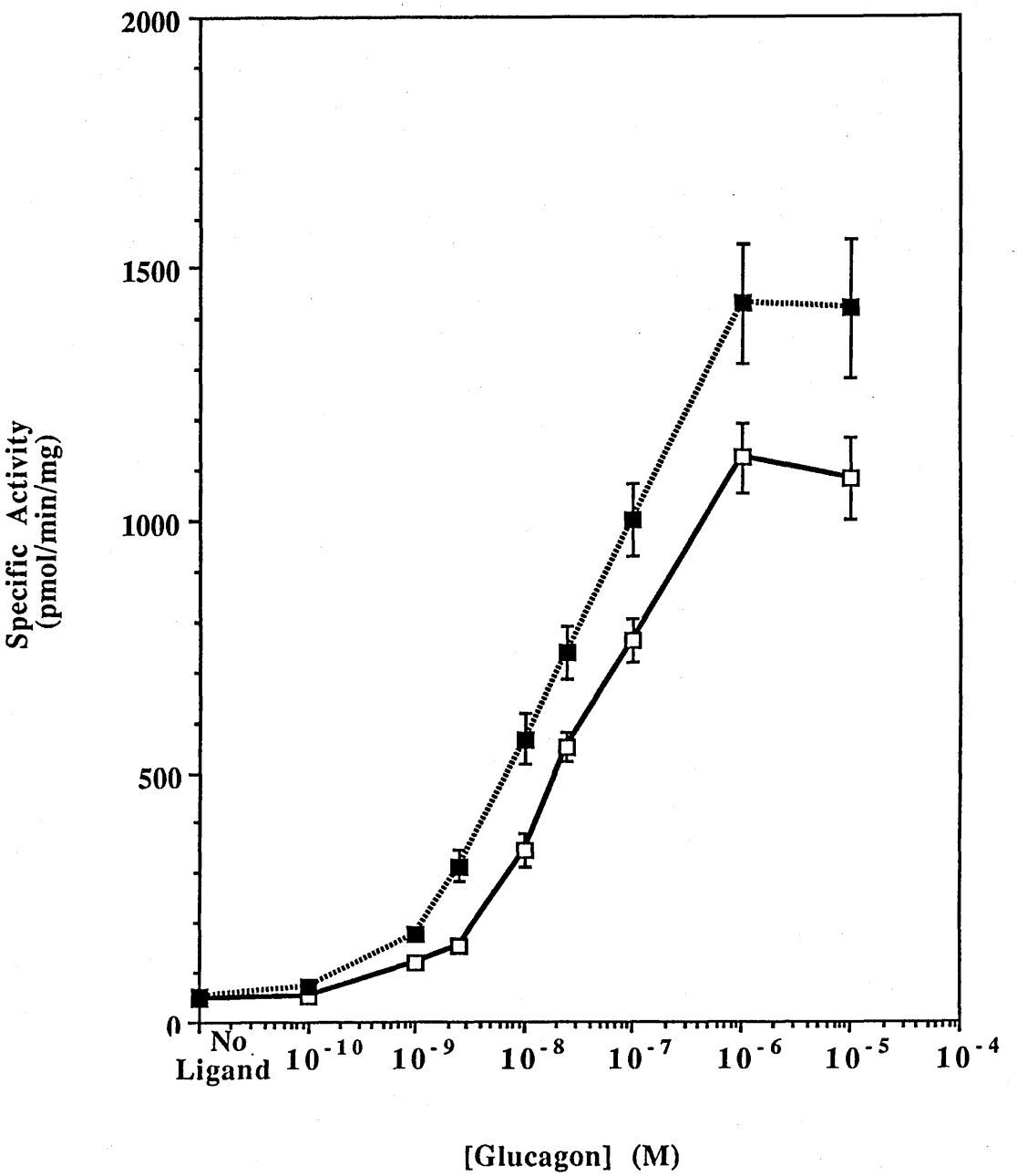

2000
1500
1000
500
0
Specific Activity
(pmol/min/mg)
No
Ligand
10-10
10-9
10-8
10-7
10-6
10-5
10-4
[Glucagon] (M)

Figure 3.7:

Effect Of Pertussis Toxin Treatment On The Dose-Response Curve For Glucagon Stimulation Of Adenylate Cyclase Activity In Liver Plasma Membranes From Diabetic (*db/db*) Animals

Sucrose-purified liver plasma membranes prepared from diabetic (*db/db*) (O) and pertussis toxin-treated (●) animals were assayed for adenylate cyclase activity for 15 minutes at 30°C in the presence of 100 μM GTP and increasing concentrations of glucagon as described in Section 2.7.1. The [^{32}P]cyclic AMP formed was measured by the double column method described in Section 2.7.2. The figure shows results pooled from four experiments performed on different membrane preparations and values are presented as MEAN ± S.D.

The activities obtained with 100 μM GTP alone are given in Table 3.2.

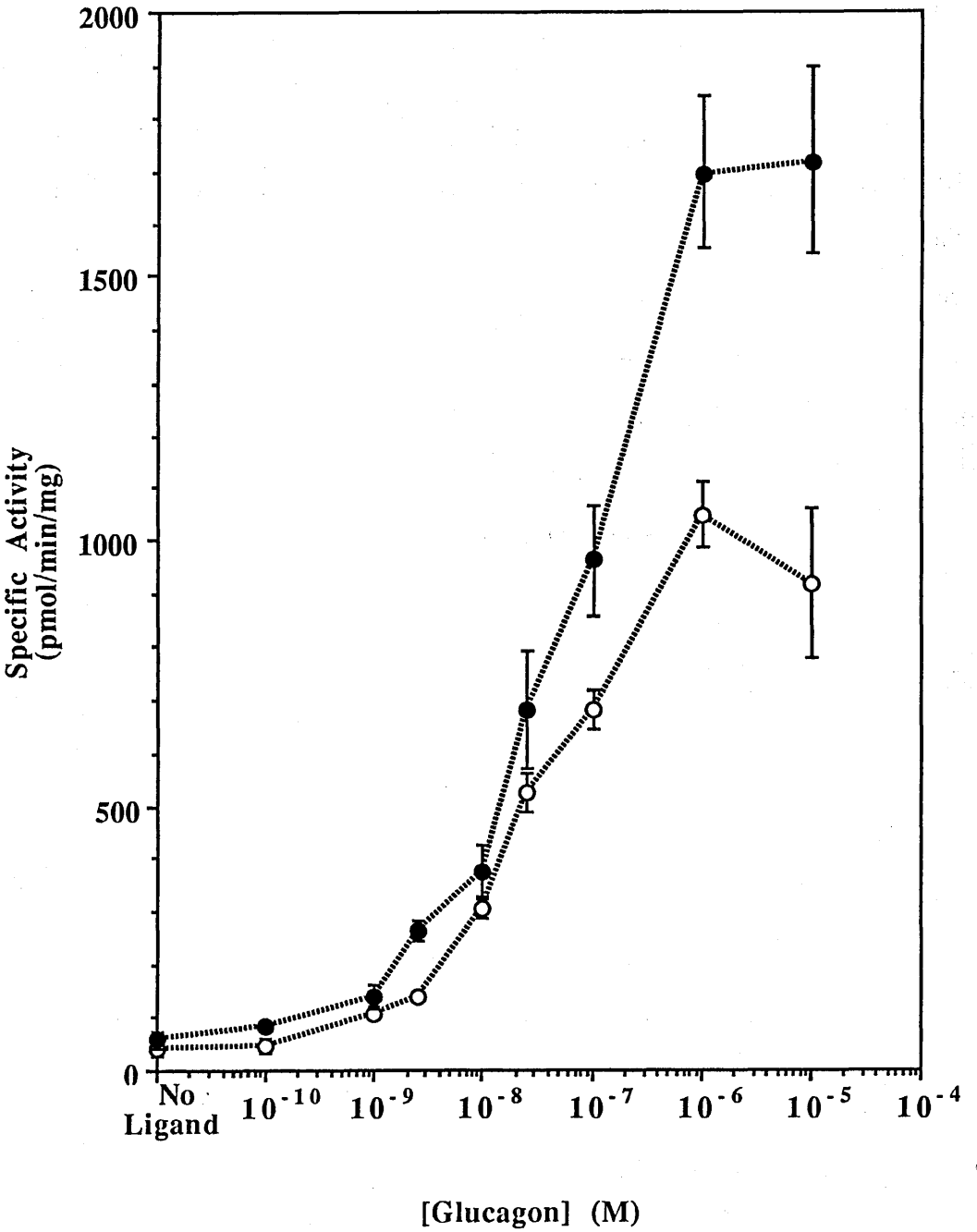

2000
1500
1000
500
0
Specific Activity
(pmol/min/mg)
No
Ligand
10-10
10-9
10-8
10-7
10-6
10-5
10-4
[Glucagon] (M)

Figure 3.8:

Dose-Response Curves For Isoproterenol Stimulation Of Adenylate Cyclase Activity In Liver Plasma Membranes From Lean And Diabetic (*db/db*) Animals

Sucrose-purified liver plasma membranes from lean (❑) and diabetic (*db/db*) (○) animals were assayed for adenylate cyclase activity for 15 minutes at 30°C in the presence of 100 μM GTP and increasing concentrations of isoproterenol as described in Section 2.7.1. The [^{32}P]cyclic AMP formed was measured by the double column method described in Section 2.7.2. The figure shows results pooled from four experiments performed on different membrane preparations and values are presented as MEAN ± S.D.

The activities obtained with 100 μM GTP alone are given in Table 3.2.

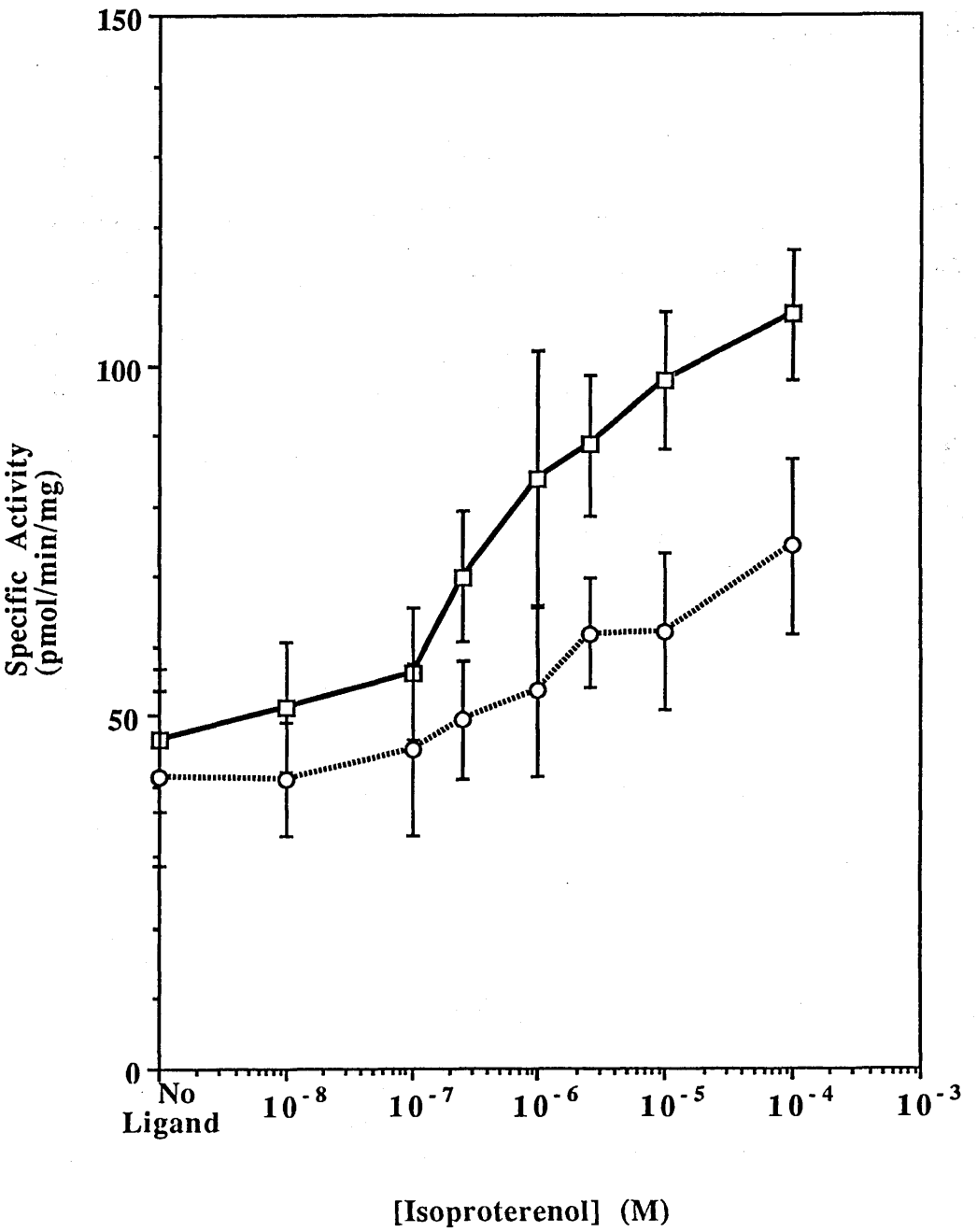

150
100
50
0
Specific Activity
(pmol/min/mg)
No
Ligand
10-8
10-7
10-6
10-5
10-4
10-3
[Isoproterenol] (M)

Figure 3.9:

Effect Of Pertussis Toxin On The Dose-Response Curve For Isoproterenol Stimulation Of Adenylate Cyclase Activity In Liver Plasma Membranes From Lean Animals

Sucrose-purified liver plasma membranes from lean (❑) and pertussis toxin-treated lean (■) animals were assayed for adenylate cyclase activity for 15 minutes at 30°C with 100 μM GTP and increasing concentrations of isoproterenol as described in Section 2.7.1. The [^{32}P]cyclic AMP formed was measured by the double column method described in the Section 2.7.2. The figure shows results pooled from four experiments performed on different membrane preparations with values presented as MEAN ± S.D.

The activities obtained with 100 μM GTP alone are given in Table 3.2.

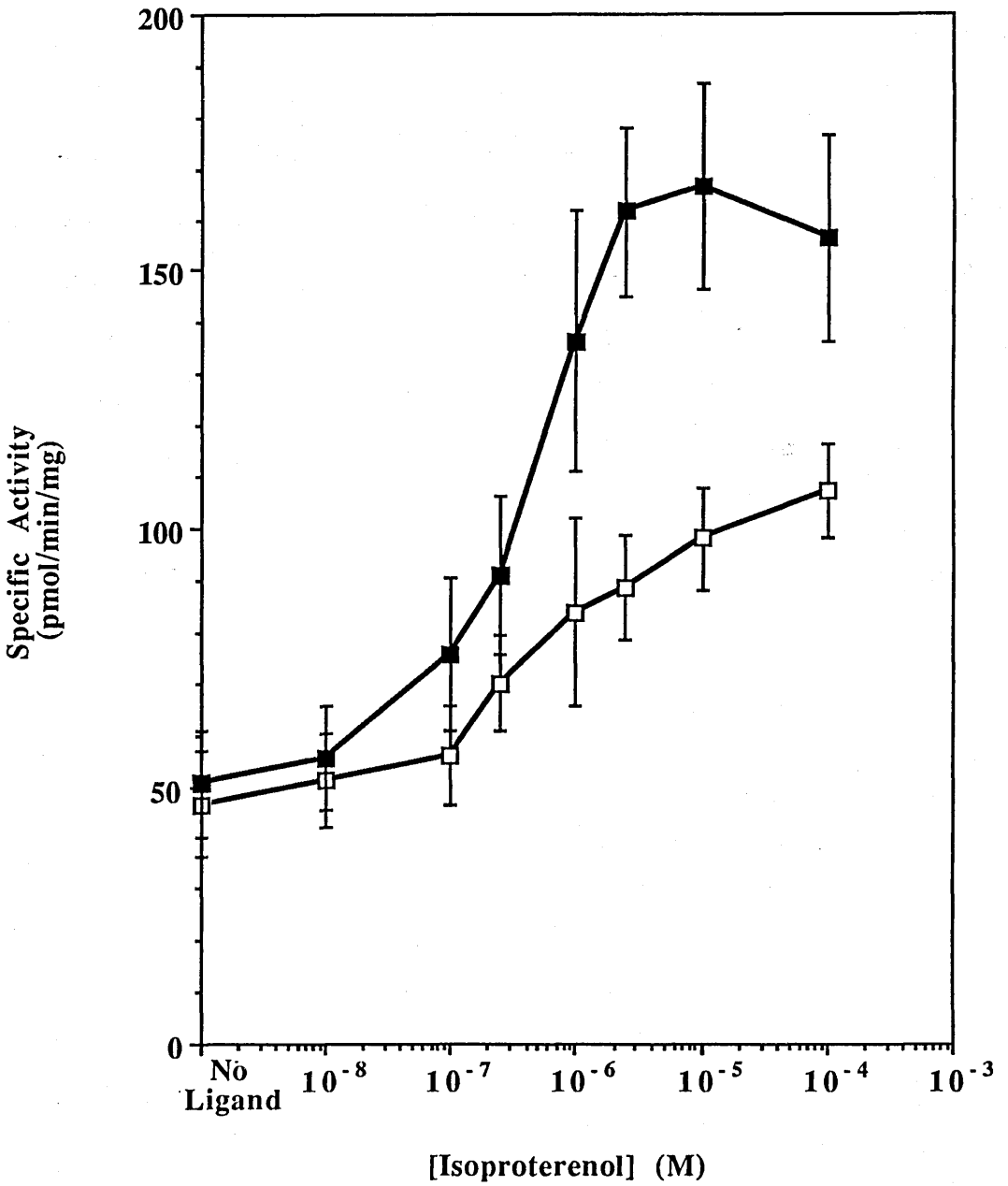

200
150
100
50
0
Specific Activity
(pmol/min/mg)
No
Ligand
10-8
10-7
10-6
10-5
10-4
10-3
[Isoproterenol] (M)

Figure 3.10:

Effect Of Pertussis Toxin Treatment On The Dose-Response Curve For Isoproterenol Stimulation Of Adenylate Cyclase Activity In Liver Plasma Membranes From Diabetic (*db/db*) Animals

Sucrose-purified liver plasma membranes from diabetic (*db/db*) (O) and pertussis toxin-treated diabetic (●) animals were assayed for adenylate cyclase activity for 15 minutes at 30°C in the presence of 100 μM GTP and increasing concentrations of isoproterenol as described in Section 2.7.1. The [^{32}P]cyclic AMP formed was measured by the double column method described in Section 2.7.2. The figure shows results pooled from four experiments performed on different membrane preparations and values are presented as MEAN ± S.D.

The activities obtained with 100 μM GTP alone are given in Table 3.2.

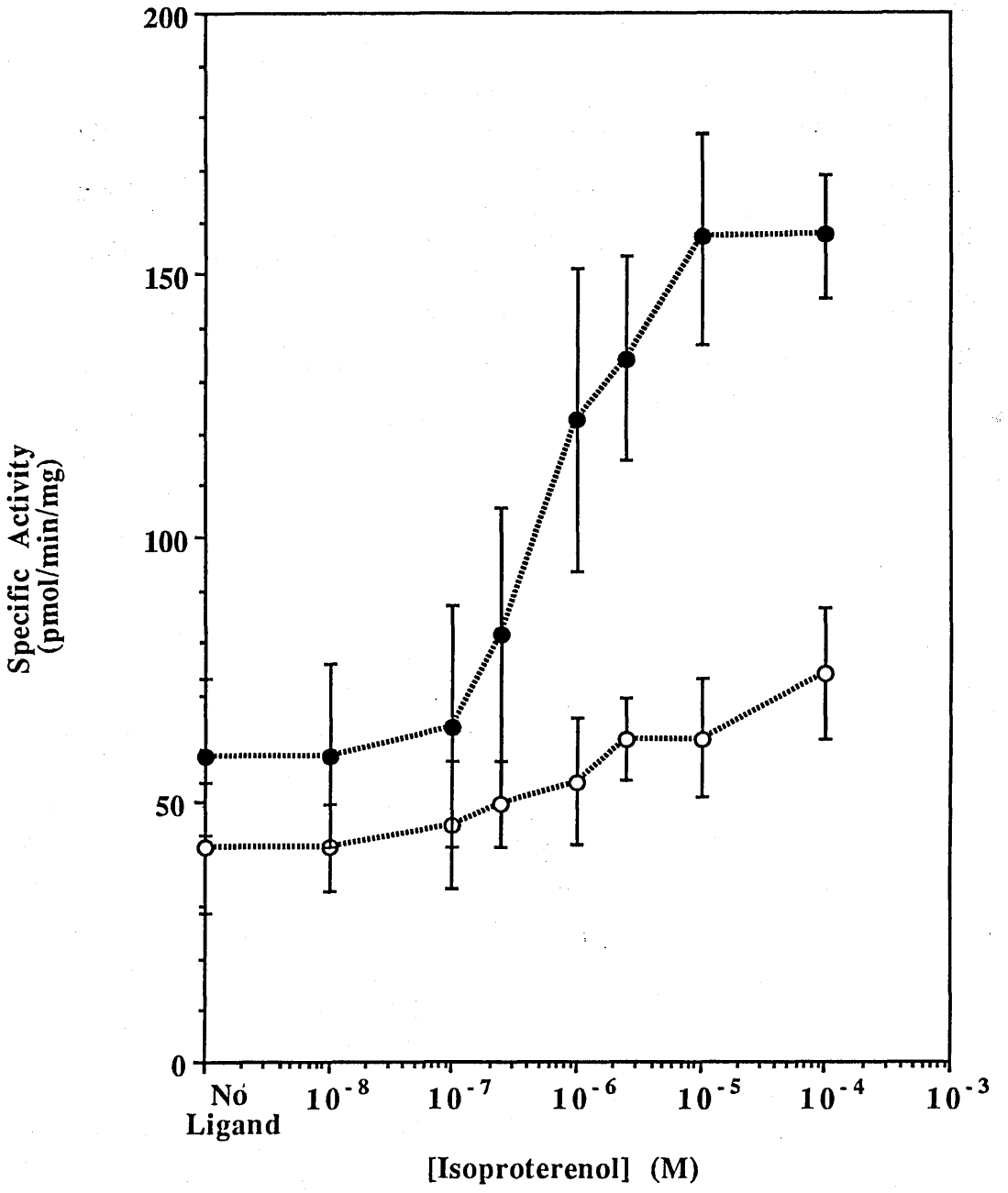

200
150
100
50
0
Specific Activity
(pmol/min/mg)
No
Ligand
10-8
10-7
10-6
10-5
10-4
10-3
[Isoproterenol] (M)

Table 3.2:

Hormonal Stimulation Of Adenylate Cyclase Activity In Mouse Liver Plasma Membranes

Sucrose-purified liver plasma membranes from lean, pertussis toxin-treated lean, diabetic (*db/db*) and pertussis toxin-treated diabetic animals were assayed for adenylate cyclase activity in the presence of either 100 μM GTP, or 100 μM GTP together with 10 μM glucagon or with 100 μM isoproterenol as described in Section 2.7.1. The [^{32}P]cyclic AMP formed was measured by the double column method described in Section 2.7.2. Specific activities are given as pmol/min/mg of protein and expressed as MEAN ± S.D. for four experiments using different membrane preparations.

(Fold activations above GTP-stimulated activities are in parentheses). EC_{50} is the hormone concentration at which half-maximal activation was achieved. 'PTx' denotes the fact that animals were treated with pertussis toxin.

The activities obtained with GTP alone were 43.5 ± 10.6 (lean), 38.2 ± 9.4 (lean-PTx), 50.5 ± 8.2 (diabetic) and 58.3 ± 15.2 (diabetic-PTx) (MEAN ± S.D. from eight experiments).

Table 3.2:

Animal & Treatment	Glucagon	EC_{50} (nM)	Isoproterenol	EC_{50} (μM)
Lean	1081 ± 79 (23.2)	30 ± 7	107 ±9 (2.3)	0.3 ± 0.1
Lean-PTx	1420 ± 136 (28.1)	27 ± 3	156 ± 20 (3.1)	0.6 ± 0.4
Diabetic	918 ± 139 (22.3)	25 ± 4	74 ± 12 (1.8)	0.6 ± 0.3
Diabetic-PTx	1719 ± 177 (29.5)	22 ± 3	157 ± 11 (2.7)	0.9 ± 0.3

Figure 3.11:

Immunological Detection Of $G_i\alpha$-2 But Not $G_i\alpha$-1 In Liver Plasma Membranes From Lean And Diabetic (*db/db*) Animals

Sucrose-purified liver plasma membranes were prepared and subjected to SDS-PAGE under conditions allowing the resolution of $G_i\alpha$-1 and $G_i\alpha$-2 before transfer to nitrocellulose paper and immunoblotting with antiserum SG1, which recognizes $G_i\alpha$-1 and $G_i\alpha$-2, as described in Sections 2.8.4 and 2.9. The tracks contain the following:- lane 1, 400 μg liver plasma membrane protein from lean animals; lane 2, 400 μg liver plasma membrane protein from diabetic (*db/db*) animals; lane 3, 60 μg adipocyte membrane protein from lean animals. This immunoblot is one of two such experiments performed with different membrane preparations from lean and diabetic (*db/db*) animals which yielded identical results.

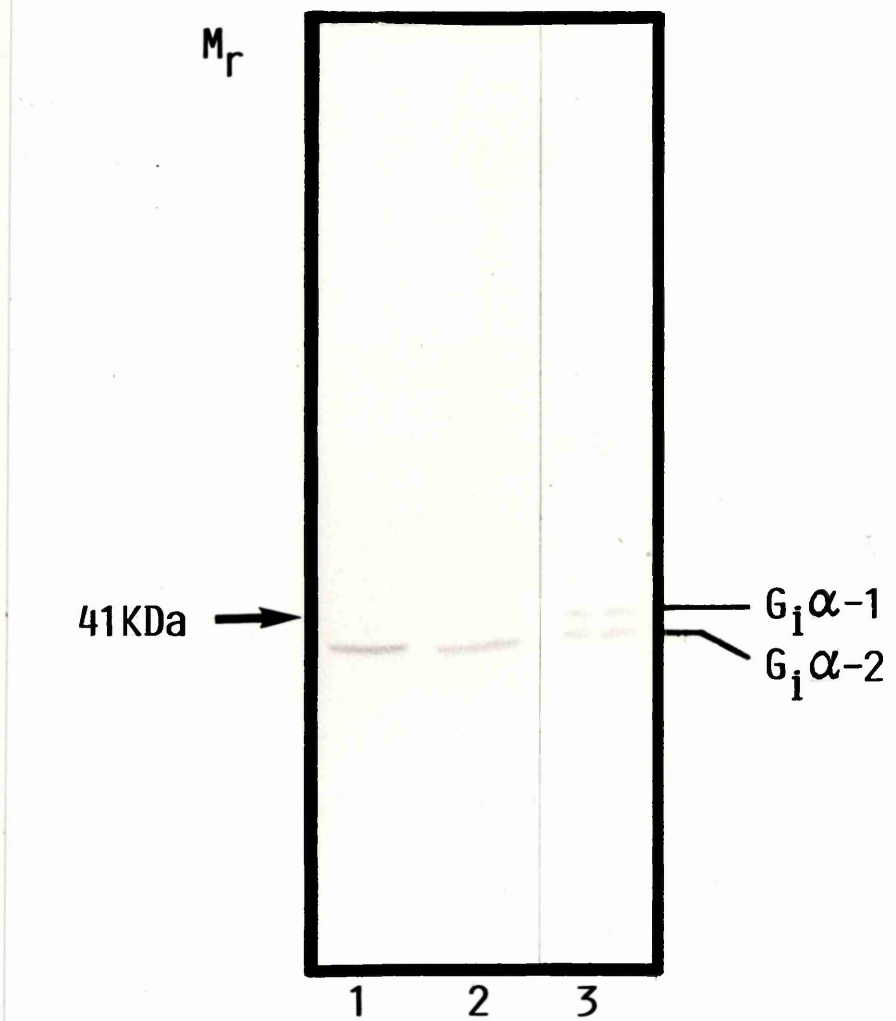

Mr
41KDa
Gi α-1
Gi α-2
1
2
3

Figure 3.12:

Immunoblotting Of $G_i\alpha$-2 In Liver Plasma Membranes From Lean And Diabetic (*db/db*) Animals

Sucrose-purified liver plasma membranes were prepared and subjected to SDS-PAGE under 'normal' conditions before transfer to nitrocellulose paper and immunoblotting using antiserum SG1 as the primary antiserum as described in Sections 2.8 and 2.9. The tracks contain the following:- lane 1, 50 μg membrane protein from lean animals; lane 2, 100 μg membrane protein from lean animals; lane 3, 200 μg membrane protein from lean animals; lane 4, 300 μg membrane protein from lean animals; lane 5, 50 μg membrane protein from diabetic (*db/db*) animals; lane 6, 100 μg membrane protein from diabetic (*db/db*) animals; lane 7, 200 μg membrane protein from diabetic (*db/db*) animals; lane 8, 300 μg membrane protein from diabetic (*db/db*) animals. The immunoblot shown is representative of four performed with different membrane preparations from lean and diabetic animals.

Comparison of levels of $G_i\alpha 2$ in lean and db/db liver plasma membranes

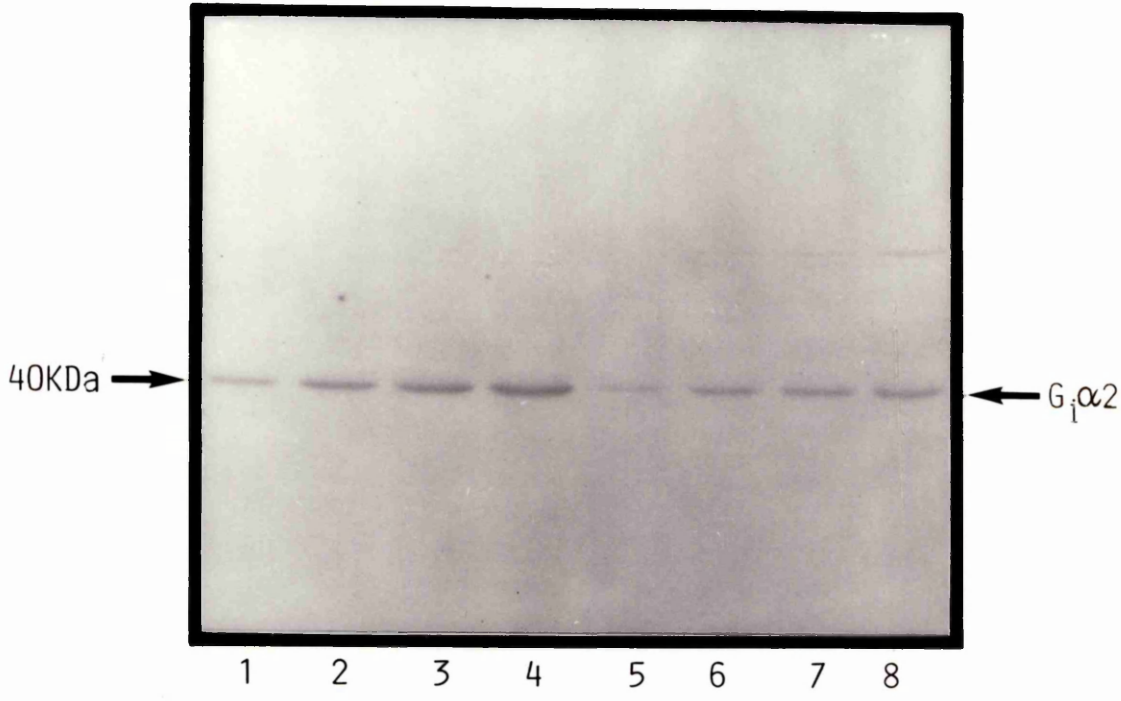


Figure 3.13:

Immunoblotting Of $G_i\alpha$-3 In Liver Plasma Membranes From Lean And Diabetic (*db/db*) Animals

Sucrose-purified liver plasma membranes were prepared and subjected to SDS-PAGE under 'normal' conditions before transfer to nitrocellulose paper and immunoblotting using antiserum I3B as the primary antiserum as described in Sections 2.8 and 2.9. The tracks contain the following:- lane 1, 50 μg membrane protein from lean animals; lane 2, 100 μg membrane protein from lean animals; lane 3, 200 μg membrane protein from lean animals; lane 4, 300 μg membrane protein from lean animals; lane 5, 50 μg membrane protein from diabetic (*db/db*) animals; lane 6, 100 μg membrane protein from diabetic (*db/db*) animals; lane 7, 200 μg membrane protein from diabetic (*db/db*) animals; lane 8, 300 μg membrane protein from diabetic (*db/db*) animals. The immunoblot shown is representative of four performed with different membrane preparations from lean and diabetic animals.

Comparison of levels of $G_i\alpha 3$ in liver plasma membranes from control and db/db mice

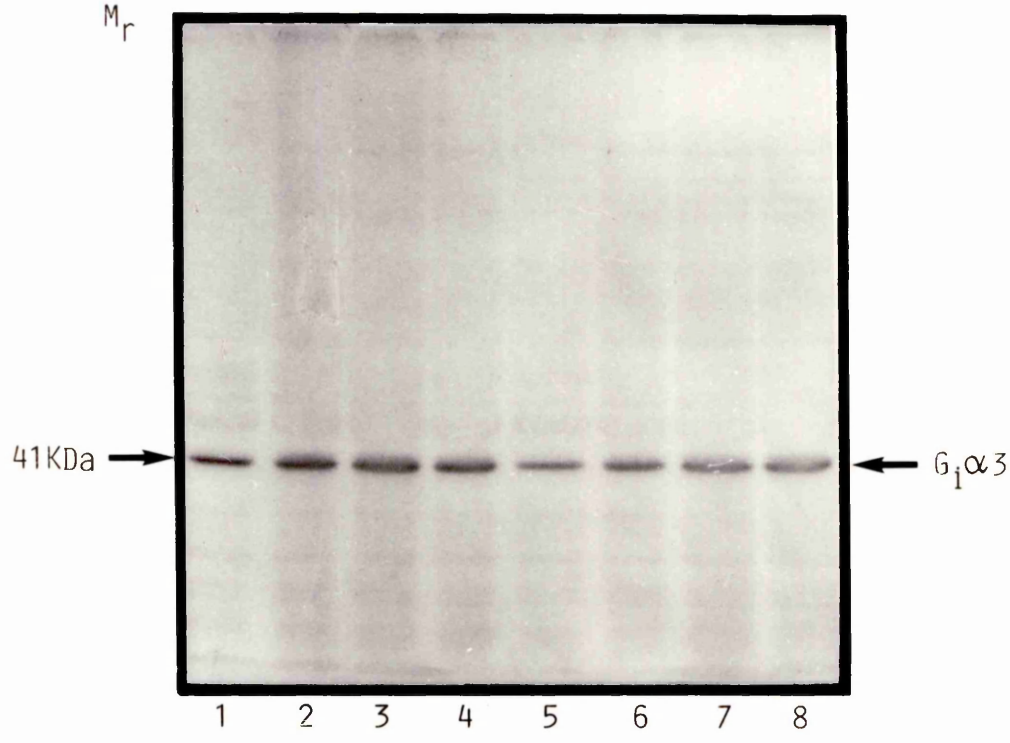
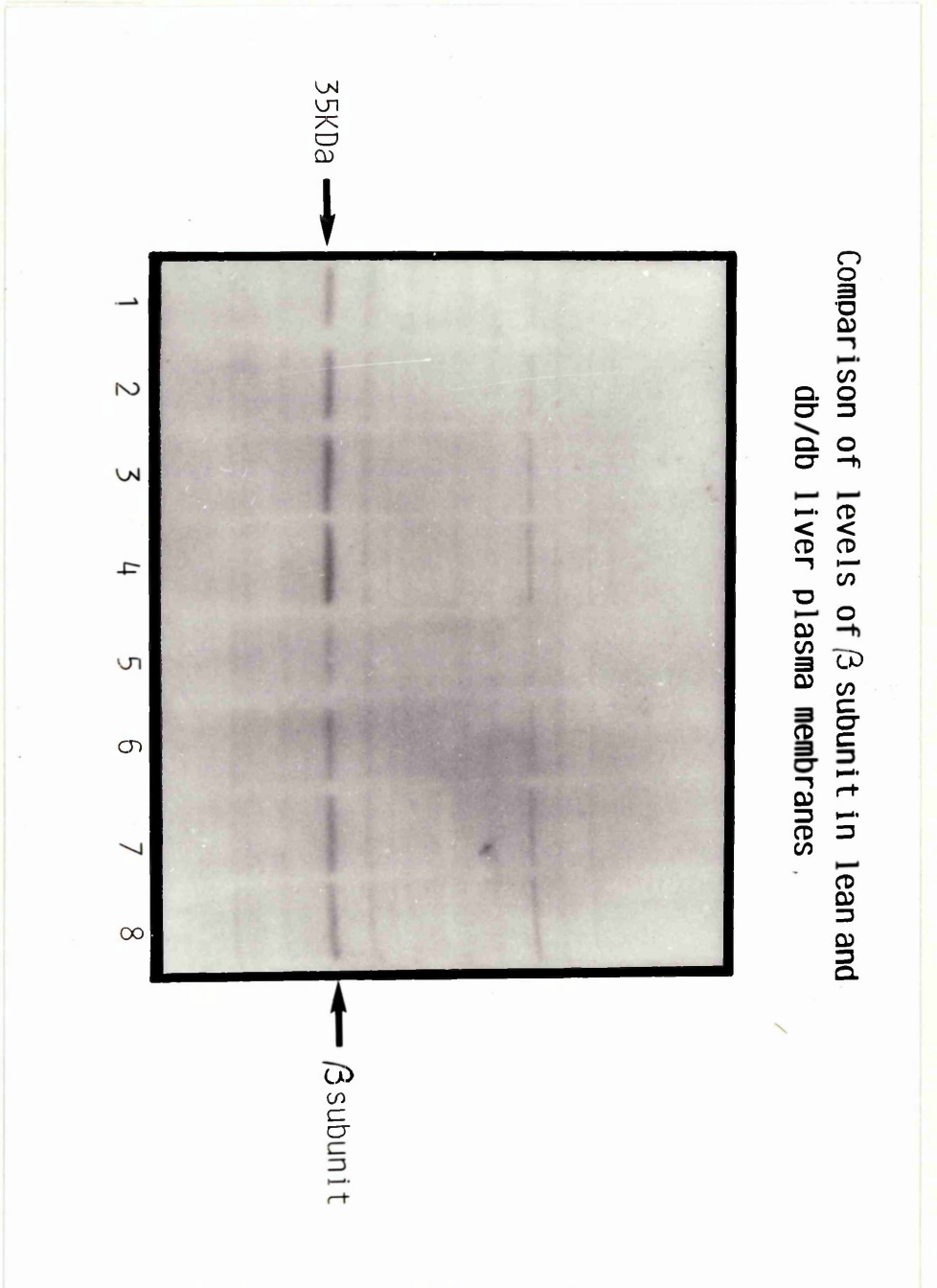


Figure 3.14:

Immunoblotting Of β-Subunits In Liver Plasma Membranes From Lean And Diabetic (*db/db*) Animals

Sucrose-purified liver plasma membranes were prepared and subjected to SDS-PAGE under 'normal' conditions before transfer to nitrocellulose paper and immunoblotting using antiserum BN1 as the primary antiserum as described in Sections 2.8 and 2.9. The tracks in the immunoblot shown in *panel a* contain the following:- lane 1, 50 μg membrane protein from lean animals; lane 2, 100 μg membrane protein from lean animals; lane 3, 200 μg membrane protein from lean animals; lane 4, 300 μg membrane protein from lean animals; lane 5, 50 μg membrane protein from diabetic (*db/db*) animals; lane 6, 100 μg membrane protein from diabetic (*db/db*) animals; lane 7, 200 μg membrane protein from diabetic (*db/db*) animals; lane 8, 300 μg membrane protein from diabetic (*db/db*) animals. The immunoblot shown is representative of four performed with different membrane preparations from lean and diabetic animals. *Panel b* shows a plot of band intensity versus amount of protein loaded for membranes from lean and diabetic animals in the blot shown in *panel a.*

Panel a



Comparison of levels of β subunit in lean and db/db liver plasma membranes.

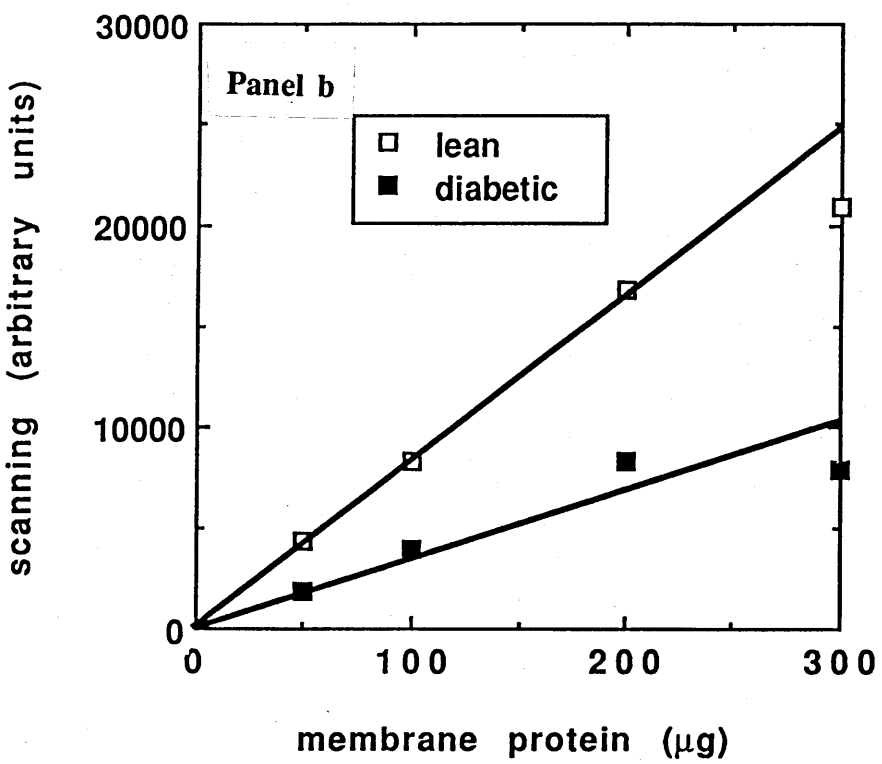

Panel b
lean
diabetic
30000
20000
10000
0
0
100
200
300
scanning (arbitrary units)
membrane protein (μg)

Figure 3.15:

Immunoblotting Of $G_s\alpha$ In Liver Plasma Membranes From Lean And Diabetic (*db/db*) Animals

Sucrose-purified liver plasma membranes were prepared and subjected to SDS-PAGE under 'normal' conditions before transfer to nitrocellulose paper and immunoblotting using antiserum CS1 as the primary antiserum as described in Sections 2.8 and 2.9. The tracks contain the following:- lane 1, 25 μg membrane protein from lean animals; lane 2, 50 μg membrane protein from lean animals; lane 3, 75 μg membrane protein from lean animals; lane 4, 25 μg membrane protein from diabetic (*db/db*) animals; lane 5, 50 μg membrane protein from diabetic (*db/db*) animals; lane 6, 75 μg membrane protein from diabetic (*db/db*) animals. The immunoblot shown is representative of four performed with different membrane preparations from lean and diabetic animals.

Comparison of levels of $G_s\alpha$ in lean and db/db liver plasma membranes

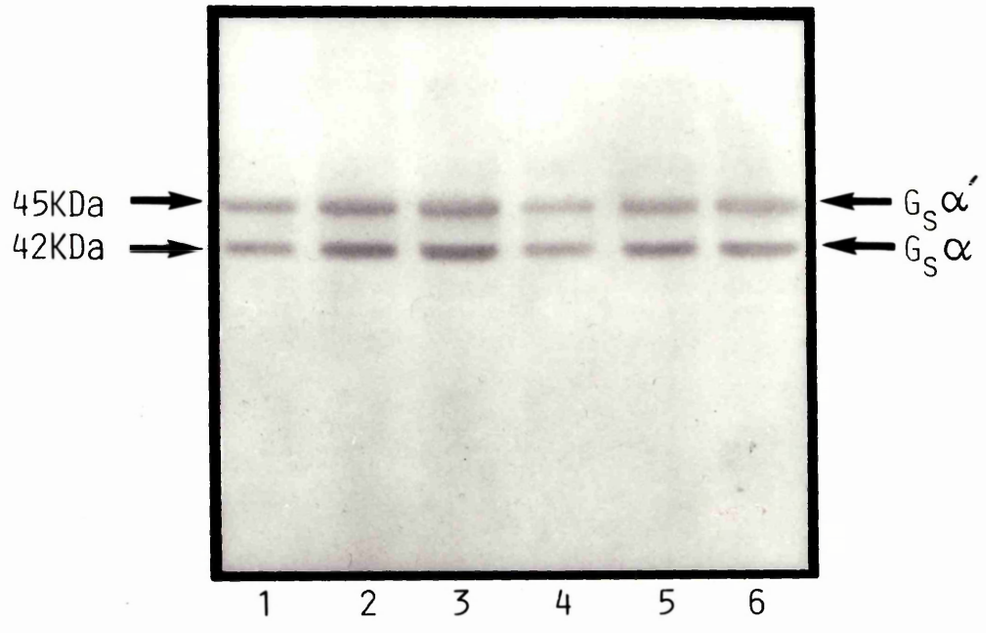


Table 3.3:

Comparative Levels Of G-Protein Subunits In Liver Plasma Membranes From Lean And Diabetic (*db/db*) Animals

Sucrose-purified liver plasma membranes from lean and diabetic (*db/db*) animals were prepared and subjected to SDS-PAGE before transfer to nitrocellulose and immunoblotting with the primary antisera described in the appropriate figure legends and in. Sections 2.8 and 2.9. Data express levels of G-protein components in diabetic (*db/db*) animals as a percentage of those found in lean animals. In each case, a range of amounts of membrane protein from lean and diabetic (*db/db*) animals was loaded on a single gel and comparisons made over conditions where linear increases in absorption were noted for increasing amounts of membrane protein applied when the blots were densitometrically scanned after visualisation with a peroxidase-conjugated second antibody. For each G-protein subunit, data are expressed as MEAN ± S.D. for four immunoblots performed with different membrane preparations. * denotes a significant difference between lean and diabetic animals, *i.e.* $p<0.005$.

G-Protein Subunit	Level As A Percentage Of That In Lean Animals (100%)
$G_i\alpha$-2	30 ± 10*
$G_i\alpha$-3	53 ± 6*
β-subunit	33 ± 8*
$G_s\alpha$ (42 kDa)	102 ± 8
$G_s\alpha$ (45 kDa)	95 ± 10

Figure 3.16:

Effect Of Pertussis Toxin Treatment On The Immunodetection Of $G_i\alpha$-2 And $G_i\alpha$-3 In Liver Plasma Membranes From Lean And Diabetic (*db/db*) Animals

200 μg sucrose-purified liver plasma membranes from lean (lanes 1 & 2) and diabetic animals (lanes 3 & 4) that either had (lanes 1 & 3) or had not (lanes 2 & 4) been treated with pertussis toxin *in vivo* were prepared and subjected to SDS-PAGE, transferred to nitrocellulose and immunoblotted using SG1 and I3B as primary antisera as described in Sections 2.8 and 2.9. These antisera allow the detection of $G_i\alpha$-2 and $G_i\alpha$-3 respectively. The immunoblots shown are representative of two experiments performed with different membrane preparations.

Western blot analysis of $G_i\alpha$ subunits
in pertussis toxin-treated mice

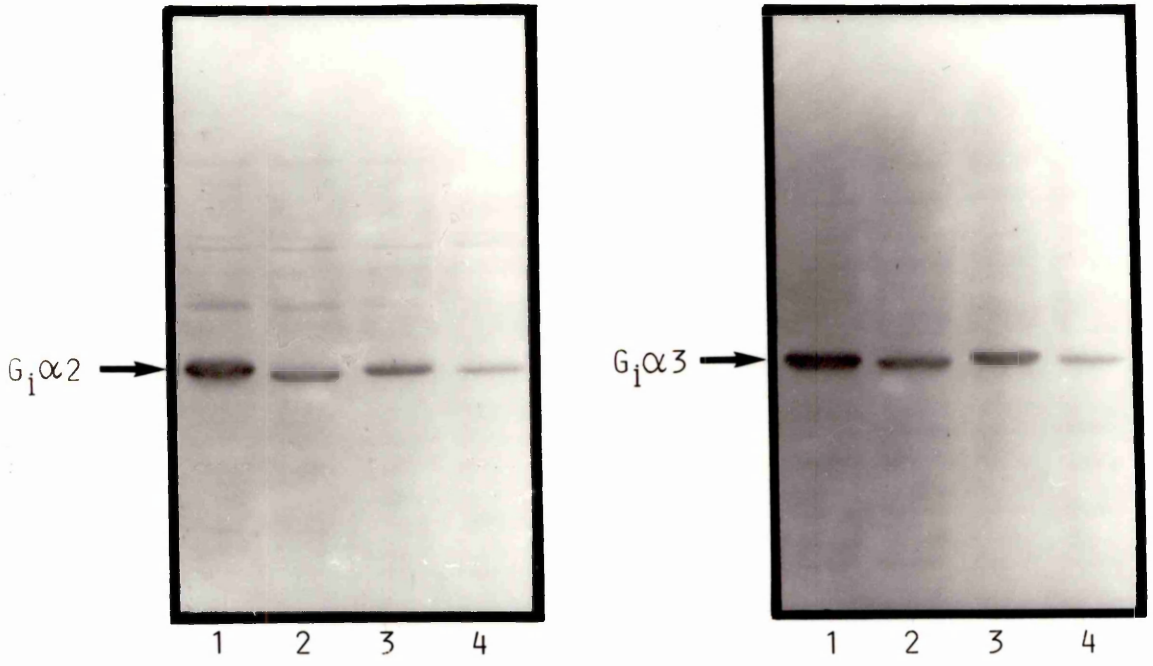


Figure 3.17:

Effect Of Pertussis Toxin Treatment On The Immunodetection Of $G_s\alpha$- And β-Subunits In Liver Plasma Membranes From Lean And Diabetic (*db/db*) Animals

Sucrose-purified liver plasma membranes prepared from lean (lanes 1 & 3) and diabetic (*db/db*) (lanes 2 & 4) animals which either had (lanes 3 & 4) or had not (lanes 1 & 2) been treated with pertussis toxin *in vivo* were prepared and subjected to SDS-PAGE, transferred to nitrocellulose and immunoblotted using antisera CS1 and BN1 as primary antisera as described in Sections 2.8 and 2.9. These antisera allow the detection of $G_s\alpha$- and β-subunits respectively. When immunoblotting with antiserum CS1, 40 μg of membrane protein were loaded per lane. 200 μg of membrane protein were used when immunoblotting with antiserum BN1. The immunoblots shown are representative of two experiments performed with different membrane preparations.

Western blot analysis of $G_s\alpha$ and β subunits
in pertussis toxin-treated mice

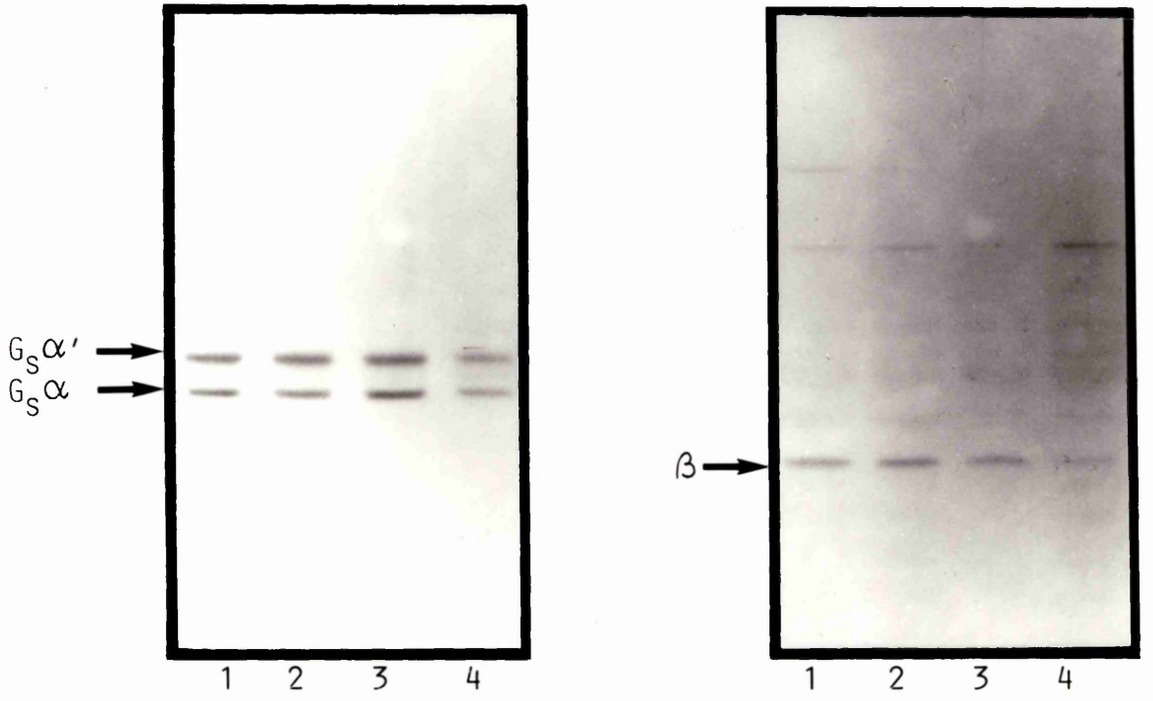


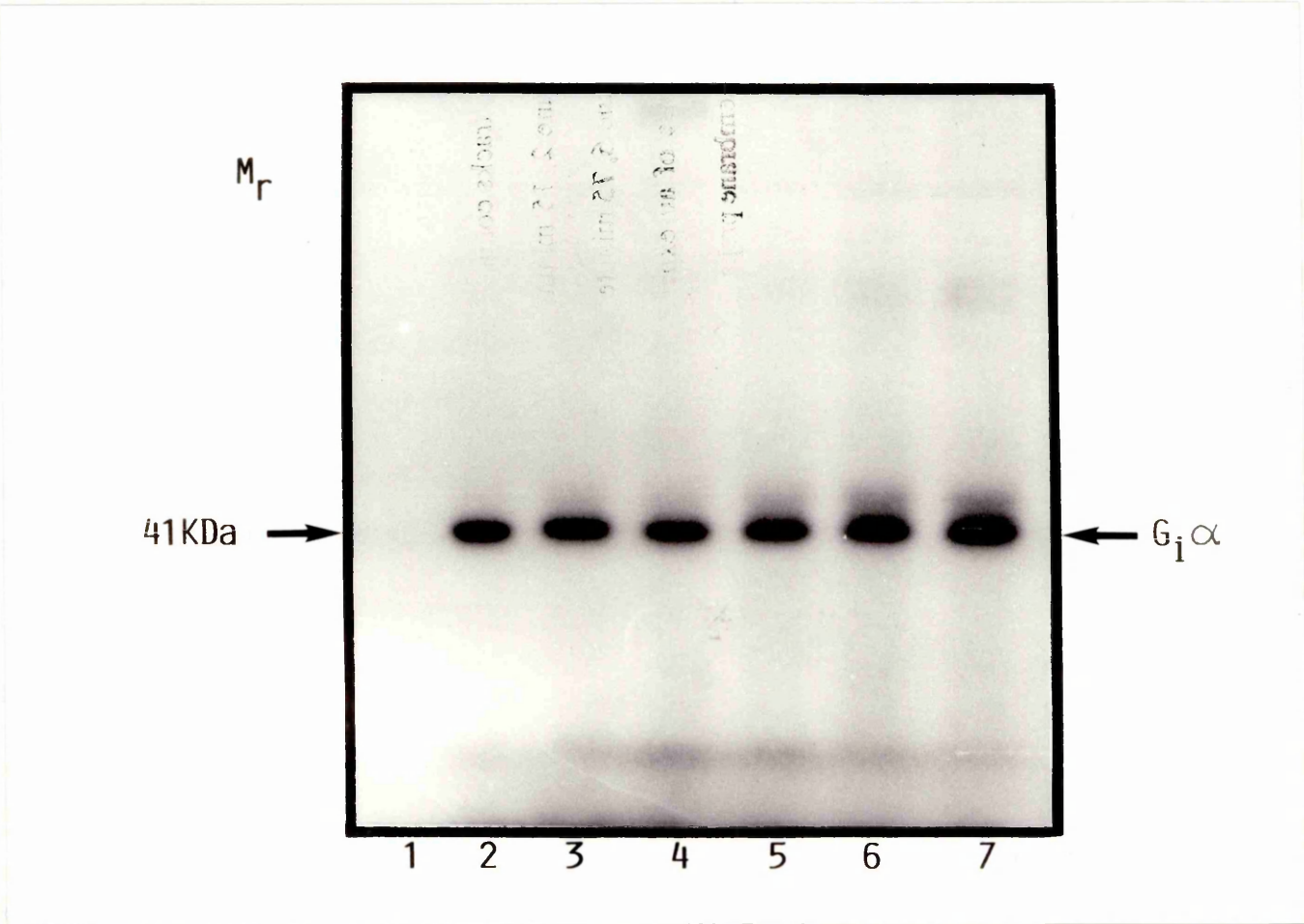
Figure 3.18:

Time-Course Of Pertussis Toxin-Catalysed ADP-Ribosylation Of Liver Plasma Membranes From Lean Animals

Pertussis toxin-catalysed ADP-ribosylation of 100 μg of sucrose-purified liver plasma membranes from lean animals was carried out *in vitro* with thiol-preactivated toxin and [α-^{32}P]NAD^+ at 30°C for various times before the reaction was stopped. The samples were then prepared for and subjected to SDS-PAGE before drying down of the gel for autoradiography as described in Section 2.11.

The tracks contain samples ribosylated for the following times:- lane 1, 0 minutes; lane 2, 15 minutes; lane 3, 30 minutes; lane 4, 45 minutes; lane 5, 60 minutes; lane 6, 75 minutes; lane 7, 90 minutes. The autoradiograph shown is a 2 day exposure of an experiment which is representative of three performed on different membrane preparations which gave essentially identical results.

The graph overleaf shows a plot of band intensity versus incubation time for the autoradiograph.


M_r
41KDa
$G_i\alpha$
1
2
3
4
5
6
7

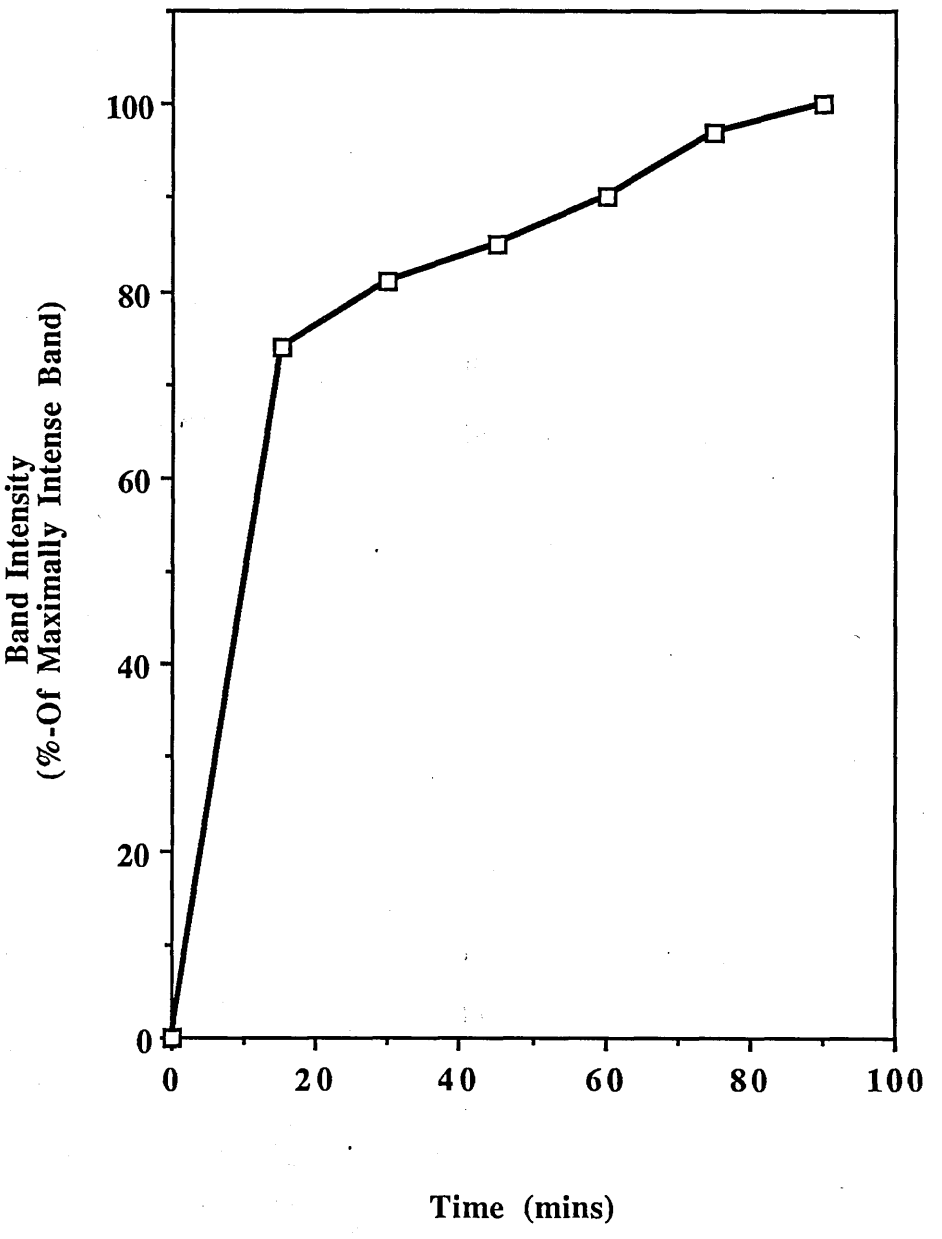

Band Intensity
(%-Of Maximally Intense Band)
100
80
60
40
20
0
0
20
40
60
80
100
Time (mins)

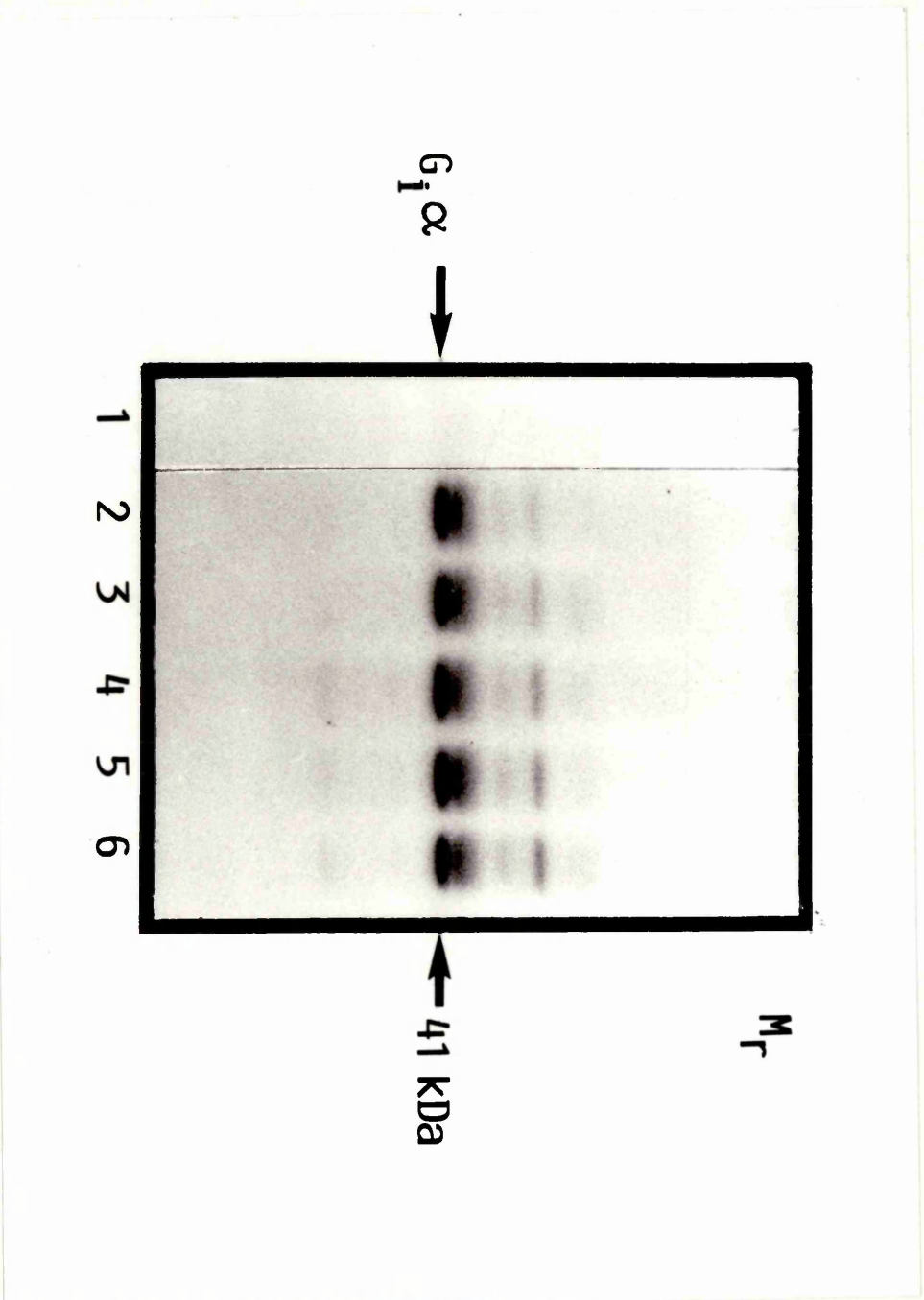
Figure 3.19:

Time-Course Of Pertussis Toxin-Catalysed ADP-Ribosylation Of Liver Plasma Membranes From Diabetic (*db/db*) Animals

Pertussis toxin-catalysed ADP-ribosylation of 100 μg of sucrose-purified liver plasma membranes from diabetic (*db/db*) animals was carried out *in vitro* with thiol-preactivated toxin and [α-^{32}P] NAD^+ at 30°C for various times before the reaction was stopped. The samples were then prepared for and subjected to SDS-PAGE before drying down of the gel for autoradiography as described in Section 2.11.

The tracks contain samples ribosylated for the following times:- lane 1, 0 minutes; lane 2, 15 minutes; lane 3, 30 minutes; lane 4, 45 minutes; lane 5, 60 minutes; lane 6, 90 minutes. The autoradiograph shown represents a 5 day exposure of an experiment which is representative of three performed on different membrane preparations which gave essentially identical results.

The graph overleaf shows a plot of band intensity versus incubation time for the autoradiograph.


$G_i\alpha$
1
2
3
4
5
6
41 kDa
M_r

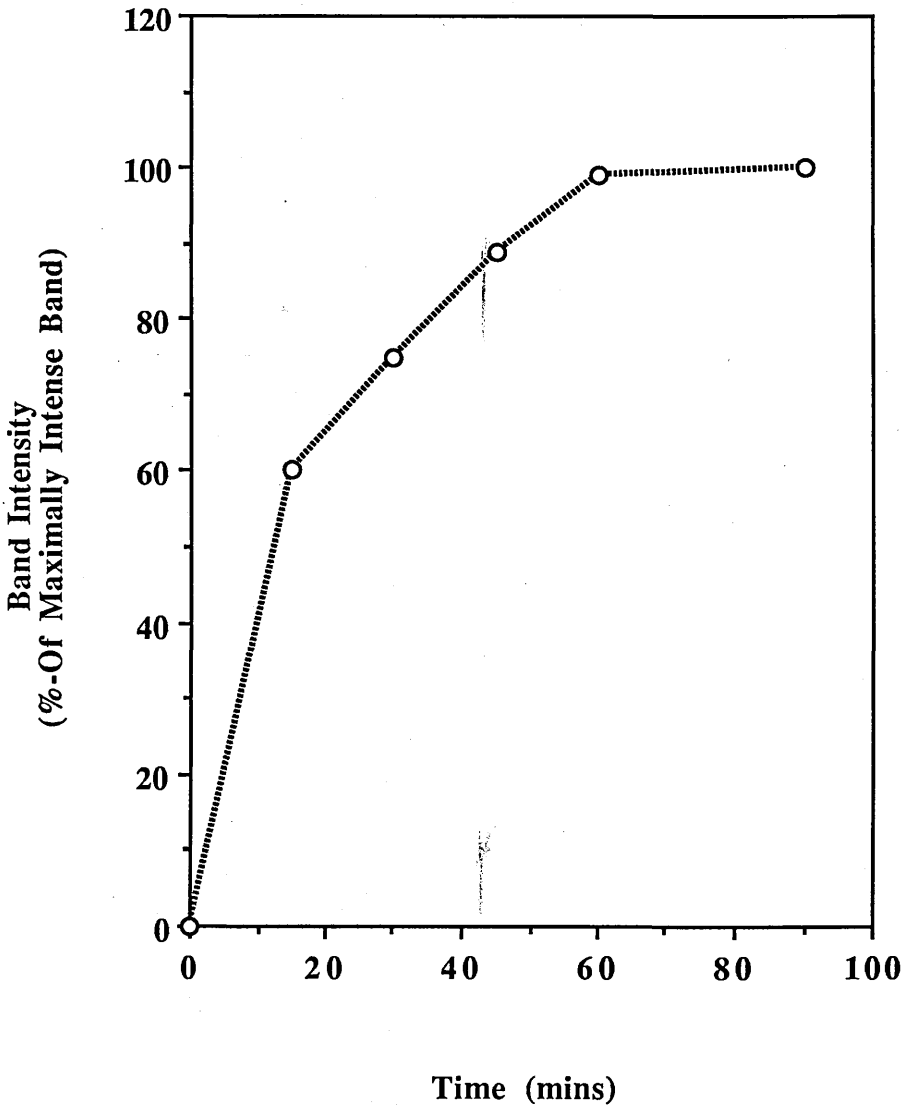

120
100
80
60
40
20
0
0
20
40
60
80
100
Band Intensity
(%-Of Maximally Intense Band)
Time (mins)

Figure 3.20:

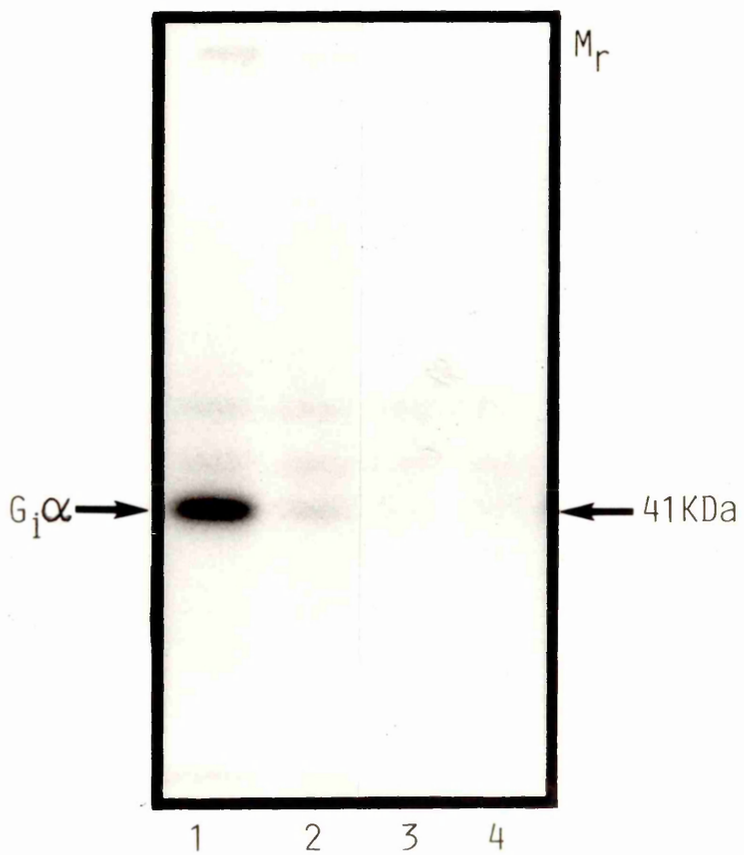
Pertussis Toxin-Catalysed ADP-Ribosylation Of Mouse Liver Plasma Membranes

Pertussis toxin-catalysed ADP-ribosylation of 100 μg of sucrose-purified liver plasma membranes from lean and diabetic (*db/db*) animals which either had or had not been treated with pertussis toxin was carried out *in vitro* with thiol-preactivated toxin and [α-^{32}P] NAD^+ at 30°C for 45 minutes before the reaction was stopped. The samples were then prepared for and subjected to SDS-PAGE before drying down of the gel for autoradiography as described in Section 2.11.

The tracks in *panel a* contain the following:- lane 1, 100 μg liver plasma membranes from lean animals; lane 2, 100 μg liver plasma membranes from diabetic (*db/db*) animals; lane 3, 100 μg liver plasma membranes from lean animals treated with pertussis toxin; lane 4, 100 μg liver plasma membranes from diabetic (*db/db*) animals treated with pertussis toxin. The autoradiograph shown is a 2 day exposure of an experiment which is representative of four performed on different membrane preparations which gave essentially identical results. *Panel b* shows the gel from *panel a* after staining for protein using Coomassie Brilliant Blue R. This shows that equal amounts of membrane protein were added in each lane, and that neither the diabetic state nor pertussis toxin treatment *in vivo* had any gross effect on the electrophoretic profile of the liver plasma membrane preparations.

Panel a

Pertussis toxin-catalysed ADP-ribosylation
of mouse liver plasma membranes



Panel b

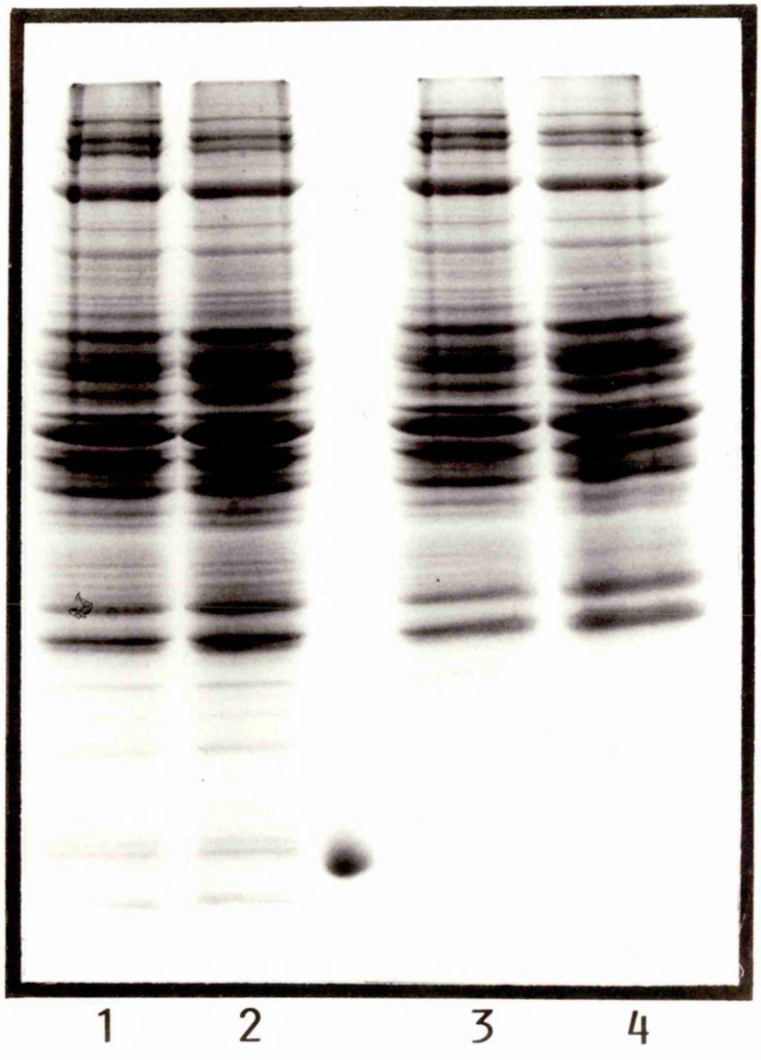


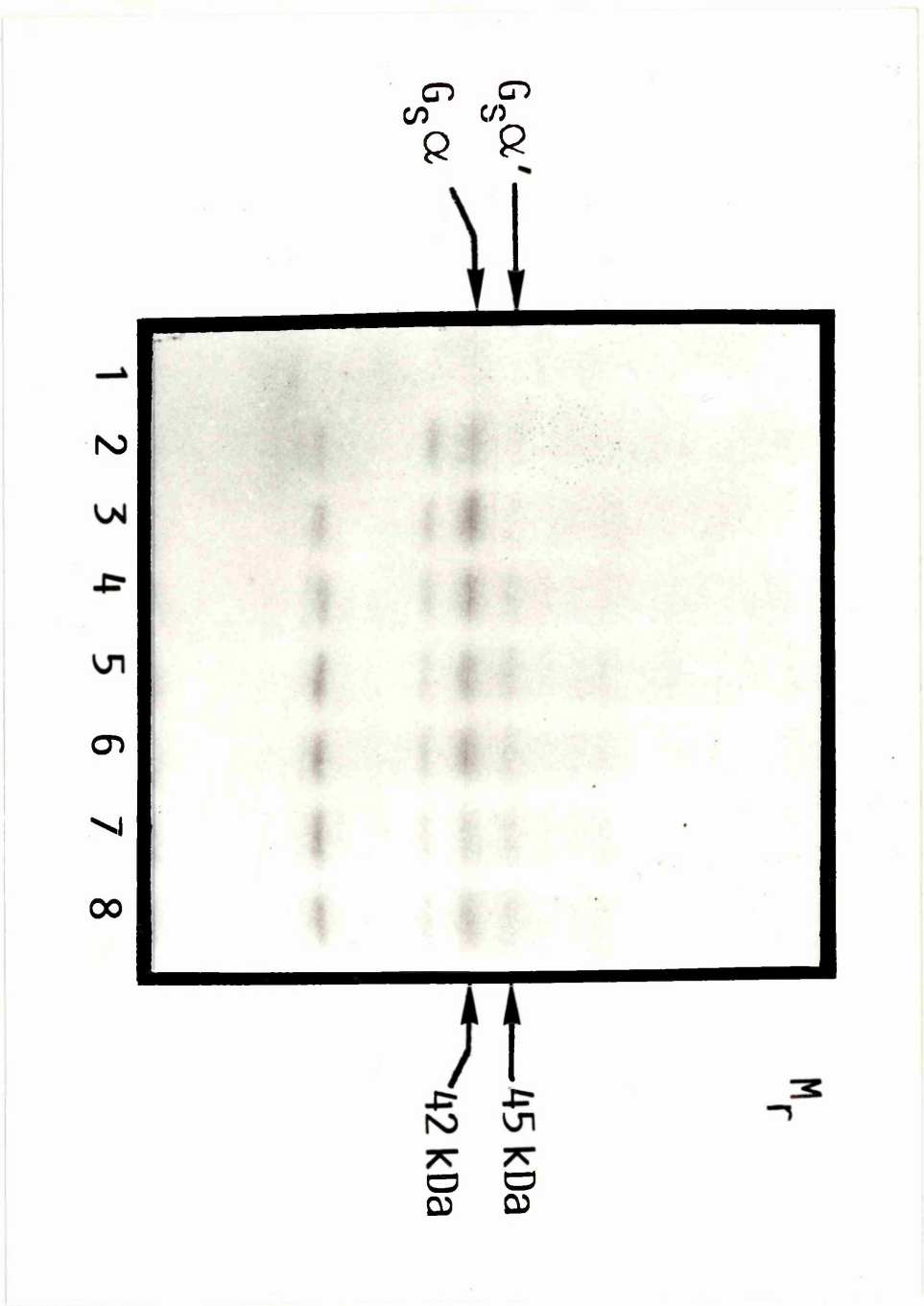
Figure 3.21:

Time-Course Of Cholera Toxin-Catalysed ADP-Ribosylation Of Liver Plasma Membranes From Lean Animals

Cholera toxin-catalysed ADP-ribosylation of 100 μg of sucrose-purified liver plasma membranes from lean animals was carried out *in vitro* with thiol-preactivated toxin and [α-^{32}P]NAD^+ at 30°C for various times before the reaction was stopped. The samples were then prepared for and subjected to SDS-PAGE before drying down of the gel for autoradiography as described in Section 2.10.

The tracks contain samples ribosylated for the following times:- lane 1, 0 minutes; lane 2, 5 minutes; lane 3, 10 minutes; lane 4, 15 minutes; lane 5, 20 minutes; lane 6, 25 minutes; lane 7, 30 minutes; lane 8, 40 minutes. The autoradiograph shown is a 2 day exposure of an experiment which is representative of three performed on different membrane preparations which gave essentially identical results.

The graph overleaf shows plots of band intensity versus incubation time for the two forms of $G_s\alpha$ in the autoradiograph.


$G_s\alpha$
$G_s\alpha'$
1 2 3 4 5 6 7 8
42 kDa
45 kDa
M_r

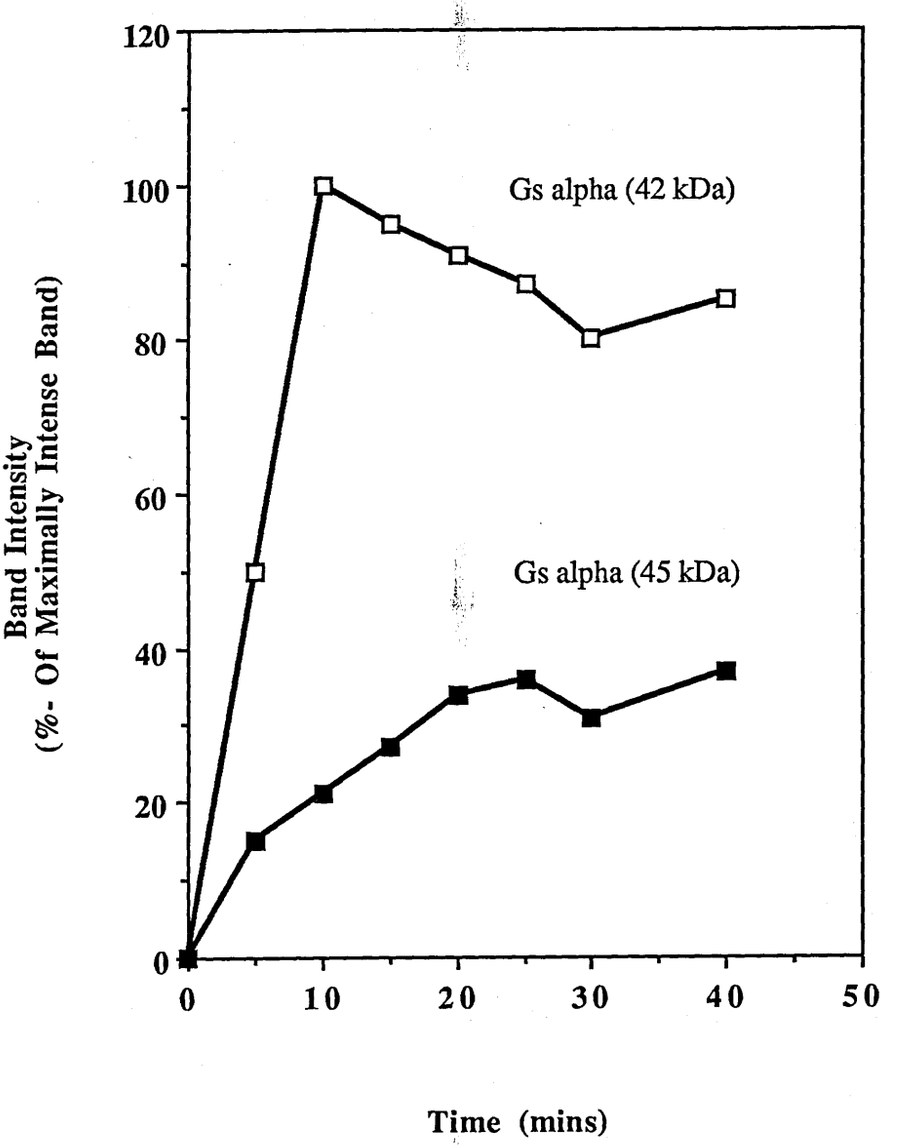

120
100
80
60
40
20
0
0
10
20
30
40
50
Gs alpha (42 kDa)
Gs alpha (45 kDa)
Band Intensity
(%- Of Maximally Intense Band)
Time (mins)

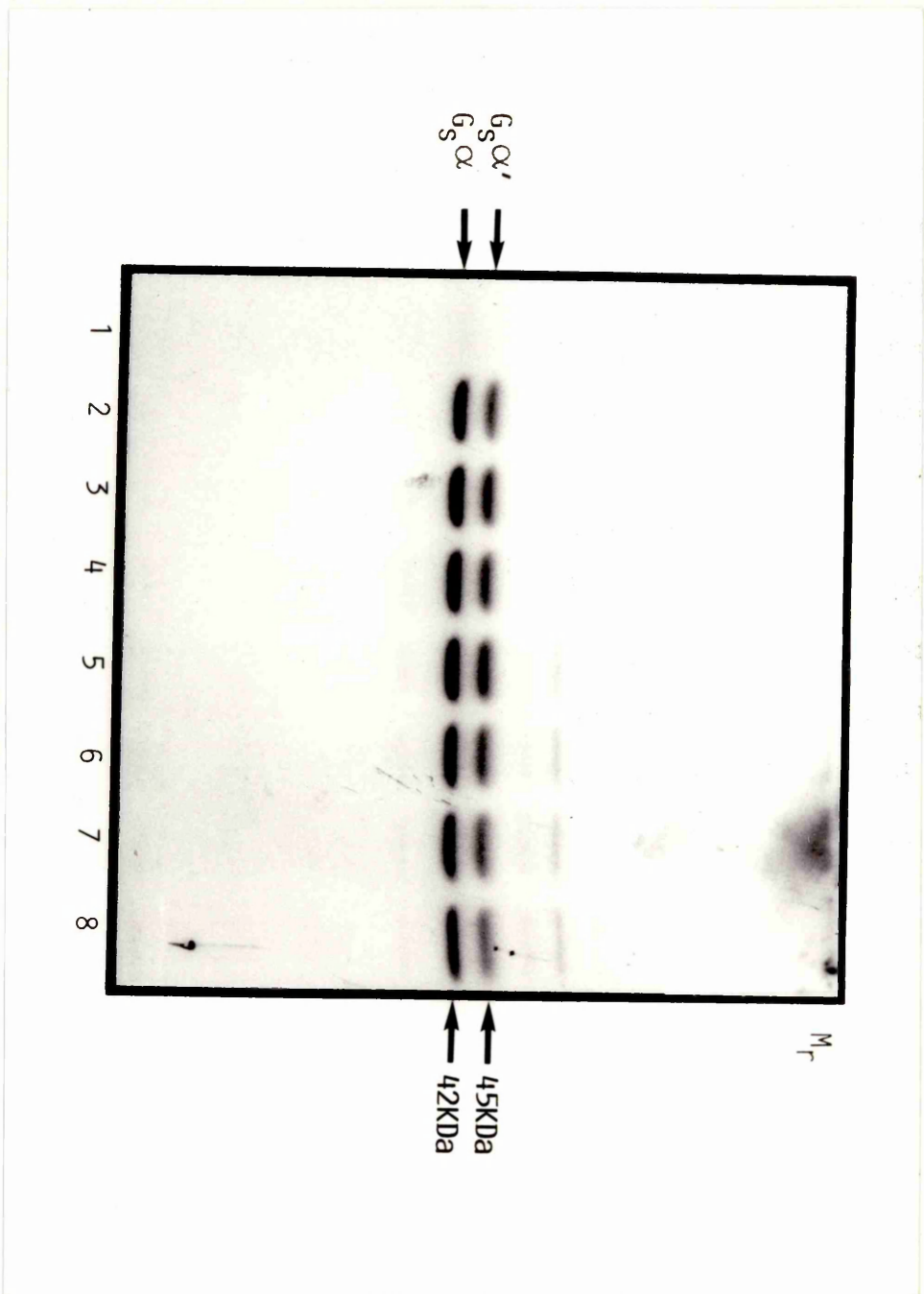
Figure 3.22:

Time-Course Of Cholera Toxin-Catalysed ADP-Ribosylation Of Liver Plasma Membranes From Diabetic (*db/db*) Animals

Cholera toxin-catalysed ADP-ribosylation of 100 μg of sucrose-purified liver plasma membranes from diabetic (*db/db*) animals was carried out *in vitro* with thiol-preactivated toxin and [α-^{32}P]NAD^{+} at 30°C for various times before the reaction was stopped. The samples were then prepared for and subjected to SDS-PAGE before drying down of the gel for autoradiography as described in Section 2.10.

The tracks contain samples ribosylated for the following times:- lane 1, 0 minutes; lane 2, 5 minutes; lane 3, 10 minutes; lane 4, 15 minutes; lane 5, 20 minutes; lane 6, 25 minutes; lane 7, 30 minutes; lane 8, 40 minutes. The autoradiograph shown represents a 2 day exposure of an experiment which is representative of three performed on different membrane preparations which gave essentially identical results.

The graph overleaf shows plots of band intensity versus incubation time for the two forms of $G_s\alpha$ in the autoradiograph.


$G_s\alpha'$
$G_s\alpha$
1
2
3
4
5
6
7
8
M_r
45KDa
42KDa

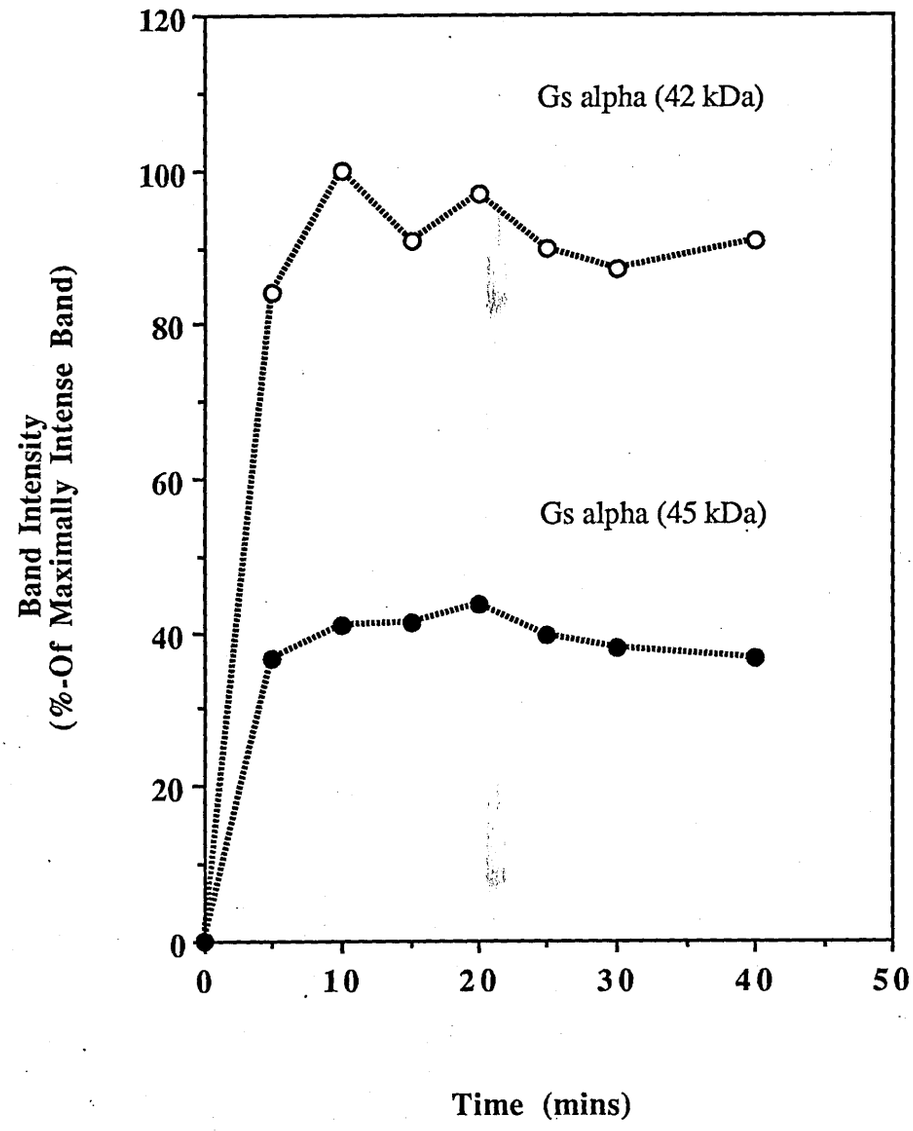

120
100
80
60
40
20
0
Gs alpha (42 kDa)
Gs alpha (45 kDa)
Band Intensity
(%-Of Maximally Intense Band)
0
10
20
30
40
50
Time (mins)

Figure 3.23:

Cholera Toxin-Catalysed ADP-Ribosylation Of Mouse Liver Plasma Membranes

Cholera toxin-catalysed ADP-ribosylation of 100 μg of sucrose-purified liver plasma membranes from lean and diabetic (*db/db*) animals was carried out *in vitro* with thiol-preactivated toxin and [α-^{32}P]NAD^+ at 30°C for 15 minutes before the reaction was stopped. The samples were then prepared for and subjected to SDS-PAGE before drying down of the gel for autoradiography as described in Section 2.10.

The tracks contain the following samples:- lane 1, 100 μg liver membranes from lean animals incubated without cholera toxin; lane 2, 100 μg liver membranes from diabetic animals; lane 3, 100 μg liver membranes from lean animals. The autoradiograph shown is a 2 day exposure of an experiment which is representative of four performed on different membrane preparations which gave essentially identical results.

Cholera toxin-catalysed ADP-ribosylation
of control and db/db liver plasma membranes

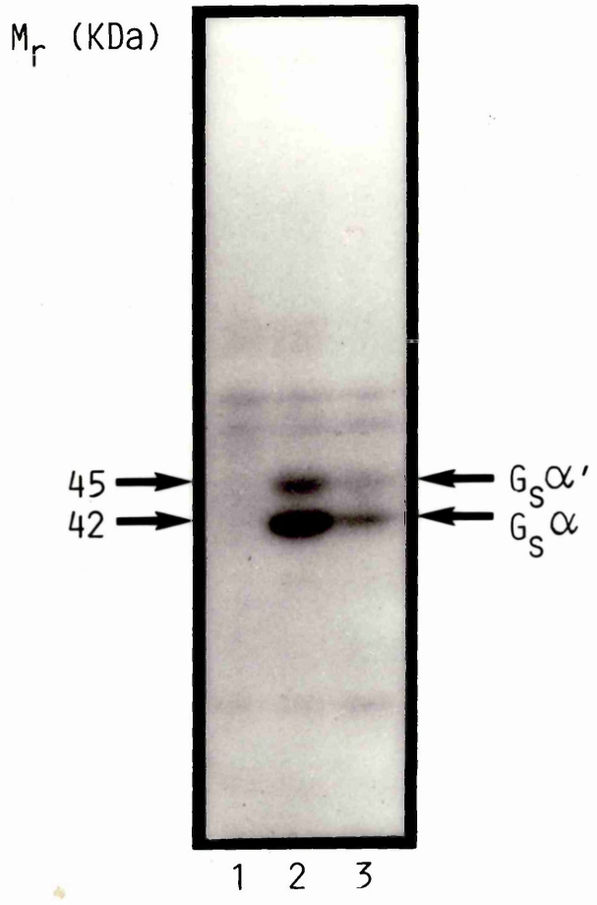


Table 3.4:

Bacterial Toxin-Catalysed ADP-Ribosylation Of Mouse Liver Plasma Membranes

Sucrose-purified liver plasma membranes from lean and diabetic (*db/db*) animals were subjected to ADP-ribosylation by cholera and pertussis toxins as described in Sections 2.10 and 2.11 and the legends to Figures 3.20 and 3.23. Non-saturating autoradiographs were densiometrically scanned as described in Section 2.10. Data are expressed as %-absorption of the relevant G-protein subunit in the diabetic samples compared with lean membranes (100%) and are presented as MEAN ± S.D. of four experiments performed with different membrane preparations from lean and diabetic (*db/db*) animals .* denotes a significant difference between lean and diabetic animals, *i.e.* $p<0.001$.

G-Protein Subunit	Level As a Percentage (%) Of That Found In Lean Animals (100%)
$G_i\alpha$ (41 kDa)	14 ± 5*
$G_s\alpha$ (42 kDa)	364 ± 57*
$G_s\alpha$ (45 kDa)	393 ± 80*

Figure 3.24:

Dose-Response Curves For Gpp(NH)p Stimulation Of Adenylate Cyclase Activity In The Presence Of 1 μM Glucagon In Liver Plasma Membranes From Lean And Diabetic (*db/db*) Animals

Sucrose-purified liver plasma membranes from lean (❑) and diabetic (*db/db*) (O) animals were assayed for adenylate cyclase activity for 15 minutes at 30°C in the presence of 1 μM glucagon and increasing concentrations of Gpp(NH)p as described in Section 2.7.1. The [^{32}P]cyclic AMP formed was measured by the double column method described in Section 2.7.2. The figure shows an experiment representative of three performed on different membrane preparations which gave quantitatively similar results.

In this experiment, the specific activities obtained in the presence of 1 μM glucagon alone were 111.9 ± 9.0 (lean) and 141.5 ± 2.1 (diabetic) pmol/min/mg.

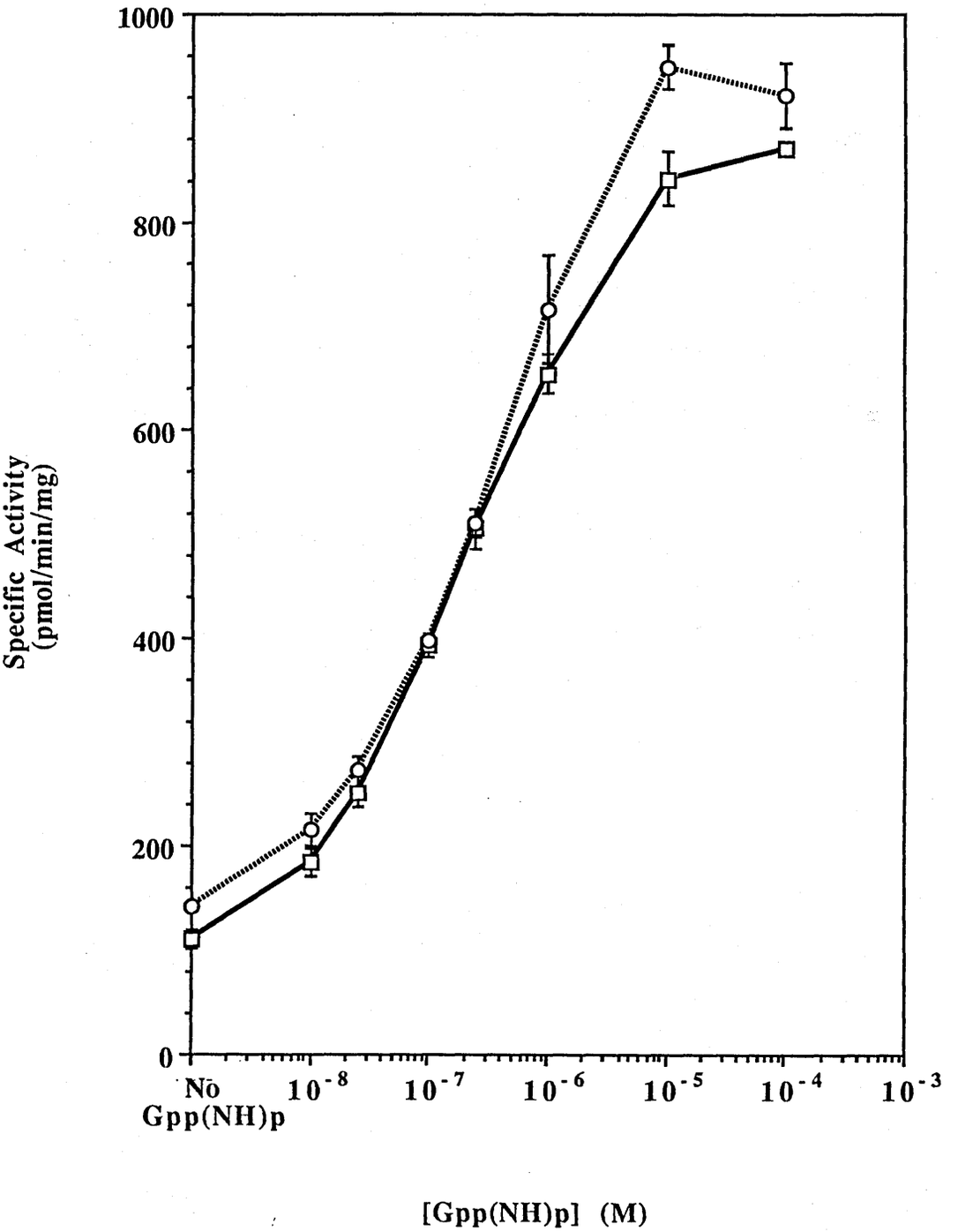

1000
800
600
400
200
0
Specific Activity
(pmol/min/mg)
No
Gpp(NH)p
10-8
10-7
10-6
10-5
10-4
10-3
[Gpp(NH)p] (M)

Figure 3.25:

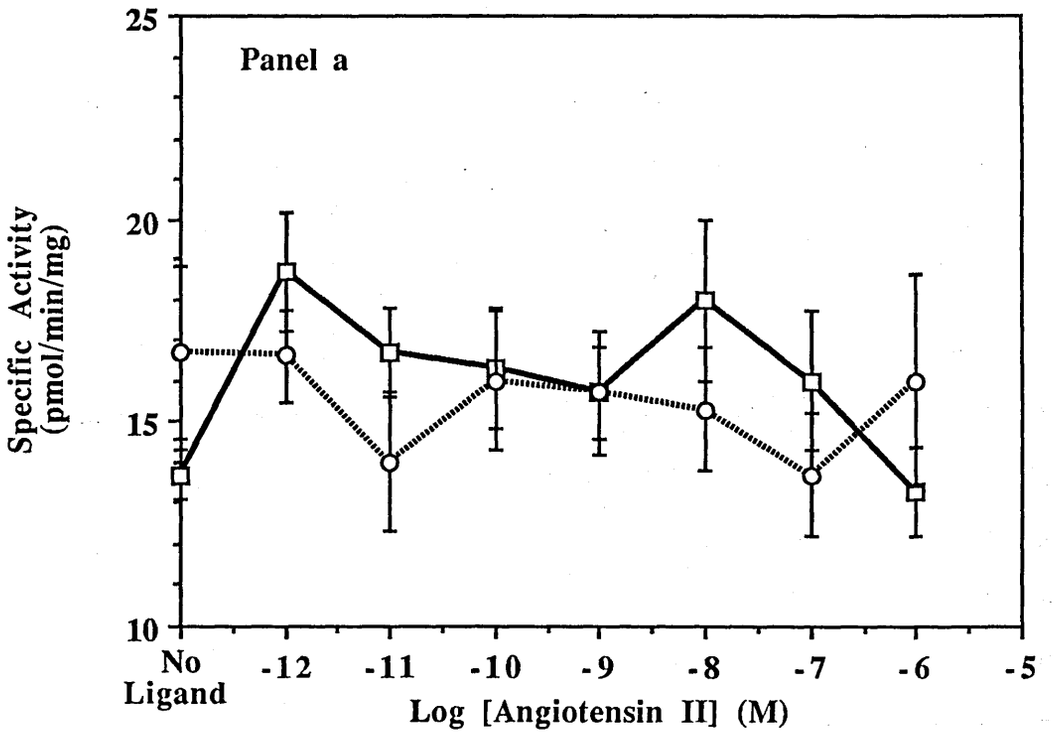
Dose-Response Curves For Angiotensin II- And Noradrenaline-Mediated Inhibition Of Adenylate Cyclase Activity In Liver Plasma Membranes From Lean And Diabetic (*db/db*) Animals

Sucrose-purified liver plasma membranes from lean (❑) and diabetic (*db/db*) (○) animals were assayed for adenylate cyclase activity for 15 minutes at 30°C in the presence of 100 μM GTP and increasing concentrations of angiotensin II (*panel a*) or noradrenaline (*panel b*) as described in Section 2.7.1. The [^{32}P]cyclic AMP formed was measured by the double column method described in Section 2.7.2.

For experiments using angiotensin II as an inhibitory ligand, membranes were resuspended in 10 mM Tris-HCl, 5 mM EDTA and 0.05% (v/v) β-mercaptoethanol to a concentration of 1.5 mg/ml. The membranes were then assayed for adenylate cyclase activity as described in Sections 2.7.1 and 2.7.2 with the inclusion of 200 mM lithium chloride in the incubation medium. These conditions have been shown by others to maximize angiotensin II-mediated inhibition of adenylate cyclase activity in liver membranes (Pobiner *et al.*, 1985; Lynch *et al.*, 1989).

The figure shows experiments representative of three performed for each inhibitory ligand on different membrane preparations which gave quantitatively similar results.

In the presence of 100 μM GTP alone, the specific activities obtained in the experiment in *panel a* were 13.7 ± 0.6 (lean) and 16.7 ± 2.1 (diabetic) pmol/min/mg. For the experiment shown in *panel b*, the specific activities obtained in the presence of 100 μM GTP were 48.0 ± 4.6 (lean) and 52.0 ± 2.0 (diabetic) pmol/min/mg.


Panel a
Specific Activity (pmol/min/mg)
25
20
15
10
No Ligand
-12
-11
-10
-9
-8
-7
-6
-5
Log [Angiotensin II] (M)

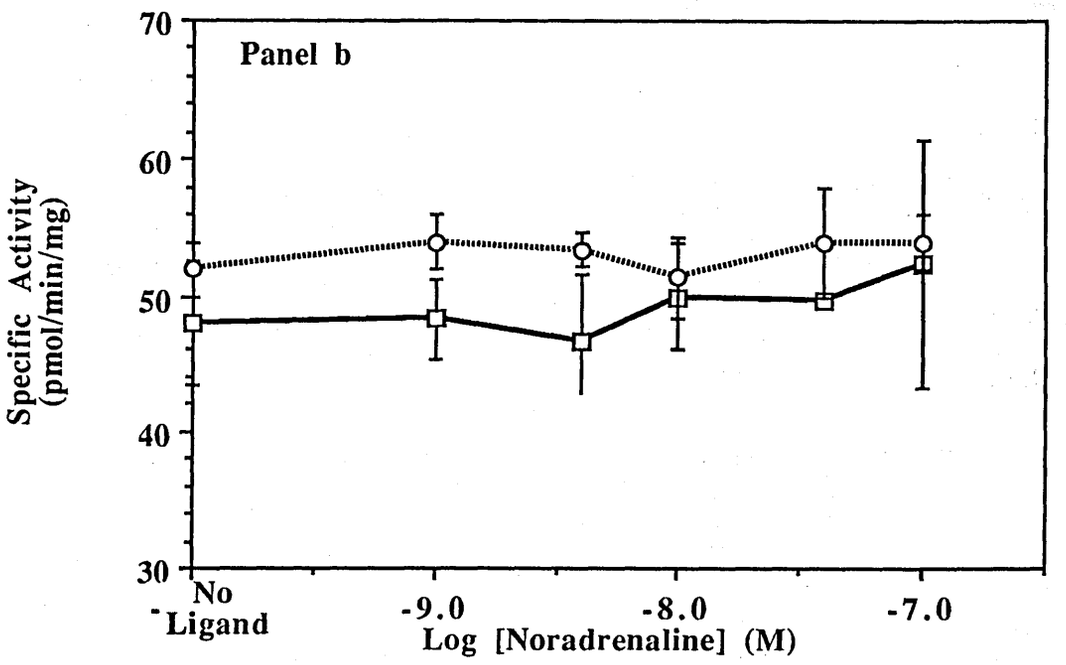

Panel b
Specific Activity (pmol/min/mg)
70
60
50
40
30
No Ligand
-9.0
-8.0
-7.0
Log [Noradrenaline] (M)

Figure 3.26:

Dose-Response Curves For Gpp(NH)p Stimulation Of Adenylate Cyclase Activity In The Presence Of 100 μM Forskolin And 11% (v/v) Ethanol In Liver Plasma Membranes From Lean And Diabetic (*db/db*) Animals

Sucrose-purified liver plasma membranes from lean (❑) and diabetic (*db/db*) (O) animals were assayed for adenylate cyclase activity for 15 minutes at 30°C in the presence of 100 μM forskolin, 11% (w/v) ethanol and increasing concentrations of Gpp(NH)p as described in Section 2.7.1. The [^{32}P]cyclic AMP formed was measured by the double column method described in Section 2.7.2. The figure shows results pooled from three experiments performed on different membrane preparations and values are presented as MEAN ± S.D.

Under these conditions, basal activities were 28.3 ± 6.0 (lean) and 34.5 ± 7.2 (diabetic) pmol/min/mg, whilst forskolin-stimulated activities were 91.3 ± 4.2 (lean) and 128.7 ± 6.7 (diabetic) pmol/min/mg.

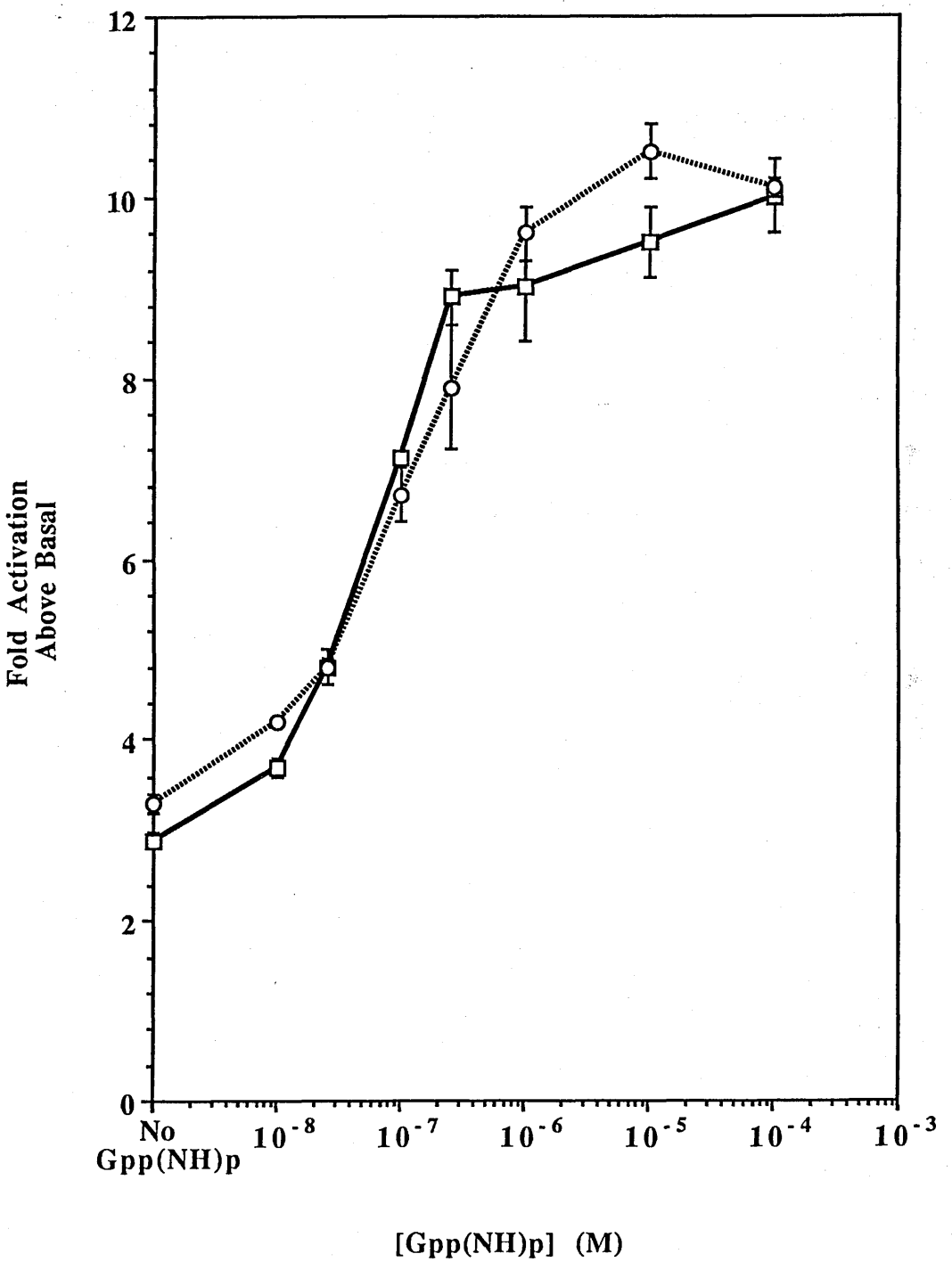

12
10
8
6
4
2
0
Fold Activation
Above Basal
No
Gpp(NH)p
10-8
10-7
10-6
10-5
10-4
10-3
[Gpp(NH)p] (M)

Figure 3.27:

Dose-Response Curves For Gpp(NH)p Inhibition Of Adenylate Cyclase Activity In The Presence Of 100 μM Forskolin And 1% (v/v) Ethanol In Liver Plasma Membranes From Lean And Diabetic (*db/db*) Animals

Sucrose-purified liver plasma membranes from lean (❑) and diabetic (*db/db*) (O) animals were assayed for adenylate cyclase activity for 5 minutes at 30°C in the presence of 100 μM forskolin, 1% (w/v) ethanol and increasing concentrations of Gpp(NH)p as described in Section 2.7.1. The [^{32}P]cyclic AMP formed was measured by the double column method described in Section 2.7.2. The figure shows an experiment representative of three performed on different membrane preparations which gave quantitatively similar results.

Basal activities in this experiment were 5.5 ± 2.1 (lean) and 5.9 ± 3.0 (diabetic) pmol/min/mg. Forskolin-stimulated activities were 150.9 ± 10.2 (lean) and 149.0 ± 15.0 (diabetic) pmol/min/mg.

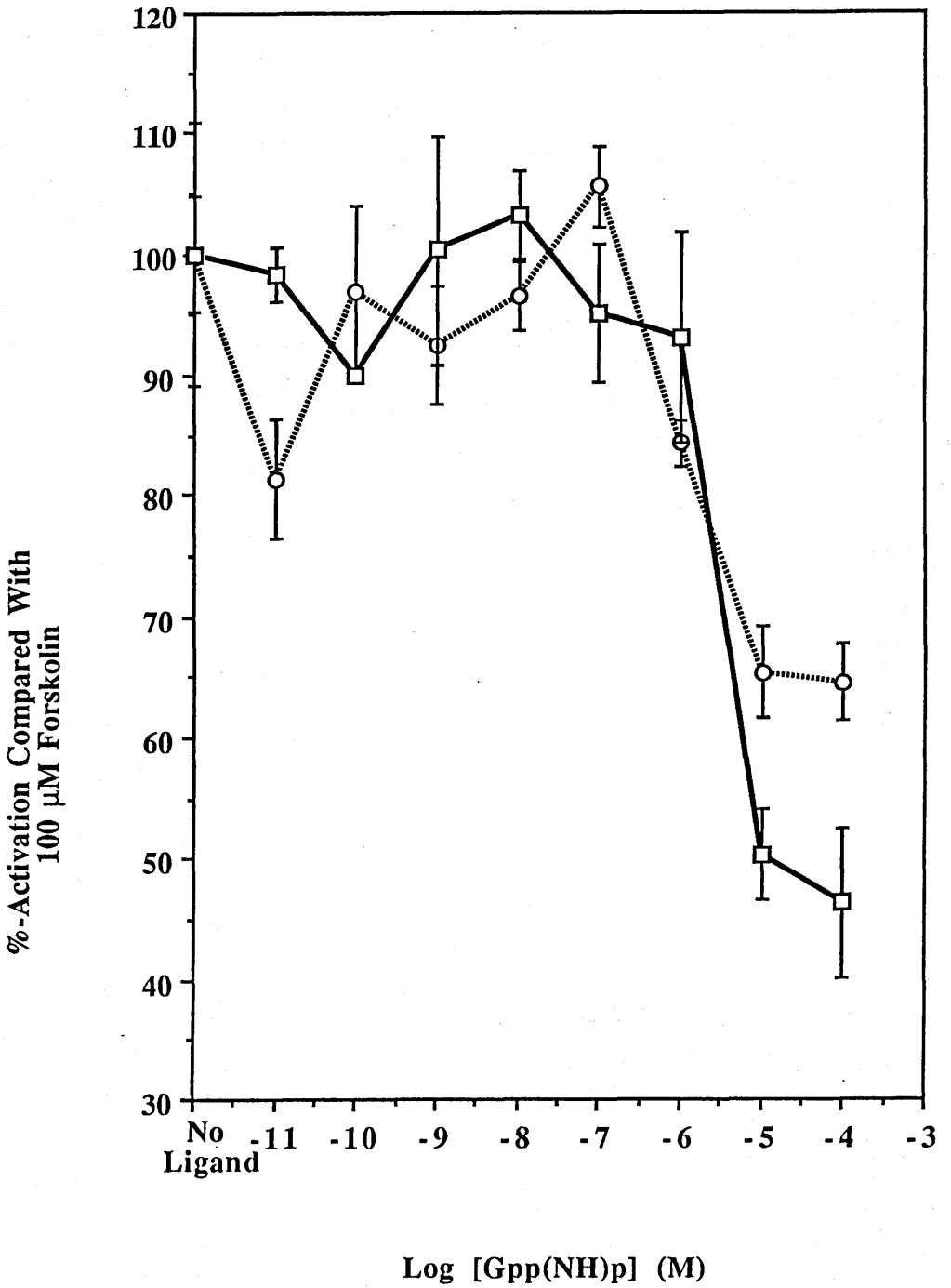

120
110
100
90
80
70
60
50
40
30
No Ligand
-11
-10
-9
-8
-7
-6
-5
-4
-3
%-Activation Compared With 100 µM Forskolin
Log [Gpp(NH)p] (M)

Figure 3.28:

Effect Of Pertussis Toxin Treatment On The Dose-Response Curve For Gpp(NH)p Inhibition Of Adenylate Cyclase Activity In The Presence Of 100 μM Forskolin And 1% (v/v) Ethanol In Liver Plasma Membranes From Lean Animals

Sucrose-purified liver plasma membranes from lean (❑) and pertussis toxin-treated lean (■) animals were assayed for adenylate cyclase activity for 5 minutes at 30°C in the presence of 100 μM forskolin, 1% (w/v) ethanol and increasing concentrations of Gpp(NH)p as described in Section 2.7.1. The [^{32}P]cyclic AMP formed was measured by the double column method described in Section 2.7.2. The figure shows an experiment representative of three performed on different membrane preparations which gave quantitatively similar results.

Basal activities in this experiment were 4.9 ± 2.2 (lean) and 5.0 ± 1.0 (pertussis toxin-treated lean) pmol/min/mg. Forskolin-stimulated activities were 157.2 ± 13.0 (lean) and 149.3 ± 10.0 (pertussis toxin-treated lean) pmol/min/mg.

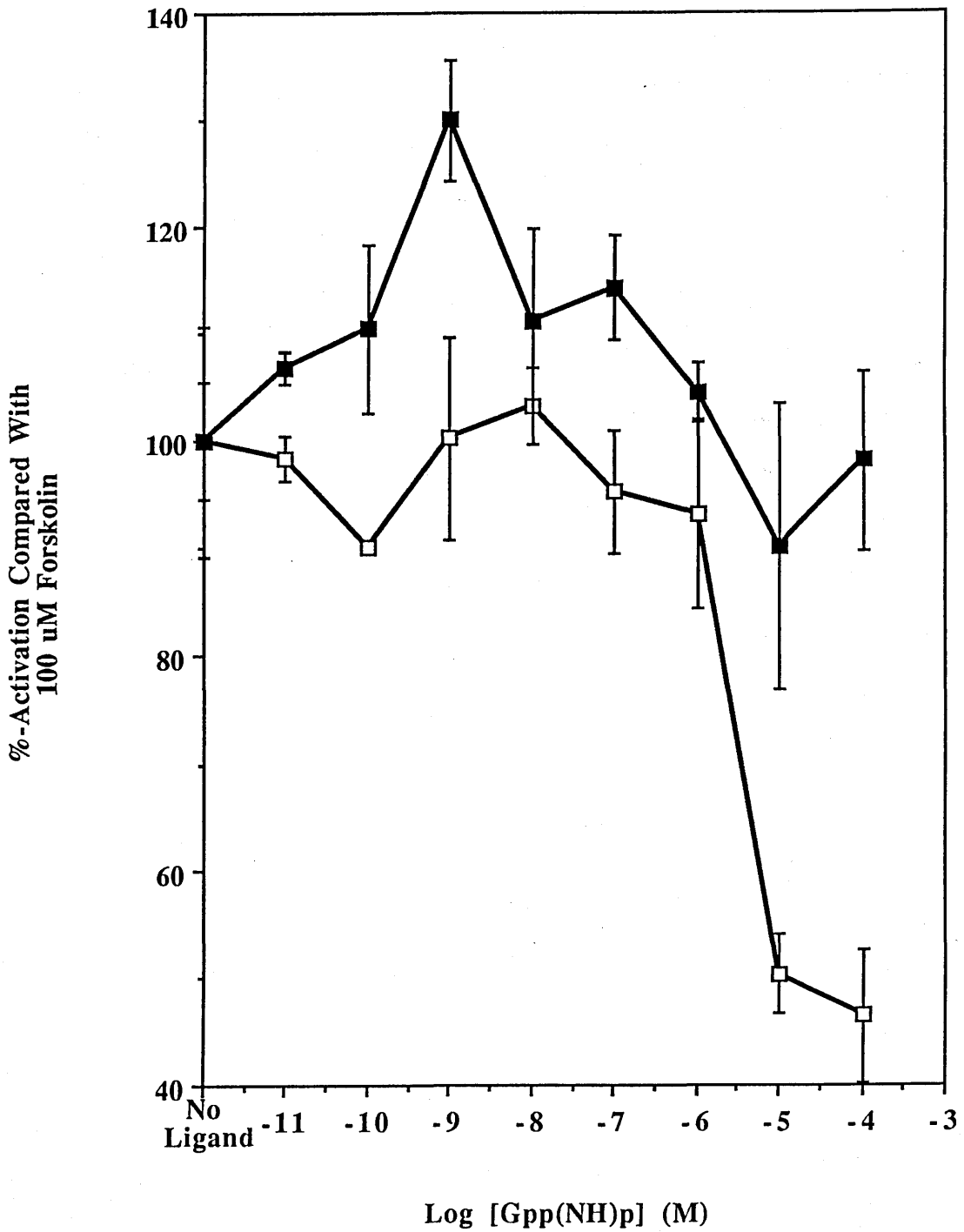

140
120
100
80
60
40
%-Activation Compared With 100 uM Forskolin
No Ligand
-11
-10
-9
-8
-7
-6
-5
-4
-3
Log [Gpp(NH)p] (M)

Figure 3.29:

Effect Of Pertussis Toxin Treatment On The Dose-Response Curve For Gpp(NH)p Inhibition Of Adenylate Cyclase Activity In The Presence Of 100 μM Forskolin And 1% (v/v) Ethanol In Liver Plasma Membranes From Diabetic (*db/db*) Animals

Sucrose-purified liver plasma membranes from diabetic (*db/db*) (○) and pertussis toxin-treated diabetic (●) animals were assayed for adenylate cyclase activity for 5 minutes at 30°C in the presence of 100 μM forskolin, 1% (w/v) ethanol and increasing concentrations of Gpp(NH)p as described in Section 2.7.1. The [^{32}P]cyclic AMP formed was measured by the double column method described in Section 2.7.2. The figure shows an experiment representative of three performed on different membrane preparations which gave quantitatively similar results.

Basal activities in this experiment were 5.9 ± 3.0 (diabetic) and 4.2 ± 1.2 (pertussis toxin-treated diabetic) pmol/min/mg. Forskolin-stimulated activities were 149.0 ± 15.0 (diabetic) and 141.5 ± 9.8 (pertussis toxin-treated diabetic) pmol/min/mg.

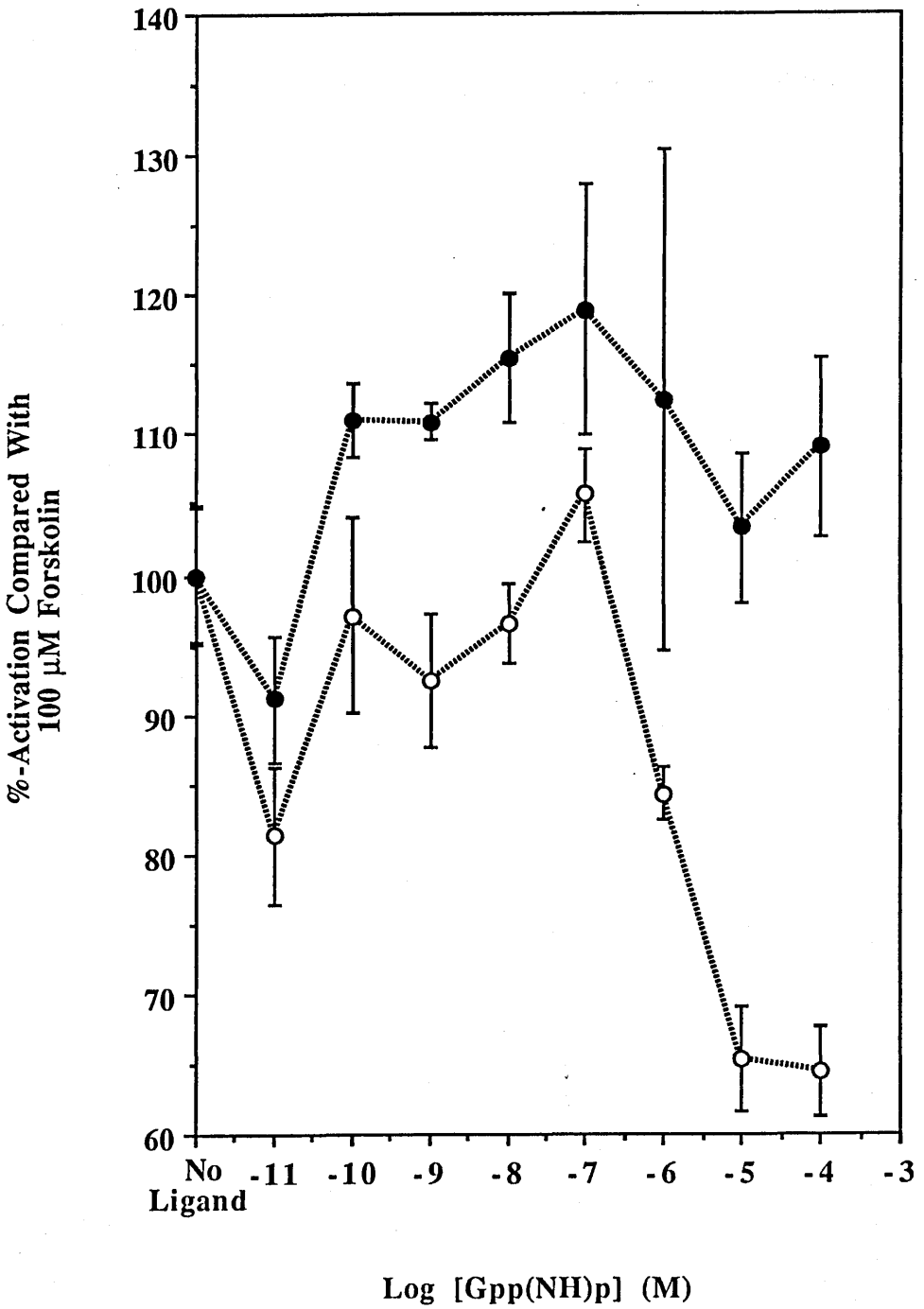

140
130
120
110
100
90
80
70
60
%-Activation Compared With
100 µM Forskolin
No Ligand
-11
-10
-9
-8
-7
-6
-5
-4
-3
Log [Gpp(NH)p] (M)

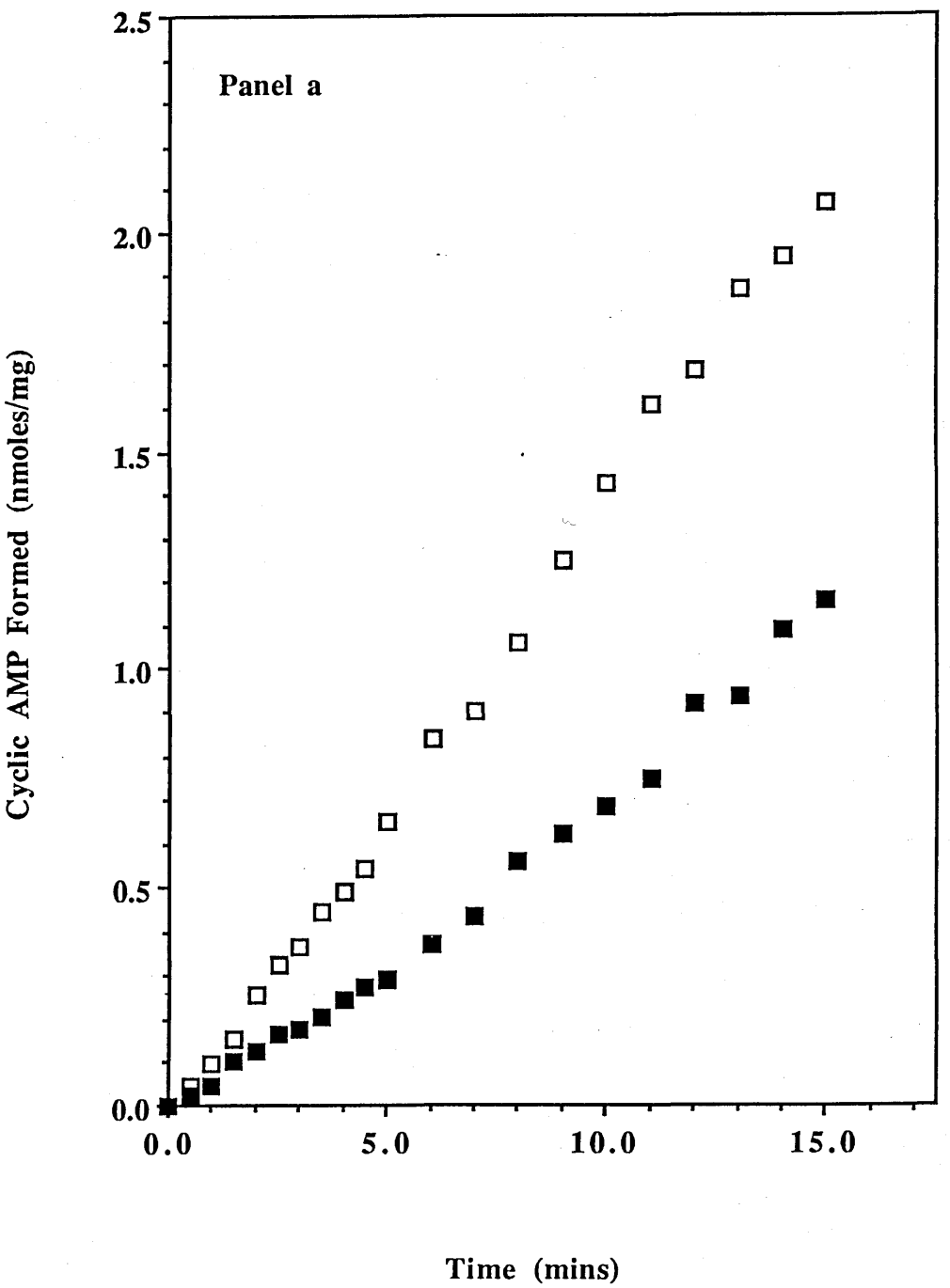
Figure 3.30:

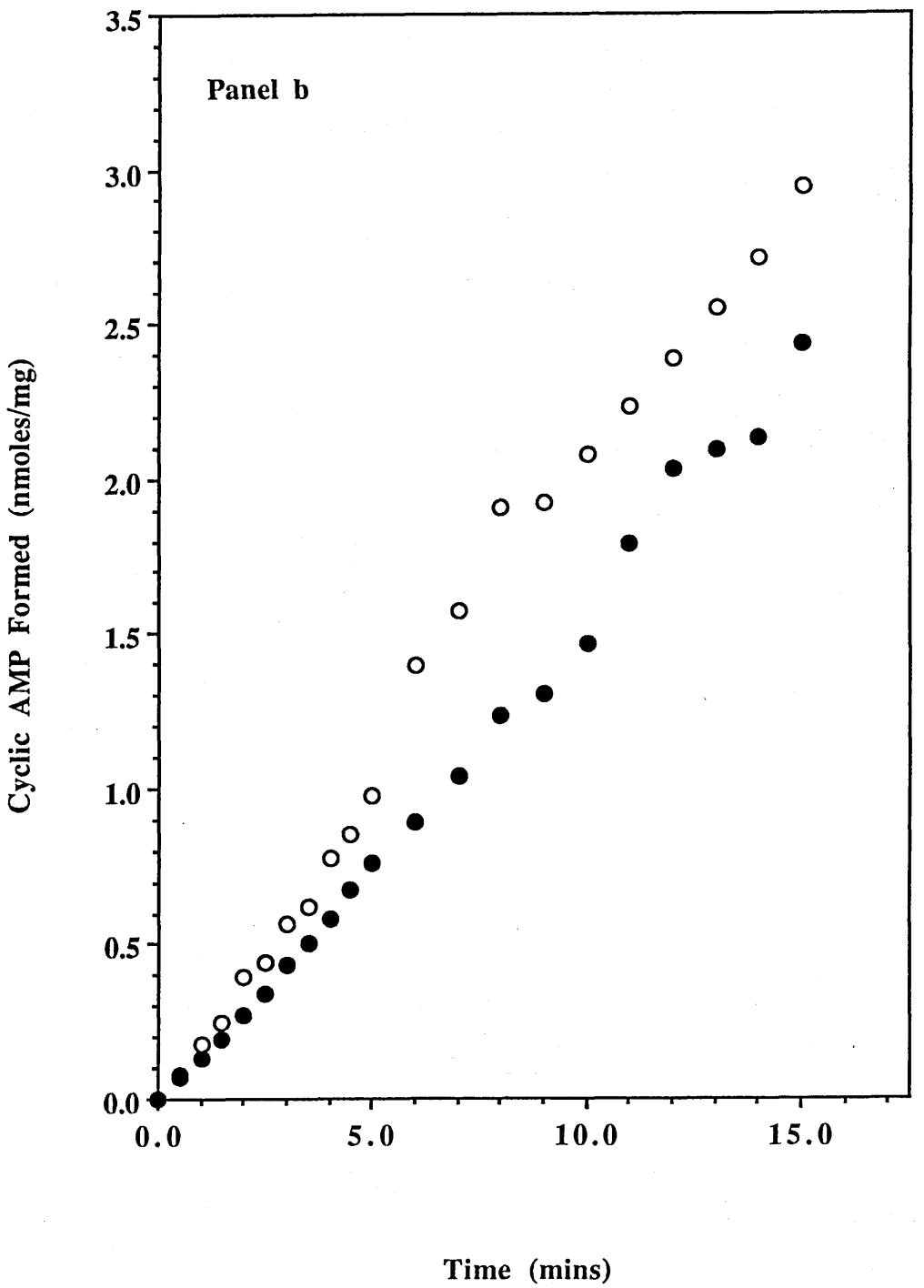
Time Courses For The Effects Of Gpp(NH)p On Forskolin-Stimulated Adenylate Cyclase Activity

Time courses were performed in a final reaction volume of 1.25 ml in a 6 ml plastic scintillation vial stirred continuously in a 30°C water bath by means of an underwater stirrer (Cambridge Instruments). The reaction mixture contained the components described in Section 2.7.1 with the addition of [α-^{32}P]ATP at a final concentration of 2μCi/50 μl. This mixture was stirred for 5 minutes at the desired temperature before the addition of membranes to initiate the reaction. At 30 or 60 second intervals, 50 μl aliquots were withdrawn and added to 100 μl aliquots of the "stopping solution" described in Section 2.7.2 before the addition of 800 μl distilled water and purification of the [^{32}P]cyclic AMP formed by sequential column chromatography (Section 2.7.2).

Panel a and *panel b* show the inhibitory effect of the addition of 100 μM Gpp(NH)p (closed symbols) on 100 μM forskolin-stimulated activity (open symbols). The experiment in *panel a* employed membranes from lean animals (□, ■), while *panel b* used membranes from diabetic (*db/db*) animals (○, ●). *Panel c* demonstrates the effect of the addition of 100 μM Gpp(NH)p on 100 μM forskolin-stimulated activity in membranes from lean animals which had been treated with pertussis toxin *in vivo* (△,▲).

These experiments were performed on the same day using the same batches of reagents, and are representative of two such groups of experiments performed with different membrane preparations which gave quantitatively similar results.


Panel a
2.5
2.0
1.5
1.0
0.5
0.0
0.0
5.0
10.0
15.0
Cyclic AMP Formed (nmoles/mg)
Time (mins)


Panel b
Cyclic AMP Formed (nmoles/mg)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.0
5.0
10.0
15.0
Time (mins)

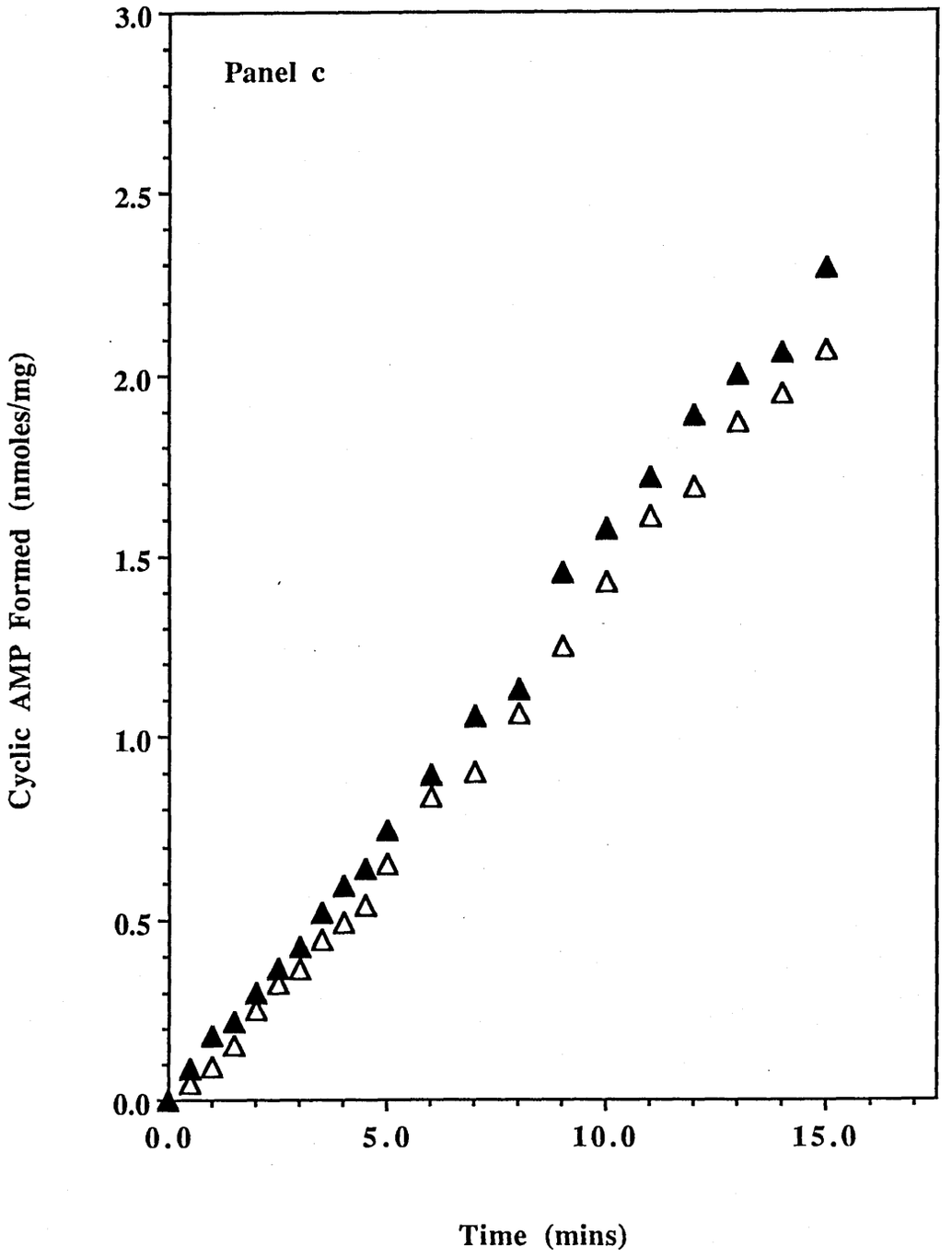

Panel c
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.0
5.0
10.0
15.0
Cyclic AMP Formed (nmoles/mg)
Time (mins)

Figure 3.31:

Effect Of Increasing Ethanol Concentrations On Forskolin-Stimulated Adenylate Cyclase Activity In Liver Plasma Membranes From Lean Animals

Sucrose-purified liver plasma membranes from lean animals (□) were assayed for adenylate cyclase activity for 15 minutes at 30°C in the presence of 100 μM forskolin and increasing concentrations of absolute ethanol as described in Section 2.7.1. The [^{32}P]cyclic AMP formed was measured by the double column method described in Section 2.7.2. The figure shows an experiment representative of three performed on different membrane preparations which gave quantitatively similar results.

In this experiment, basal and forskolin-stimulated activities in the presence of a minimal concentration of ethanol were 4.0 ± 1.1 and 120.5 ± 11.9 pmol/min/mg respectively.

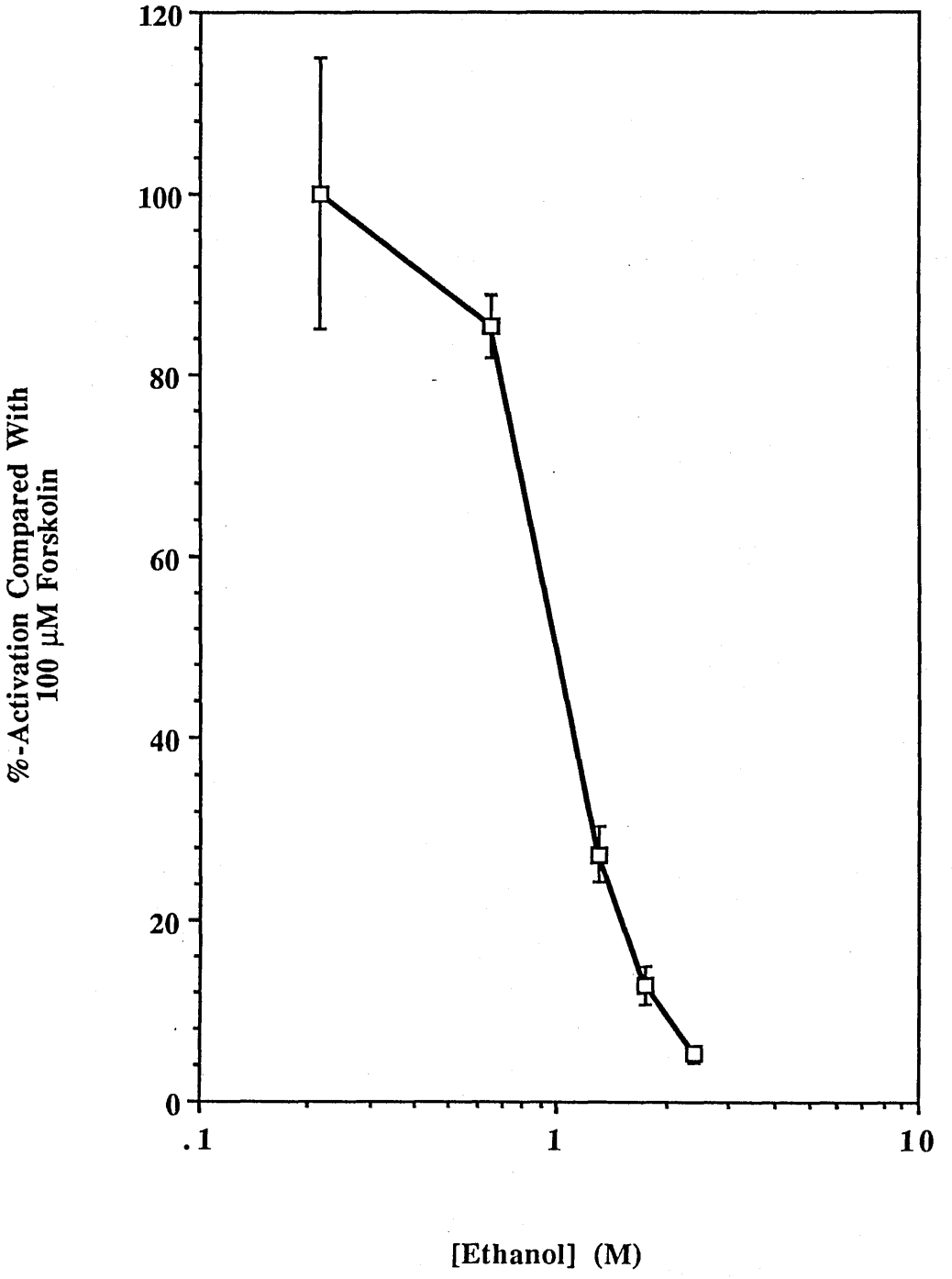
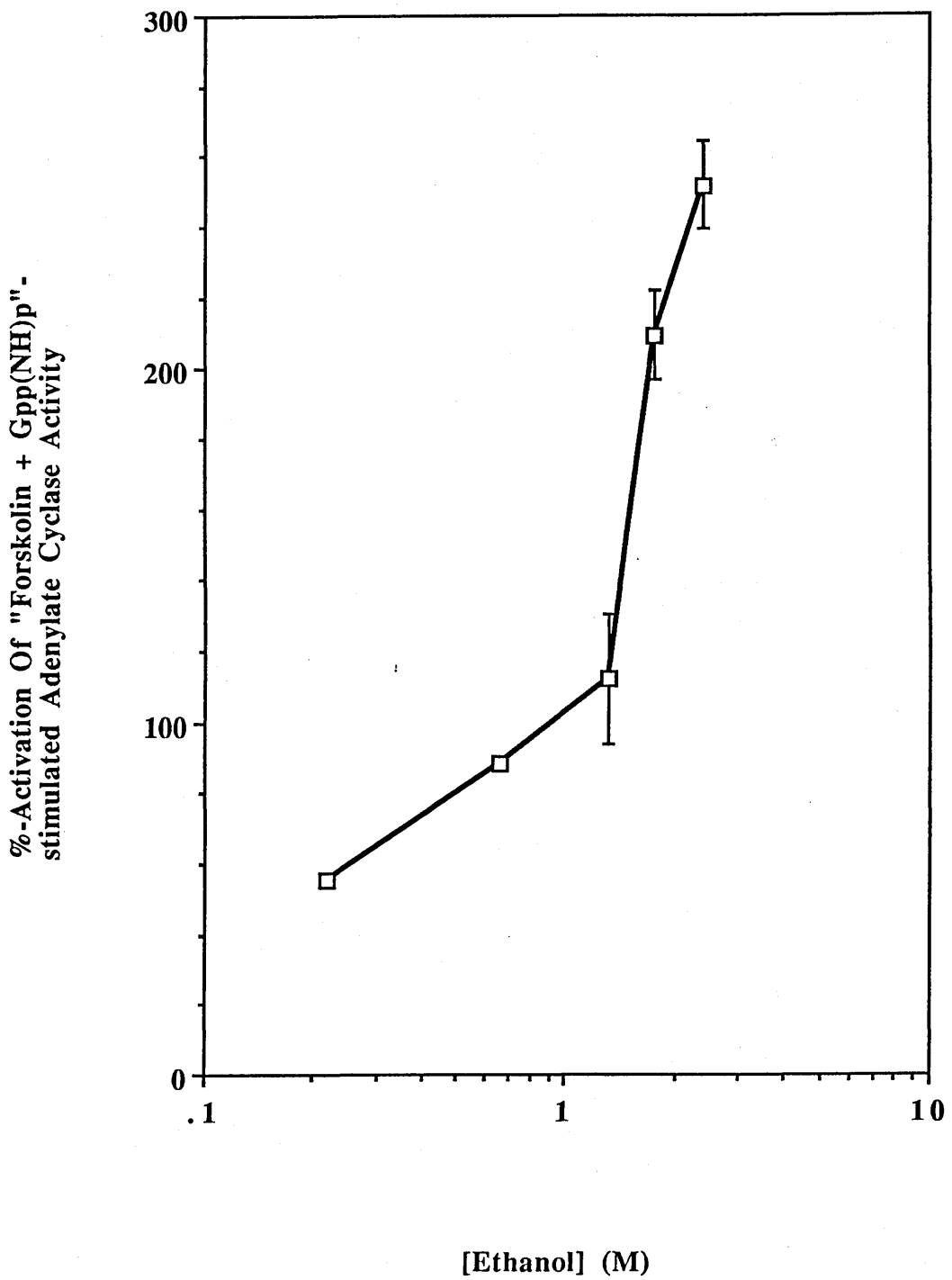

120
100
80
60
40
20
0
.1
1
10
%-Activation Compared With
100 µM Forskolin
[Ethanol] (M)

Figure 3.32:

Effect Of Increasing Ethanol Concentrations On 'Forskolin + Gpp(NH)p'-Stimulated Adenylate Cyclase Activity In Liver Plasma Membranes From Lean Animals

Sucrose-purified liver plasma membranes from lean animals (☐) were assayed for adenylate cyclase activity for 15 minutes at 30°C in the presence of 100 μM forskolin, 100 μM Gpp(NH)p and increasing concentrations of absolute ethanol as described in Section 2.7.1. The [^{32}P]cyclic AMP formed was measured by the double column method described in Section 2.7.2. The figure shows an experiment representative of three performed on different membrane preparations which gave quantitatively similar results.

In this experiment, basal and 'forskolin + Gpp(NH)p'-stimulated activities in the presence of a minimal concentration of ethanol were 4.0 ± 2.2 and 86.5 ± 4.3 pmol/min/mg respectively.


%-Activation Of "Forskolin + Gpp(NH)p"-
stimulated Adenylate Cyclase Activity
300
200
100
0
.1
1
10
[Ethanol] (M)

3.2 RESULTS & DISCUSSION

Initially it was decided to test for any potential defects in adenylate cyclase regulation by activating the adenylate cyclase system of membranes from lean and diabetic animals with maximal doses of a range of ligands capable of activating the system at various loci (Table 3.1). Basal activities were the same in both lean and diabetic animals (Table 3.1). Furthermore, the addition of a saturating dose of forskolin, a diterpene capable of binding to and activating the catalytic unit of adenylate cyclase directly (Seamon & Daly, 1981), resulted in the same fold activation of adenylate cyclase activity over basal in membranes from both lean and diabetic animals (Table 3.1). This data implied that the functioning of the adenylate cyclase catalytic unit was unaltered by the diabetic state. Similarly, the fold activations of adenylate cyclase activity above basal after stimulation with maximally effective concentrations of GTP, Gpp(NH)p and NaF, which act on the system at the level of G-protein-mediated activation of the catalytic unit, were also the same for membranes from lean and diabetic animals (Table 3.1).

To characterise the responses to Gpp(NH)p and NaF further, dose-response curves were performed for each of these ligands (Figures 3.1 and 3.2). Whereas the dose-response curves for NaF stimulation of adenylate cyclase were identical for membranes from lean and diabetic animals (EC_{50}=1.5 ± 0.5 mM for lean animals, 2.0 ± 0.4 mM for diabetic animals: data expressed as MEAN ± S.D. for three experiments on different membrane preparations) (Figure 3.1 and Table 3.1), the dose-response curves for Gpp(NH)p activation of cyclase activity were quite different (Figure 3.2). Although Gpp(NH)p was capable of stimulating adenylate cyclase activity to the same extent in membranes from lean and diabetic animals (Table 3.1), the EC_{50} value for stimulation was lower in membranes from diabetic animals (60 ± 8 nM for diabetic animals compared with 158 ± 10 nM for lean animals: data expressed as MEAN ± S.D for three separate experiments on different membrane preparations).

Considering the fact that both Gpp(NH)p and NaF are supposed to act at the

level of G-protein activation, it is difficult to resolve the altered kinetics of cyclase activation by Gpp(NH)p but not NaF in liver membranes from diabetic animals compared with those from lean animals. It is possible that the differences observed are related to the different mechanisms by which NaF and Gpp(NH)p activate G-protein α-subunits. From studies on the retinal-specific G-protein transducin, it has been proposed that NaF forms a $[AlF_4]^-$ complex which binds to the guanine nucleotide binding site of GDP-bound holomeric α-subunits and mimics the terminal γ-phosphate group of GTP, thereby inducing activation (Bigay *et al.*, 1985). However, this model has been contested for NaF-mediated regulation of adenylate cyclase activity in platelet membranes due to the inability of NaF, but not Gpp(NH)p, to cause a reduction in the agonist binding affinity of α_2-adrenergic receptors (Stadel & Crooke, 1988). From these observations it was suggested that Gpp(NH)p and NaF mediate G-protein activation by distinct routes (Stadel & Crooke, 1988). Gpp(NH)p activation of a G-protein necessarily requires the release of GDP from the guanine nucleotide binding site before a molecule of Gpp(NH)p can bind. Such distinct modes of activation are presumably reflected in the markedly different maximal activities with which Gpp(NH)p and NaF can stimulate adenylate cyclase (Table 3.1). These, in turn, may reflect different conformations of Gpp(NH)p- and NaF-activated $G_s\alpha$-subunits which may be differentially susceptible to altered regulatory conditions in liver membranes from diabetic animals compared with controls. In support of this idea is the observation made by Lynch *et al.* (1989) that guanine nucleotide-stimulated, but not NaF-stimulated, adenylate cyclase activity was enhanced in liver plasma membranes prepared from rats that had been pretreated with pertussis toxin (to inactivate G_i) compared with the activity seen with membranes from sham-injected controls. However, a reduction in a tonic inhibitory effect of G_i in liver plasma membranes from diabetic animals was not responsible for the altered response to Gpp(NH)p observed here as membranes from pertussis toxin-treated diabetic mice retained

their greater sensitivity to activation by Gpp(NH)p compared to membranes from pertussis toxin-treated lean mice (Figures 3.3 and 3.4).(EC_{50}=100 ± 9 nM for PTx-treated diabetic animals compared with 355 ± 45 nM for PTx-treated lean animals: data are presented for three experiments performed on different membrane preparations). Nevertheless, when such experiments were performed in the presence of 1 μM glucagon, no such difference in sensitivity was observed (250 ± 15 nM for lean animals, 220 ± 10 nM for diabetic animals: data presented for three experiments performed on different membrane preparations) and the maximal stimulations elicited were similar between membranes from lean and diabetic animals (870 ± 50 pmol/min/mg for lean animals, 922 ± 33 pmol/min/mg for diabetic animals: data presented for three experiments performed on different membrane preparations) (Figure 3.24).

There are several potential explanations for the enhanced sensitivity of liver membranes from diabetic animals to activation by Gpp(NH)p in the absence of stimulatory receptor activation. Altered expression of G-protein subunits could potentially affect G_s dissociation in several ways. For example, an altered ratio of expression of $G_s\alpha$-subunits compared with β-subunits might alter the ability of holomeric G_s to dissociate into its constituent subunits. Indeed, a decreased activation of G_s in the presence of increasing concentrations of βγ-subunits has been noted by others (Gilman, 1987; Katada *et al.*, 1984c). However, in comparative immunoblotting studies of $G_s\alpha$-subunits using antiserum CS1 there was no alteration in the expression of the two forms of $G_s\alpha$ present between liver membranes from lean and diabetic animals (Table 3.3 and Figure 3.15). In contrast, there was a 70% reduction in the expression of β-subunits present in diabetic samples compared with leans (Table 3.3 and Figure 3.14). This could potentially provide an explanation for the observed difference in the response to Gpp(NH)p observed in diabetic membranes compared with controls by the following argument. As detailed above, this effect cannot have been due to an

altered tonic inhibitory effect of G_i in membranes from diabetic animals as the enhanced sensitivity of membranes from diabetic animals to stimulation by Gpp(NH)p was retained after pertussis toxin treatment *in vivo* (Figures 3.3 and 3.4), which abolished G_i function (Figures 3.28 and 3.29). Therefore the enhanced sensitivity of adenylate cyclase to stimulation by Gpp(NH)p is likely to be due to an alteration in G_s activation. Gpp(NH)p activates G_s by binding to the guanine nucleotide binding site such that a conformational change is induced which yields the free activated Gpp(NH)p-bound α-subunit (Birnbaumer *et al.*, 1985b). This activation of G_s can be attenuated by the addition of βγ-subunit complexes (Birnbaumer *et al.*, 1985b), and so the observation that membranes from diabetic animals were more sensitive to activation by Gpp(NH)p may be a reflection of the reduction in expression of β-subunits, and the concomitant reduction in levels of βγ-subunits. That such a sensitizing effect was not observed for NaF-mediated stimulation of adenylate cyclase might reflect the different conformations which Gpp(NH)p and NaF induce, with Gpp(NH)p-bound G_s being more susceptible to the reduction in concentration of βγ-subunits in membranes from diabetic animals.

The validity of such an argument rests on an appreciation of the relative expressions of other G-protein subunits capable of complexing with βγ-subunits. To address this question, at least in part, comparative immunoblotting analyses were performed with antisera SG1 and I3B to compare the relative expressions of different G_iα-subunits expressed in liver plasma membranes. As discussed in Section 2.9, in non-retinal tissues SG1 is capable of recognising the α-subunits of G_i1 and G_i2 which can only be resolved under the specific electrophoretic conditions described in Section 2.8. Figure 3.11 shows that whereas both G_iα-1 and G_iα-2 were expressed in membranes prepared from mouse white adipose tissue, only one band corresponding to G_iα-2 was expressed in liver membranes from both lean and diabetic animals. This is consistent with studies on the expression of various G-protein components in rat liver, where neither mRNA or protein corresponding to G_iα-1 was detected (Suki *et al.*, 1987; Bushfield *et al.*,

1990c). Hence SG1 could be used as a specific probe for $G_i\alpha$-2 in liver plasma membranes from both lean and diabetic animals. Comparative immunoblotting studies with SG1 demonstrated that the expression of $G_i\alpha$-2 was decreased by approximately 70% in membranes from diabetic animals compared with leans (Figure 3.12 and Table 3.3). Using antiserum I3B in similar immunoblotting experiments demonstrated that the expression of $G_i\alpha$-3 was also reduced in liver plasma membranes from diabetic animals compared with leans, but not to the same extent as $G_i\alpha$-2 (Figure 3.13 and Table 3.3).

Considering the concomitant reductions in expression of $G_i\alpha$-2 and $G_i\alpha$-3 with β-subunits, it was necessary to prove whether such alterations in expression could alter the equilibria between holomeric and activated G-protein subunits in the presence of guanine nucleotides. This question was addressed by using the abilities of cholera and pertussis toxins to catalyse the NAD^+-dependent ADP-ribosylation of G_s and G_i respectively. Cholera toxin can catalyse the ADP-ribosylation of either holomeric or activated $G_s\alpha$-subunits, but the latter are the preferred substrates (Ribeiro-Neto *et al.*, 1987). In contrast, pertussis toxin can only catalyse the ADP-ribosylation of holomeric GDP-bound $G_i\alpha$-subunits (Tsai *et al.*, 1984). Pertussis toxin-catalysed ADP-ribosylation of 100 μg of membranes from lean and diabetic animals in the presence of [α-^{32}P]NAD^+ resulted in the labelling of three bands (Figure 3.20). However, only labelling of the band with a molecular weight of 41 kDa was absolutely dependent on the presence of pertussis toxin, and presumably this represented a mixture of $G_i\alpha$-2 and $G_i\alpha$-3 previously identified by immunoblotting (Figures 3.12 and 3.13). The labelling of this band was reduced by some 86% in membranes from diabetic animals compared with controls (Figure 3.20 and Table 3.4) and presumably this reflects the reductions in levels of $G_i\alpha$-2 and $G_i\alpha$-3 observed by immunoblotting (Table 3.3). Comparing Figures 3.18 and 3.19, it can be seen that the rates at which the ribosylation reaction reached equilibrium were similar, with equilibrium being attained after 45 minutes for

membranes from both lean and diabetic animals (Figure 3.14, Table 3.3). Therefore, the incubation time chosen for the comparative labelling experiments (Figure 3.20) was such that the labelling of $G_i\alpha$-subunits had reached their maximum in membranes from both lean and diabetic animals, *i.e.* after 45 minutes (Figure 3.18 and 3.19).

When analogous experiments were performed using cholera toxin and [^{32}P] NAD^+, five bands were labelled of which two (42 and 45 kDa bands) were labelled in a toxin-dependent manner in membranes from both lean and diabetic animals (Figure 3.23). Presumably these cholera toxin substrates correspond to the two forms of $G_s\alpha$-subunits recognised previously by immunoblotting (Figure 3.15). However in contrast to the results obtained in the comparative immunoblotting experiments, when no difference in expression between control and diabetic samples was noted (Figure 3.15 and Table 3.3), the labelling of the two forms of $G_s\alpha$ was enhanced by some 3.5- to 4-fold in diabetic samples (Figure 3.23 and Table 3.4). The time courses of the ADP-ribosylation reactions in liver membranes from lean and diabetic animals showed that ribosylation was maximal for both sets of membranes under the conditions in which comparative labelling was measured (Figures 3.21 and 3.22). In contrast to the pertussis toxin-catalysed ADP-ribosylation experiments, membranes from diabetic animals reached equilibrium more rapidly in the cholera toxin-catalysed ADP-ribosylation compared with membranes from lean animals. This is particularly marked for the 45 kDa form of $G_s\alpha$, which takes approximately 15 minutes longer to reach equilibrium in membranes from lean animals compared with those from diabetic animals (Figure 3.21 compared with Figure 3.22). Two obvious potential explanations for these effects are (i) liver membranes from diabetic animlals contained relatively more active ADP-ribosylation factor (ARF), the low molecular weight GTP-binding protein whose presence is essential for cholera toxin to catalyse the ADP-ribosylation of its substrates (Kahn & Gilman, 1984) and, since there was no

means available by which to measure ARF activity, this possibility cannot be excluded; (ii) alternatively, the observed reduction in expression of β-subunits in liver membranes from diabetic animals compared with lean animals may also effect the ADP-ribosylation reaction by increasing the concentration of dissociated $G_s\alpha$-subunits. In the ribosylation mixture, GTP was present at a concentration of 500 μM, *i.e.* G_s was saturated with GTP as it would be under physiological conditions (Birnbaumer *et al.*, 1985). Now, it has been noted for G_i and G_o that G-proteins are capable of traversing through their regulatory cycle in the presence of GTP but in the absence of agonist-bound receptor (Costa & Herz, 1989). In the case of G_s, this dissociation would be promoted by the reduction in concentration of βγ-subunits in diabetic samples, thereby causing an increase in the proportion of free GTP-bound $G_s\alpha$-subunits. As these free subunits are the preferred substrates for cholera toxin-catalysed modification compared with the holomeric form (Ribeiro-Neto *et al.*, 1987), this situation would lead to the enhanced rate and extent of cholera toxin-catalysed ADP-ribosylation of $G_s\alpha$ in the absence of any change in expression of $G_s\alpha$. In order to test this hypothesis thoroughly, it would be necessary to test whether the addition of increasing concentrations of βγ-subunits caused a reduction in the cholera toxin-catalysed labelling of $G_s\alpha$ in diabetic samples. However, no purified β-subunits were available to me during the course of these studies.

Despite the observed reductions in expression of various G-protein subunits, and the possible alterations in equlilibria between holomeric and dissociated G-protein subunits, there was no effect of these changes on either the sensitivity or responsiveness of adenylate cyclase to stimulation by glucagon (Figure 3.5, Table 3.2). The reasons for this are unclear as one might have expected an enhanced sensitivity to stimulation by glucagon in membranes from diabetic animals for the same reasons that account for the increased sensitivity such membranes exhibit to Gpp(NH)p (Figure 3.2). However, it is possible that there was another modification in the adenylate cyclase system from diabetic animals

which counteracted the affect of the reduced concentrations of βγ-subunits. In this respect it is worth noting that genetically diabetic (*db/db*) animals are hyperglucagonaemic (Laube *et al.*, 1973). Hence it is likely that the number of glucagon receptors present in liver plasma membranes from diabetic animals was reduced compared with lean animals due to the persistent hyperglucagonaemia causing receptor down-regulation. With regard to this point, other workers have shown that the hyperglucagonaemia caused by the induction of Type I diabetes leads to a reduction in the number of glucagon receptors present in liver plasma membranes from these animals compared with non-diabetic controls (Dighe *et al.*, 1984; Bhathena *et al.*, 1978). A reduced receptor number would tend to reduce the sensitivity of the system to activation by glucagon without necessarily affecting maximal stimulation until very high losses are elicited (Houslay *et al.*, 1980),thus tending to counteract the effect of reduced levels of βγ-subunits.

The response of adenylate cyclase to stimulation by isoproterenol was slightly reduced in membranes from diabetic animals compared with leans (Figure 3.8, Table 3.2). As has been postulated above, a reduction in β-adrenergic receptor number could explain why no enhanced sensitivity to isoproterenol was observed in membranes from diabetic animals compared with leans, but may not account for the reduction in maximal stimulation (Figure 3.8, Table 3.2). Hence the functioning of the receptor may have been altered such that its ability to couple to G_s was impaired in membranes from diabetic animals. Comprehensive receptor binding studies could address these points, as one could assess the degree of coupling between the β-adrenergic receptor and G_s by measuring the ability of non-hydrolysable GTP analogues to reduce agonist affinity for the receptor.

Studies on the effects of the reductions in expression of $G_i\alpha$-2, $G_i\alpha$-3 and β-subunits on the inhibitory regulation of adenylate cyclase activity proved difficult. Other workers have been able to measure pertussis toxin-sensitive inhibition of GTP-stimulated adenylate cyclase activity in liver plasma membranes using either

angiotensin II (Pobiner *et al.*, 1985; Lynch *et al.*, 1989) or α_2-adrenergic agonists (Bégin-Heick & Welsh, 1988). However, I was unable to observe any inhibitory effects of these ligands on adenylate cyclase activity stimulated with 100 μM GTP in either lean or diabetic samples (Figure 3.25) despite incorporating modifications to the reaction medium described by Pobiner *et al.* (1985) which have been suggested to enhance the inhibitory effect of angiotensin II (see legend to Figure 3.25 for details).

The ability of GTP and Gpp(NH)p to inhibit forskolin-stimulated adenylate cyclase activity in membranes prepared from either whole liver or hepatocytes has been reported in several publications (Gawler *et al.*, 1987; Itoh *et al.*, 1985). Since Gpp(NH)p exhibits a higher affinity for G_i than G_s, low concentrations of Gpp(NH)p can selectively activate G_i resulting in the inhibition of adenylate cyclase activity (Hildebrandt *et al.*, 1982; Hudson & Fain, 1982). As the concentration of the nucleotide increases, G_s becomes activated such that the final maximal specific activity obtained reflects the situation when both G_s and G_i are fully activated (Gawler, 1987). However, these experiments were performed in the presence of 10% (v/v) absolute ethanol, the solvent used to dilute the forskolin from its stock solution (10 mM) before addition to the assay (Gawler, 1987). Under these conditions, I was unable to detect any inhibitory phase to the Gpp(NH)p dose-response curve in the presence of 100 μM forskolin (Figure 3.26). At maximally effective concentrations of Gpp(NH)p, lean and diabetic animals produced similar fold stimulations above basal activity (10.0 ± 0.4 for lean animals, 10.1 ± 0.1 for diabetic animals; data are presented as MEAN ± S.D. for three experiments performed on different membrane preparations) with similar EC_{50} values (105 ± 63 nM for lean animals, 80 ± 28 nM for diabetic animals; data are presented as MEAN ± S.D. for three experiments performed on different membrane preparations). However, dilution of the stock forskolin solution with distilled water such that the final carry-over concentration of ethanol into the assay is 1% (v/v) produced a

completely different response (Figures 3.27, 3.28 and 3.29). In membranes from both lean and diabetic animals, increasing concentrations of Gpp(NH)p produced a dose-dependent inhibition of forskolin-stimulated adenylate cyclase activity: presumably these inhibitory effects reflected G_i functioning as they were completely abolished by pretreatment of either lean (Figure 3.28) or diabetic (Figure 3.29) animals with 1 μg of pertussis toxin for 3 days *in vivo*. The concentrations of Gpp(NH)p resulting in half-maximal inhibition of adenylate cyclase activity were similar for lean and diabetic preparations (10 ± 5 μM for lean animals, 5 ± 3 μM for diabetic animals; data presented as MEAN ± S.D for three experiments performed on different membrane preparations) although the maximal inhibition achieved was slightly greater in membranes prepared from lean animals (55 ± 6% for lean animals compared with 37 ± 3% for diabetic animals; data presented as MEAN ± S.D. for three experiments performed on different membrane preparations).

Because the inclusion of ethanol produced drastic changes in the nature of the responses of liver membranes to Gpp(NH)p in the presence of 100 μM forskolin, it was decided to determine the effects of increasing concentrations of ethanol on forskolin-stimulated activity and 'forskolin + Gpp(NH)p'-stimulated activity. Figure 3.31 shows that increasing ethanol concentrations caused a successive reduction in forskolin-stimulated adenylate cyclase activity until at an ethanol concentration of 2.5 M, only 5% of the activity attained at 0.2 M was detectable. In contrast, increasing concentrations of ethanol within the same range as used for the experiment in Figure 3.31 enhanced the specific activity obtained by forskolin and Gpp(NH)p (Figure 3.32). The mechanism by which ethanol exerted these effects is unknown, but presumably the alcohol must act at more than one point in the hormone-sensitive adenylate cyclase system to cause opposite effects on forskolin-stimulated activity depending on the presence of a guanine nucleotide.

As these inhibitory effects of Gpp(NH)p observed were distinct from those reported by others (Gawler *et al.*, 1987; Hudson & Fain, 1982), it was decided to characterise the effect in lean and diabetic preparations (Figure 3.30). For control

membrane preparations, the addition of 100 μM forskolin produced a stimulation of adenylate cyclase activity which was linear over the time-course of the incubation (Figure 3.30, *panel a*). On the inclusion of 100 μM Gpp(NH)p to this mixture, a 'lag phase' was induced over the first 5 minutes before a final steady-state velocity was attained which is lesser in magnitude than the reaction rate observed with forskolin alone (Figure 3.30, *panel a*). In membranes from diabetic animals, distinct kinetic characteristics were observed; the inclusion of Gpp(NH)p did not induce a 'lag phase' but a linear steady-state velocity which was less than the rate obtained with forskolin alone was attained (Figure 3.30, *panel b*). However, in samples from diabetic animals, this final steady-state rate was not as reduced compared with the forskolin-stimulated activity as was observed for membranes from lean animals (Figure 3.30, *panel b*). Both the 'lag phase' and the reduced steady-state velocity seen in the presence of 100 μM Gpp(NH)p were abolished by pertussis toxin pretreatment (Figure 3.30, *panel c*).

Three studies have implied that liver membranes contain "spare" G_i (Pobiner *et al.*, 1985; Lynch *et al.*, 1989; Bushfield *et al.*, 1990c). Pobiner *et al.* (1985) noted that more than 80% of the 41 kDa pertussis toxin substrate in hepatocytes had to be modified before there was any effect on angiotensin II-mediated inhibition of adenylate cyclase activity. Also, Bushfield *et al.* (1990c) noted that despite 60% reductions in expression of $G_i\alpha$-2 and $G_i\alpha$-3 compared with non-diabetic controls, there was only a small effect on the ability of P_{2y}-purinergic receptor agonists to inhibit cyclic AMP accumulation in streptozotocin-induced diabetic rat hepatocytes. In a similar study, Lynch *et al.* (1989) observed that a 50% reduction in the expression of $G_i\alpha$-2 had no effect on the ability of angiotensin II to inhibit adenylate cyclase activity in liver membranes from BB/Wor Type I diabetic rats compared with non-diabetic controls. It is not yet clear which of the pertussis toxin substrates in liver is responsible for the inhibition of adenylate cyclase activity, although indirect evidence has suggested that $G_i\alpha$-2 may be

responsible (Bushfield *et al.*, 1990a). However, the concept of a "spare" inhibitory capacity in liver plasma membranes is consistent with the observations here of a 70% reduction in levels of $G_i\alpha$-2 and a 50% reduction in $G_i\alpha$-3 causing less than a 40% reduction in the maximal inhibitory capacity in membranes from diabetic samples compared with leans.

The lack of any gross affects of reduced expressions of $G_i\alpha$-2 and $G_i\alpha$-3 on tonic inhibitory regulation of adenylate cyclase in liver membranes is further demonstrated in Figures 3.3, 3.4, 3.6, 3.7, 3.9 and 3.10. Each of these figures demonstrate that membranes from both lean and diabetic animals pretreated with pertussis toxin, resulting in the ADP-ribosylation and inactivation of G_i *in vivo*, exhibited enhanced maximal responses to Gpp(NH)p, glucagon and isoproterenol but similar EC_{50} values compared with non-treated animals (Table 3.2, Figures 3.3, 3.4, 3.6, 3.7, 3.9 and 3.10). Therefore, these results appear to be consistent with the presence of a tonic GTP-dependent inhibitory effect of G_i on stimulation of adenylate cyclase activity in lean and diabetic samples which is abolished by treatment with pertussis toxin. However, since the animals were treated for 3 days it was important to ascertain whether this treatment caused any alterations in expression of G-protein subunits which could otherwise explain the enhanced hormone- and guanine nucleotide-mediated stimulations of adenylate cyclase. Indeed, an altered expression of β-subunits in adipocyte membranes prepared from PTx-treated rats compared with untreated rats has been noted by others (Watkins *et al.*, 1989). Therefore comparative immunblotting experiments were performed on liver membranes prepared from toxin-treated and untreated animals (Figures 3.16 and 3.17). Immunoblotting with antisera SG1 and I3B, to identify $G_i\alpha$-2 and $G_i\alpha$-3 respectively, demonstrated that the immunoreactivity of both these proteins was enhanced and their mobility on SDS-PAGE gels was reduced ($G_i\alpha$-2 signal increased by 3.8±0.6-fold in lean animals, and by 4.1±0.5-fold in diabetic animals after PTx treatment; $G_i\alpha$-3 signal increased by 6.9±0.7-fold in lean animals, and by

7.8±0.9-fold in diabetic animals after PTx treatment: data presented as MEAN ± S.D for two experiments performed on different membrane preparations). The reduction in mobility observed for both $G_i\alpha$-2 and $G_i\alpha$-3 in membranes from pertussis toxin-treated lean and diabetic animals is consistent with their ADP-ribosylation (Goldsmith *et al.*, 1987). This observation is particularly important with regard to determining the extent of ADP-ribosylation of G_i proteins in liver membranes from diabetic animals *in vivo* as it was impossible to ascertain whether these proteins had been fully ribosylated *in vivo* using thiol-preactivated toxin and [^{32}P]NAD^+ because the labelling obtained with untreated diabetic animals was already small (Figure 3.20). Hence any reduction in labelling in membranes from toxin-treated diabetic animals was difficult to estimate. However, the fact that all the immunoreactivity corresponding to $G_i\alpha$-2 and $G_i\alpha$-3 exhibited a reduced mobility demonstrated that a 3 day treatment of diabetic animals with pertussis toxin was sufficient to cause the complete ribosylation of G_i proteins *in vivo* whereas a 60 minute *in vitro* treatment of membranes with preactivated toxin was not (Figure 3.20).

The reason for the enhanced immunoreactivity of $G_i\alpha$-subunits in membranes from pertussis toxin-treated animals is not clear. Ribeiro-Neto & Rodbell (1989) demonstrated that treatment of membranes from rat brain cortex with pertussis toxin caused both a reduced mobility of $G_i\alpha$-1 and $G_i\alpha$-2 and increased the immunoreactivity of these proteins to polyclonal antisera raised against their C-terminal decapeptide sequences. Of course, it was also possible that pertussis toxin-mediated inactivation of $G_i\alpha$-subunits *in vivo* increased intracellular levels of cyclic AMP in liver such that expression of these proteins was increased: isolation of the $G_i\alpha$-2 gene has shown there to be a positive cyclic AMP-responsive element upstream of the promoter sequence (Brann *et al.*, 1987). However, any increase in expression did not correlate with the presence of a functional G_i activity in liver membranes from pertussis toxin-treated animals (Figures 3.29 and 3.30) or a reduced stimulation of adenylate cyclase activity by

various ligands (Figures 3.3, 3.4, 3.6, 3.7, 3.8 and 3.9).

Pertussis toxin-treatment of diabetic animals resulted in other changes which were not observed for membranes from pertussis toxin-treated lean animals. The expression of both forms of $G_s\alpha$-subunits and β-subunits were altered in toxin-treated animals compared with untreated animals (Figure 3.17). In membranes from PTx-treated lean animals the expression of both forms of $G_s\alpha$-subunits were elevated (2.5±0.5-fold for the 45 kDa form, and 2.9±0.6-fold for the 42 kDa form; data presented as MEAN ± S.D. for two experiments performed on different membrane preparations). In contrast, levels of these $G_s\alpha$-subunits were decreased in membranes from diabetic animals which had been treated with PTx *in vivo* (20±6% reduction for the 45 kDa form, and 15±5% reduction for the 42 kDa form; data presented as MEAN ± S.D. for two experiments performed on different membrane preparations). Treatment of both lean and diabetic animals with PTx *in vivo* led to a small reduction in the expression of β-subunits (11±6% for lean animals, 28±6% reduction for diabetic animals; data presented as MEAN ± S.D. for two experiments performed on different membrane preparations). The mechanisms by which these changes in expression occur are unknown. However, the similarity between membranes from toxin-treated lean and toxin-treated diabetic animals with respect to the kinetics of hormone- (Figures 3.6, 3.7, 3.9 and 3.10) and guanine nucleotide-dependent (Figures 3.3 and 3.4) stimulation of adenylate cyclase activity implies that pertussis toxin treatment *in vivo* causes alterations in the adenylate cyclase system which extended beyond the simple modification of $G_i\alpha$-subunits and which together result in a net increase in adenylate cyclase activity.

3.3 CONCLUSIONS

Several groups have shown that insulin resistance in rodents is associated with tissue-specific alterations in the expression and functioning of various components of the hormone-sensitive adenylate cyclase system. These changes may be partly responsible for the metabolic perturbations associated with diabetic syndromes.

A common feature of the regulation of adenylate cyclase activity in liver membranes prepared from insulin-resistant Type I and Type II diabetic animals is an impaired functioning of G_i: this has been noted for the genetically obese Zucker rat (Houslay *et al.*, 1989), the genetically obese mouse (Bégin-Heick & Coleman, 1988) and the stz-induced Type I diabetic rat (Gawler *et al.*, 1987; Bushfield *et al.*, 1990c). This defect is restricted to the abolition of guanine nucleotide- but not receptor-dependent inhibition of adenylate cyclase activity (Houslay, 1989), as assessed by measuring the ability of the non-hydrolysable GTP analogue Gpp(NH)p to inhibit forskolin-stimulated adenylate cyclase activity: whilst a 30-40% maximal inhibitory effect was observed for membranes from non-diabetic controls, membranes from stz-diabetic and genetically obese rats did not display any inhibition (Gawler *et al.*, 1987; Bushfield *et al.*, 1990c). In both these systems, this attenuated functioning was attributed to a constitutively elevated PKC activity which resulted in the phosphorylation and inactivation of $G_i\alpha$-2 (Bushfield *et al.*, 1990b and c).

In contrast to these findings, a pertussis toxin-sensitive tonic inhibitory effect of G_i was found to be present in liver plasma membranes prepared from both lean and genetically diabetic (*db/db*) animals. The magnitude of this inhibition was slightly reduced in diabetic animals compared with leans, probably as a result of the large reductions in expression of the three species capable of mediating inhibitory effects, *i.e.* $G_i\alpha$-2, $G_i\alpha$-3 and β-subunits, in membranes from diabetic animals. Consistent with the presence of a tonic inhibitory effect of G_i in diabetic samples

was the enhanced stimulation of adenylate cyclase by Gpp(NH)p, isoproterenol and glucagon after preparation of membranes from lean and diabetic animals which had been treated with pertussis toxin *in vivo* to inactivate G_i. However, pertussis toxin treatment resulted in alterations in G-protein subunit expression in liver membranes from both lean and diabetic animals, and it may have produced other changes in the adenylate cyclase system which could also explain the enhanced maximal activities attained after such treatment.

One interesting observation from this work was the inability to observe Gpp(NH)p-dependent inhibition of forskolin-stimulated adenylate cyclase activity under conditions which have been reported to give reproducible inhibitory effects (Gawler, 1987). By reducing the ethanol concentration to a minimum, potent pertussis toxin-sensitive inhibition of forskolin-stimulated activity was observed, but only at Gpp(NH)p concentrations which were 2 log orders of magnitude greater than those previously reported. Time-course experiments showed that Gpp(NH)p inhibition of forskolin-stimulated adenylate cyclase activity in membranes from lean animals, but not diabetics, proceeded with a 'lag phase' of approximately 5 minutes before a steady-state rate was attained which was lesser in magnitude than that attained by forskolin alone. In membranes prepared from pertussis toxin-treated animals, Gpp(NH)p did not induce a lag phase and the steady state reaction velocity attained was similar in magnitude to that attained with forskolin alone.

Increasing the ethanol concentration present in the reaction mixture reduced the forskolin-stimulated activity, yet potentiated the activity observed with forskolin and 100 μM Gpp(NH)p. The mechanisms by which these effects of ethanol occur are unknown, but presumably it acts on more than one locus to produce such effects on cyclase stimulation.

One might expect that such gross alterations in G-protein subunit expression might have profound affects on the regulation of adenylate cyclase activity in membranes from diabetic animals compared with their lean littermates. Indeed, the

observed reduction in expression of β-subunits, and the subsequent reduction in levels of βγ-subunits that would occur, was expected to alter the equilibria between holomeric and dissociated G-protein α-subunits. As G_sα-subunits are supposed to be less abundant than G_iα-subunits in most membranes (Gilman, 1987), one might have expected a particularly large effect of the reduced expression of β-subunits on the equilibrium between holomeric and dissociated G_sα-subunits. One line of evidence supporting this hypothesis was the enhanced cholera toxin-catalysed incorporation of [^{32}P]ADP-ribose into the two forms of G_sα-subunits present in liver plasma membranes from diabetic animals compared with leans. A similar explanation may have contributed to the small reduction in rate and large reduction in the extent of pertussis toxin-catalysed ADP-ribosylation of G_iα-subunits in membranes from diabetic animals compared with leans, although these factors were difficult to assess due to the contribution of the reductions in expression of G_iα-2 and G_iα-3 to the reduction in labelling.

Despite these alterations in expression of G-protein subunits and possible changes in the ratio of holomeric to dissociated subunits, there were only two alterations in the kinetics of adenylate cyclase stimulation in diabetic samples compared with leans: an enhanced sensitivity to stimulation by Gpp(NH)p and a reduced response to isoproterenol. A similarly reduced response to isoproterenol has been noted in adipocyte membranes prepared from genetically diabetic animals, although the mechanisms by which this occurs is unknown (Bégin-Heick & Coleman, 1988; see Chapter 4). The reduced ability of the β-adrenergic agonist isoproterenol to activate adenylate cyclase in membranes from diabetic animals would suggest that this defect is not restricted to white adipose tissue. However, since the stimulation of adenylate cyclase by isoproterenol was only 2- to 2.5-fold in membranes from lean animals, β-adrenergic receptor-mediated stimulation of cyclic AMP production is unlikely to have any effect on the stimulation of gluconeogenesis and glycogenolysis, as the activation of cyclic AMP-dependent protein kinase required for these processes necessitates large increases in levels of

intracellular cyclic AMP, *i.e.* those cyclic AMP levels attained by stimulation with nanomolar concentrations of glucagon. As stimulation of adenylate cyclase by glucagon was unaffected by the diabetic state, there were two possibilities which could explain the observed reduction in isoproterenol-stimulated adenylate cyclase activity in diabetic samples: firstly, the defect was specific to the β-adrenergic receptor and may either have been the result of a large reduction in receptor number or a defect in the receptor's ability to couple to G_s. Secondly, there could have been a G-protein defect, manifested in the altered response to Gpp(NH)p, which caused the reduced stimulation by isoproterenol but did not affect the response to glucagon because the interaction of the glucagon receptor with G_s was also altered in such a manner that it counteracted any G-protein defect, for example there could have been a reduced glucagon receptor in liver membranes from diabetic animals. However, since the G-protein defect merely involved an enhanced sensitivity to guanine nucleotides in the absence of hormones, and GTP is present in vast excess of the concentrations required for G-protein activation (Birnbaumer *et al.*, 1985b), it is most likely that the reduced β-adrenergic receptor-mediated activation of adenylate cyclase was a receptor-specific defect.

Considering the aims of undertaking work on this system as they were stated in the introduction to this chapter, the results obtained have produced two conclusions. Firstly, the alterations in hepatic carbohydrate and lipid metabolism seen in genetically diabetic mice are not simply explicable by any alteration in the regulation of hormone-stimulated adenylate cyclase activity in liver membranes from diabetic animals compared with lean controls. Secondly, unlike the genetically obese mouse and obese rat systems previously studied, liver membranes from diabetic mice exhibit a functional G_i activity, albeit slightly reduced. Therefore a non-functional hepatic G_i activity, with respect to the guanine nucleotide-dependent tonic inhibitory functioning of this protein(s), is not a universal characteristic of severe insulin-resistant and obese states.

CHAPTER 4

Further Studies On The Regulation Of Adenylate Cyclase Activity In Adipocyte Membranes From Lean And Diabetic (*db*/*db*) Mice

4.1 INTRODUCTION

The most obvious characteristic of severe insulin-resistant states associated with Type II diabetes mellitus is obesity. This arises due to an increase in adipocyte volume caused by triglyceride accumulation. Such a scenario could arise due to altered regulation of any of the several steps involved in the control of triglyceride metabolism: examples include a reduced synthesis or increased degradation of cyclic AMP, a reduced activity of cyclic AMP-dependent protein kinase (PKA), a reduced phosphorylation and activation of hormone-sensitive lipase, increased lipogenesis, *etc.*

Several animal models of obesity and insulin resistance have been studied with respect to hormone-regulated lipolysis and lipogenesis. The most comprehensive studies have been performed on the genetically obese hyperglycaemic (*ob/ob*) mouse. In adipocytes prepared from these animals, there is a drastically reduced ability of catecholamines to stimulate lipolysis (Bégin-Heick & Heick, 1977; Shepherd *et al.*, 1977). This is not associated with any reduced ability of non-metabolisable cyclic AMP analogues to activate lipolysis *via* stimulation of PKA and the subsequent activation of hormone-sensitive lipase (Herberg *et al.*, 1970). Hence the defect must reside upstream of PKA activation. Consistent with this hypothesis is the reduced ability of catecholamines to elevate intracellular cyclic AMP levels (Bégin-Heick & Heick, 1977). Although adipocytes from obese animals possess a greater cyclic AMP phosphodiesterase activity compared with lean animals, hormone-stimulated lipolysis remains lower in adipocytes from obese animals in the presence of PDE inhibitors (Shepherd *et al.*, 1977). Hence, the defect must reside in a reduced ability of catecholamines to stimulate cyclic AMP synthesis. This has been confirmed by several groups who have noted a reduced stimulation of adenylate cyclase activity in isolated adipocyte membranes from obese animals compared with lean animals (Dehaye *et al.*, 1978).

Detailed studies on the kinetics of adenylate cyclase regulation have been

performed for the genetically obese mouse adipocyte system by Bégin-Heick and co-workers and have been described in several publications (Bégin-Heick, 1985; Bégin-Heick, 1986; Bégin-Heick & Coleman, 1988). As well as a reduced steady-state stimulation of adenylate cyclase by catecholamines, there is a noticeable 'lag phase', or hysteresis, before steady-state activation is reached on stimulation with GTP and β-adrenergic agonists in membranes from obese animals but not leans (Bégin-Heick, 1986). These defects are also associated with the absence of a GTP-dependent tonic inhibitory effect of G_i in adipocyte membranes from obese animals compared with leans (Bégin-Heick, 1985). However, receptor-dependent inhibition by the classical anti-lipolytic agents PIA, PGE_1 and nicotinic acid persists in membranes from obese animals (Greenberg *et al.*, 1987).

Relatively few studies have attempted to explain the reduced catecholamine-dependent stimulation of lipolysis and adenylate cyclase observed in adipocytes from genetically diabetic (*db/db*) mice (Kupiechi & Adams, 1974; Levilliers *et al.*, 1978; Bégin-Heick & Coleman, 1988). One study performed with adipocyte membranes from genetically diabetic animals showed that G_i functioning was intact with respect to GTP-dependent 'tonic' inhibitory effects, unlike genetically obese (*ob/ob*) animals, but this was not a detailed study (Bégin-Heick & Coleman, 1988). A more detailed examination of the regulation of adenylate cyclase activity in adipocytes from genetically diabetic mice was undertaken for several reasons. Firstly, it might have provided some insight into potential mechanisms by which the reduced stimulation of adenylate cyclase activity by β-adrenergic receptor agonists occurred. Secondly, it might have revealed other signalling defects which could exacerbate the reductions in catecholamine-stimulated cyclic AMP levels observed in diabetic mouse adipocytes. Finally, it was important to compare any altered regulation of adenylate cyclase activity with defects observed in other obesity model systems, such as the obese (*ob/ob*) mouse and the obese (*fa/fa*) Zucker rat. This was undertaken in order to determine whether the obese state is characterised by a

common set of defects which may help to explain the abnormal accumulation of triglyceride.

Figure 4.1:

Dose-Response Curves For Isoproterenol Stimulation In Adipocyte Membranes From Lean And Diabetic (*db/db*) Animals

Adipocyte membranes from lean (❑) and diabetic (*db/db*) (○) animals were assayed for adenylate cyclase activity for 30 minutes at 30°C in the presence of 100 μM GTP and increasing concentrations of isoproterenol as described in Section 2.7.1. The [^{32}P]cyclic AMP formed was measured by the double column method described in Section 2.7.2. The figure shows results pooled from three experiments performed on different membrane preparations and values are presented as MEAN ± S.D.

Specific activities obtained in the presence of 100 μM GTP alone were 6.3 ± 2.1 (lean) and 5.6 ± 2.2 (diabetic) pmol/min/mg (data presented as MEAN ± S.D.).

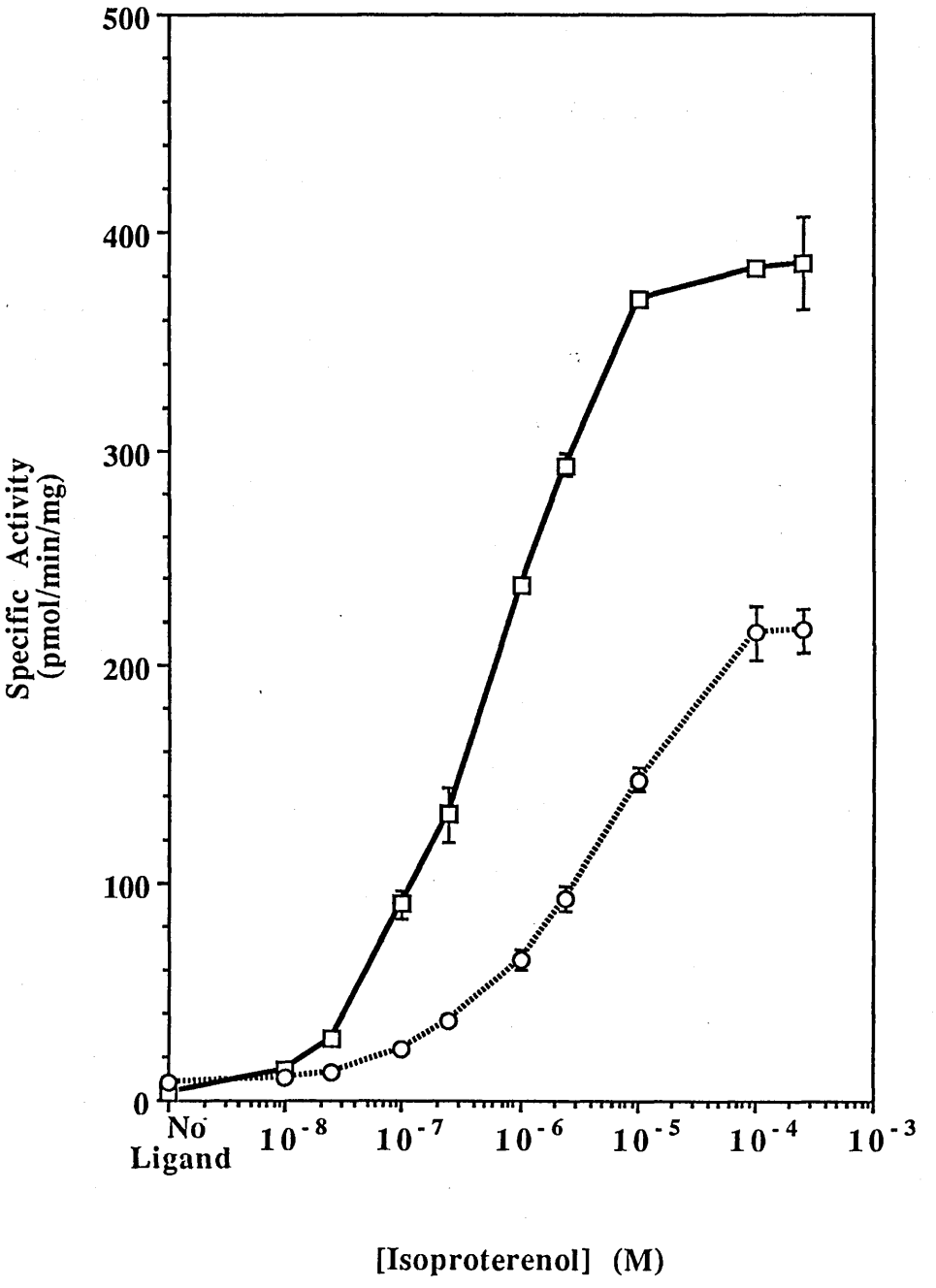

500
400
300
200
100
0
Specific Activity
(pmol/min/mg)
No
Ligand
10-8
10-7
10-6
10-5
10-4
10-3
[Isoproterenol] (M)

Table 4.1:

Adenylate Cyclase Activity In Adipocyte Membranes From Lean And Diabetic (*db/db*) Animals

Adipocyte membranes prepared from lean and diabetic (*db/db*) animals were assayed for adenylate cyclase activity in the absence (basal) and in the presence of the ligands mentioned below for 15 minutes at 30°C as described in Section 2.7.1. The [^{32}P]cyclic AMP formed was measured by the double column method described in Section 2.7.2. For experiments employing mastoparan, isoproterenol, glucagon, TSH and secretin, GTP was also present at a final concentration of 100 μM. Specific activities are in pmol/min/mg protein and data are expressed as MEAN ± S.D. for three experiments using different membrane preparations. * denotes a significant difference between lean and diabetic animals, *i.e.* $p<0.001$.

n.a.=not applicable. n.d.=not determined.

As stated in the Acknowledgements, his study was performed in collaboration with Derek Strassheim, Molecular Pharmacology Group, Institute of Biochemistry, University of Glasgow. Therefore the kinetic parameters obtained with glucagon, secretin, TSH and NaF were obtained from his experiments.

Table 4.1:

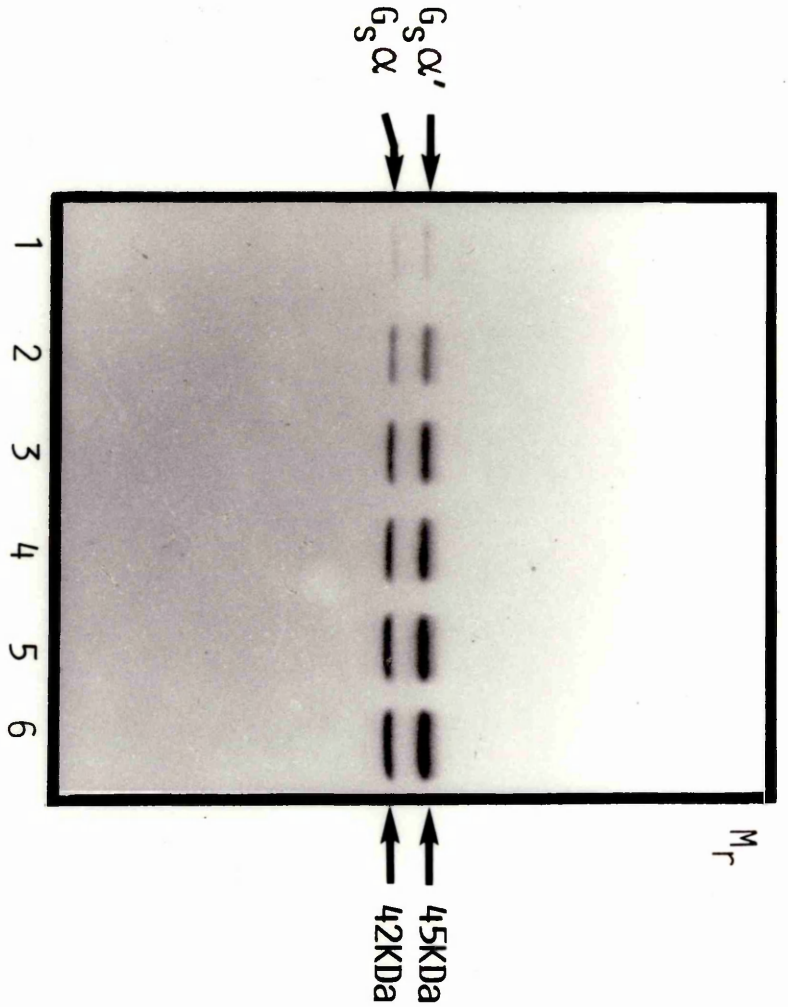
Stimulating Ligand		Lean		Diabetic (*db/db*)	
		Spec. Ac.	EC_{50} (μM)	Spec. Ac.	EC_{50} (μM)
	Basal	6.5±1.3	n.a.	6.7±1.4	n.a.
	100 μM Forskolin	180±14	n.d.	175±12	n.d.
	20 mM NaF	55±3	n.d.	51±5	n.d.
100 μM GTP +	60 μM Mastoparan	36.4±3.2	n.d.	40.2±5.3	n.d.
100 μM GTP +	100 μM Isoproterenol	302±10	0.36±0.04	160±11*	2.2±0.2*
100 μM GTP +	10 μM Glucagon	33 ± 4	0.16±0.02	16±2*	0.09±0.01
100 μM GTP +	2 μM TSH	18.6±0.9	0.079±0.008	19.0±2.1	0.081±0.009
100 μM GTP +	10 μM Secretin	23.8±1.1	0.71±0.08	13.6±0.9*	0.42±0.05

Figure 4.2:

Effect Of Increasing Protein Concentration On The Detection Of $G_s\alpha$-Subunits In Adipocyte Membranes From Lean Animals

Increasing amounts of adipocyte membrane protein prepared from lean animals were subjected to SDS-PAGE before transfer to nitrocellulose and immunoblotting with antiserum CS1 as described in Sections 2.8 and 2.9. The amounts loaded are 10 μg (lane 1), 20 μg (lane 2), 30 μg (lane 3), 40 μg (lane 4), 50 μg (lane 5) and 60 μg (lane 6). The blot shown in *panel a* is representative of three performed with different membrane preparations which produced identical results. *Panel b* shows the corresponding graph of band intensity versus amount of protein loaded for the 45kDa (□) and 42 kDa (○) forms of $G_s\alpha$.

Panel a



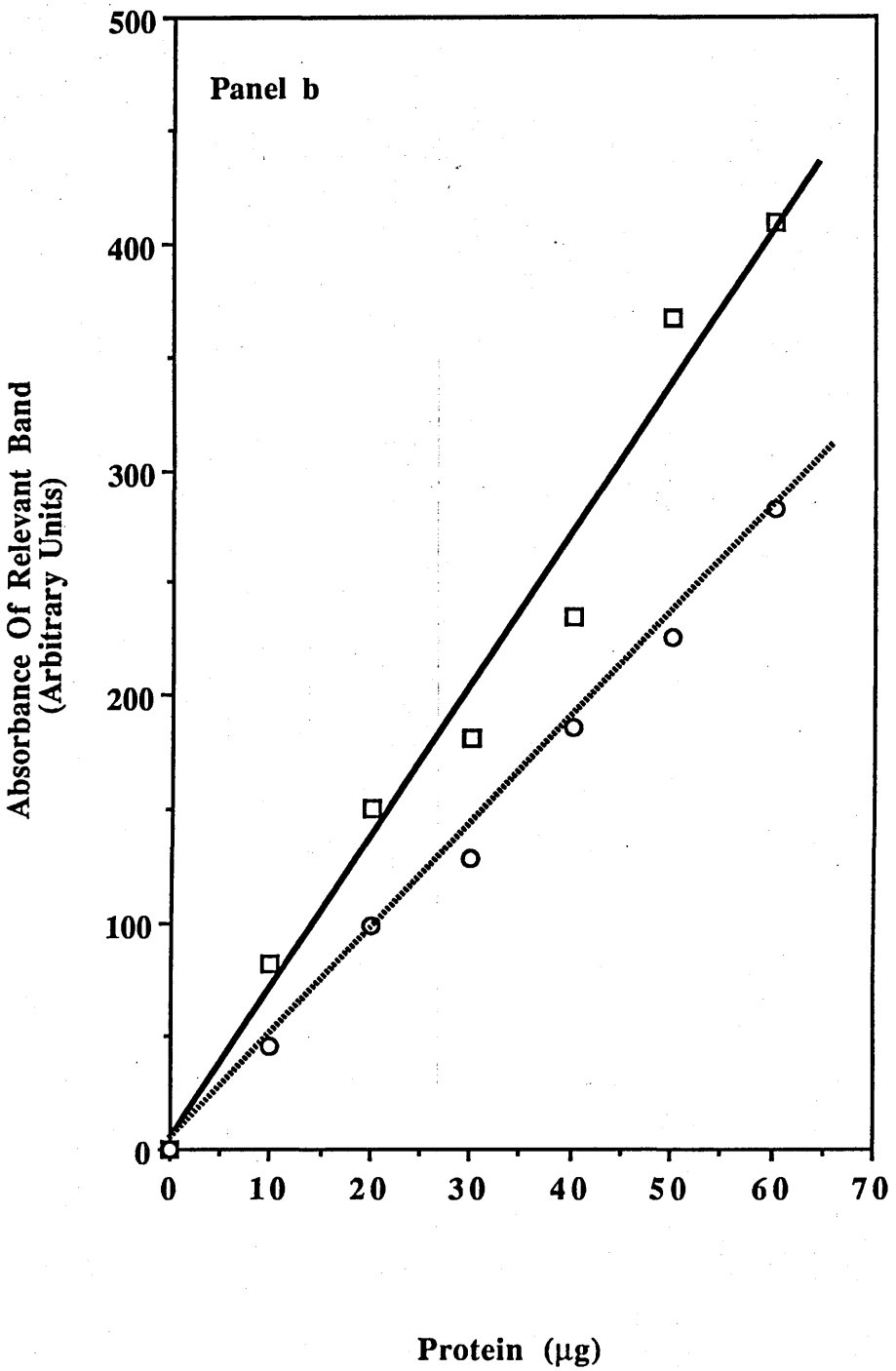
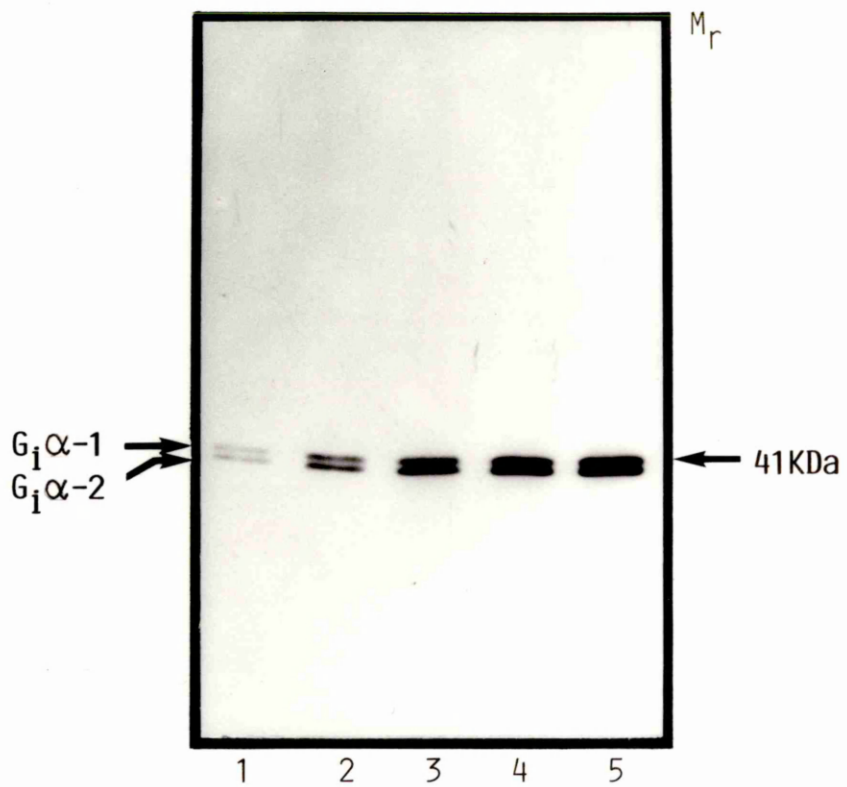

Panel b
Absorbance Of Relevant Band
(Arbitrary Units)
500
400
300
200
100
0
0
10
20
30
40
50
60
70
Protein (μg)

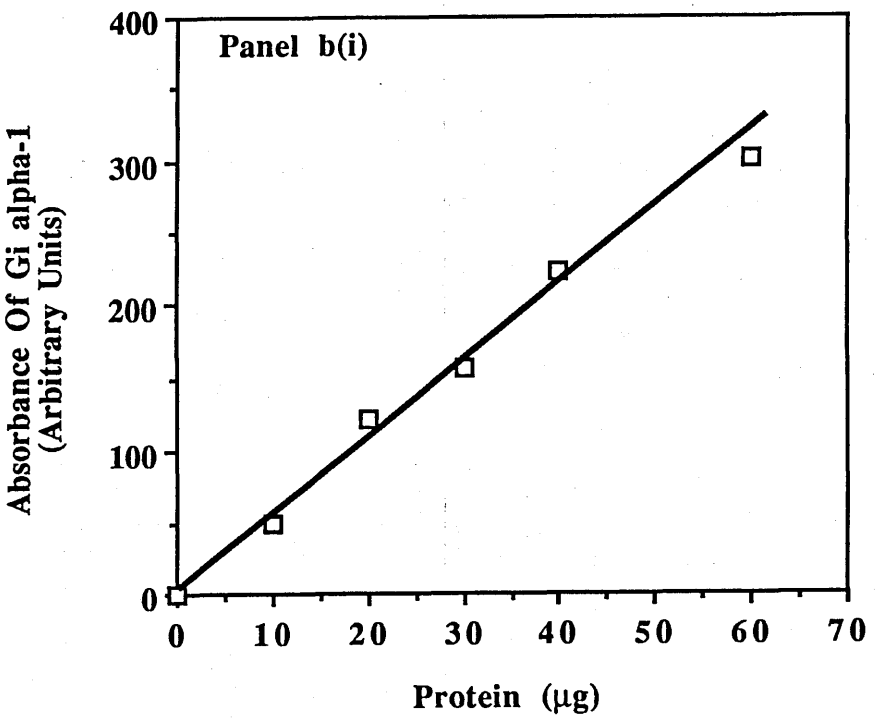
Figure 4.3:

Effect Of Increasing Protein Concentration On The Detection Of $G_i\alpha$-1 And $G_i\alpha$-2 In Adipocyte Membranes From Lean Animals

Increasing amounts of adipocyte membrane protein prepared from lean animals were subjected to SDS-PAGE under the electrophoretic conditions described in Section 2.8.4 before transfer to nitrocellulose and immunoblotting with antiserum SG1 as described in Section 2.9. The amounts loaded are 10 μg (lane 1), 20 μg (lane 2), 30 μg (lane 3), 40 μg (lane 4), and 60 μg (lane 5). The blot shown in *panel a* is representative of three performed with different membrane preparations which produced identical results. *Panel b* shows the corresponding graph of band intensity versus amount of protein loaded for both $G_i\alpha$-1 and $G_i\alpha$-2.

Panel a




Panel b(i)
Absorbance Of Gi alpha-1
(Arbitrary Units)
400
300
200
100
0
0
10
20
30
40
50
60
70
Protein (μg)

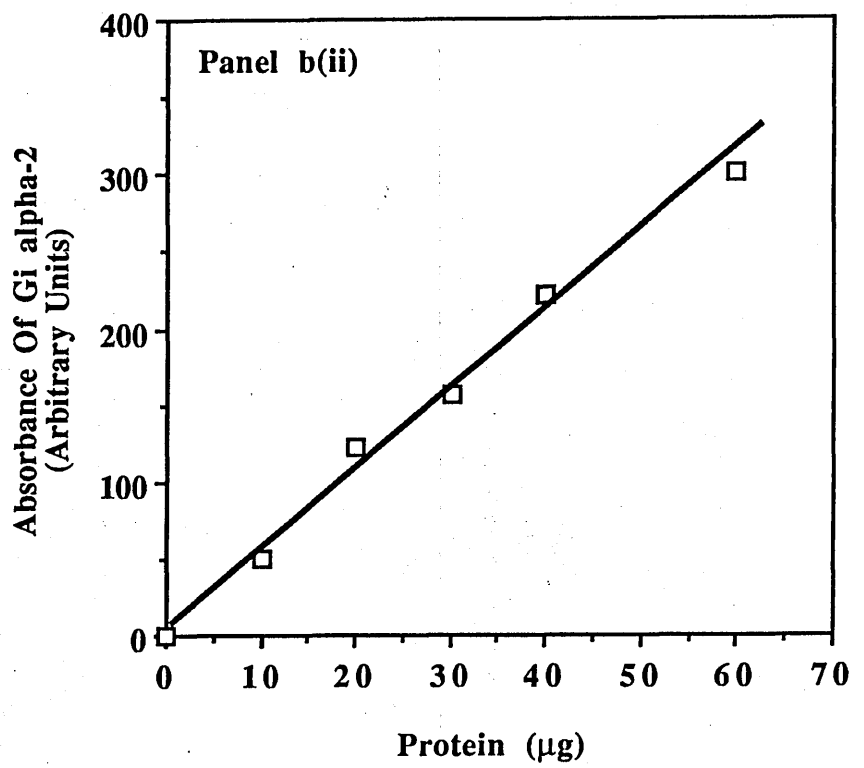
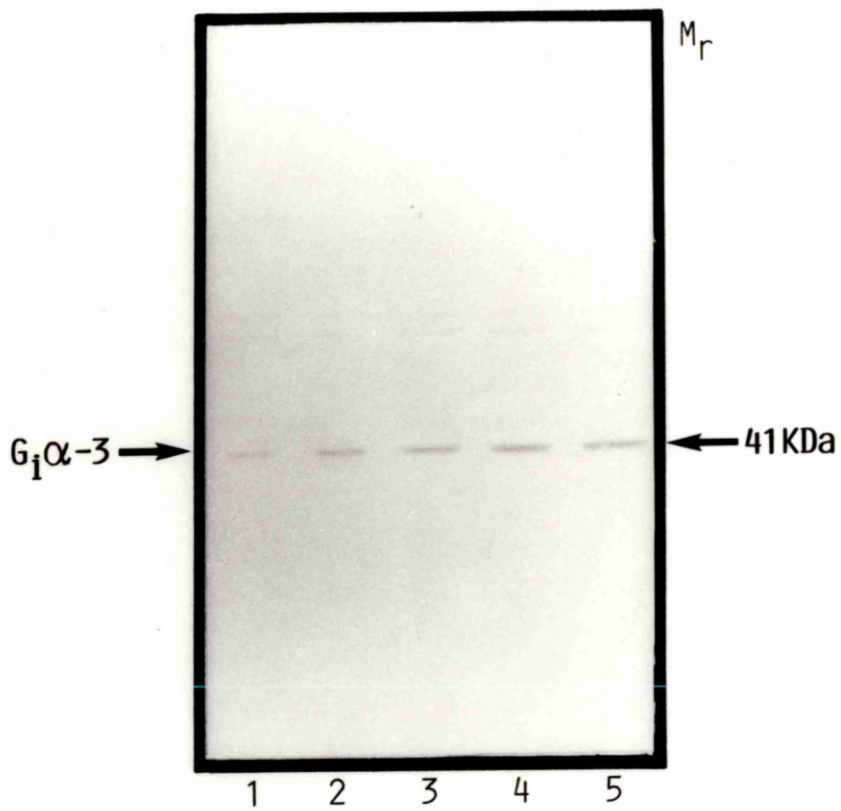

Panel b(ii)
Absorbance Of Gi alpha-2
(Arbitrary Units)
400
300
200
100
0
0
10
20
30
40
50
60
70
Protein (μg)

Figure 4.4:

Effect Of Increasing Protein Concentration On The Detection Of $G_i\alpha$-3 In Adipocyte Membranes From Lean Animals

Increasing amounts of adipocyte membrane protein prepared from lean animals were subjected to SDS-PAGE before transfer to nitrocellulose and immunoblotting with antiserum I3B as described in Sections 2.8 and 2.9. The amounts loaded are 50 μg (lane 1), 100 μg (lane 2), 150 μg (lane 3), 200 μg (lane 4) and 300 μg (lane 5). The blot shown in *panel a* is representative of three performed with different membrane preparations which produced identical results. *Panel b* shows the corresponding graph of band intensity versus amount of protein loaded.

Panel a



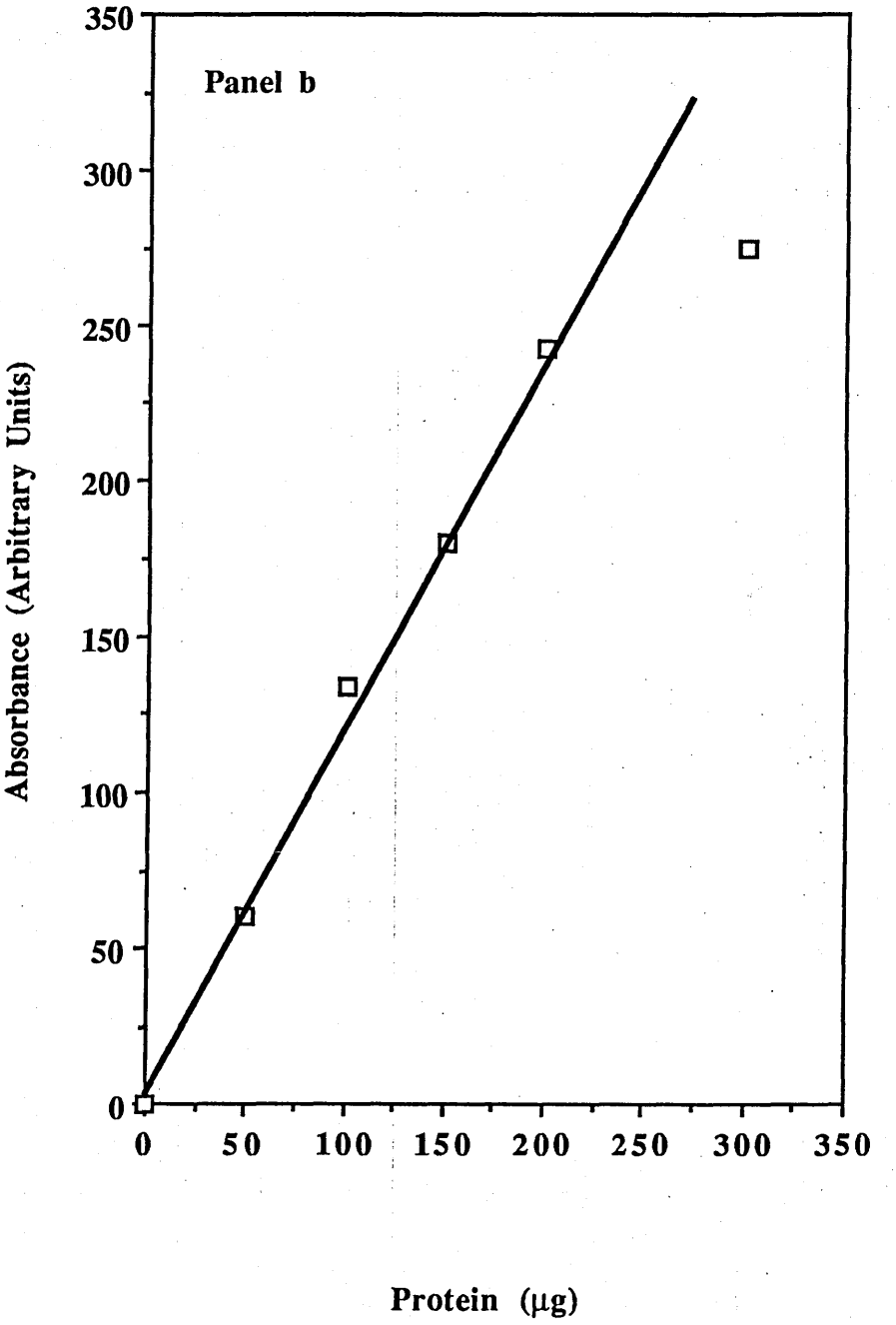
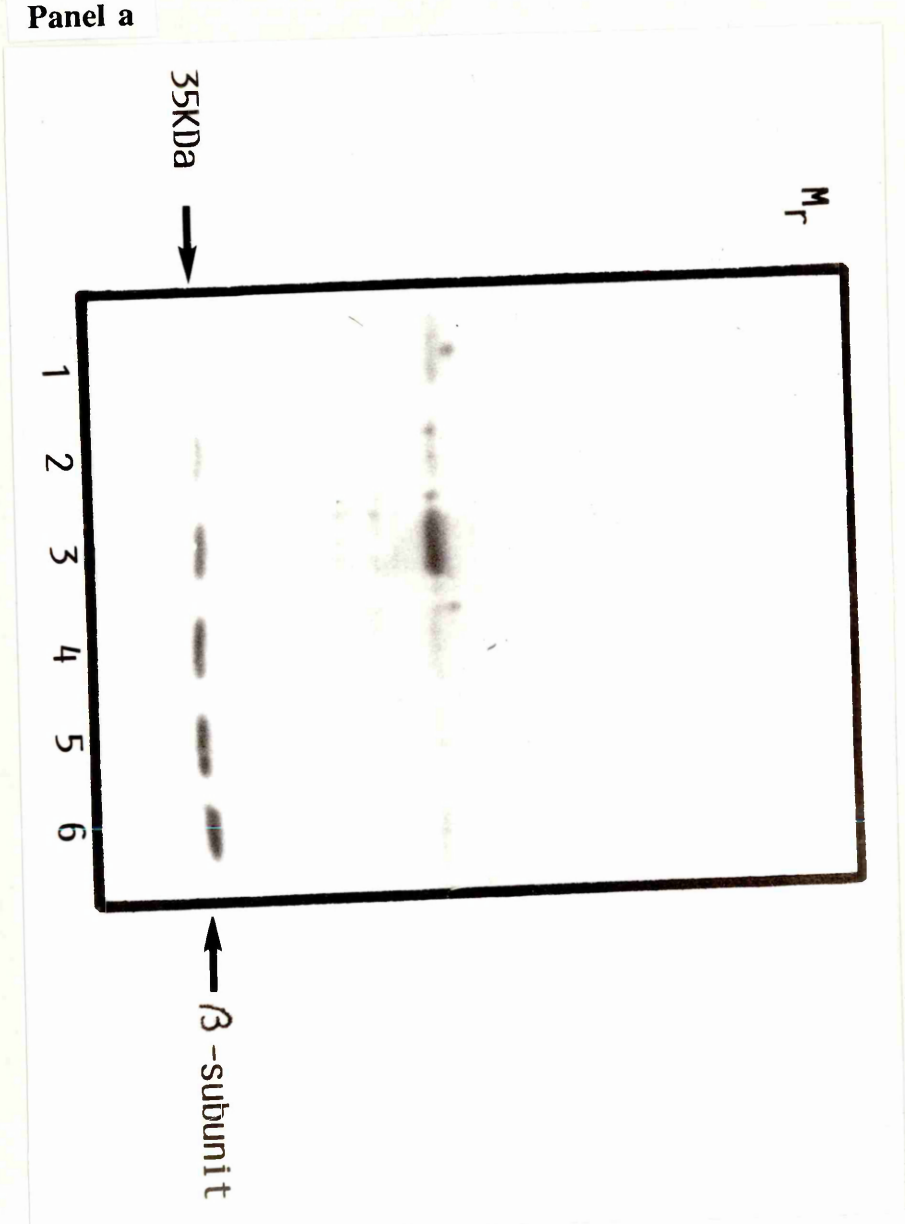

Panel b
Absorbance (Arbitrary Units)
Protein (µg)
0
50
100
150
200
250
300
350

Figure 4.5:

Effect Of Increasing Protein Concentration On The Detection Of β-Subunits In Adipocyte Membranes From Lean Animals

Increasing amounts of adipocyte membrane protein prepared from lean animals were subjected to SDS-PAGE before transfer to nitrocellulose and immunoblotting with antiserum BN1 as described in Sections 2.8 and 2.9. The amounts loaded are 20 μg (lane 1), 40 μg (lane 2), 80 μg (lane 3), 120 μg (lane 4), 160 μg (lane 5) and 200 μg (lane 6). The blot shown in *panel a* is representative of three performed with different membrane preparations which produced identical results. *Panel b* shows the corresponding graph of band intensity versus amount of protein loaded.

Panel a



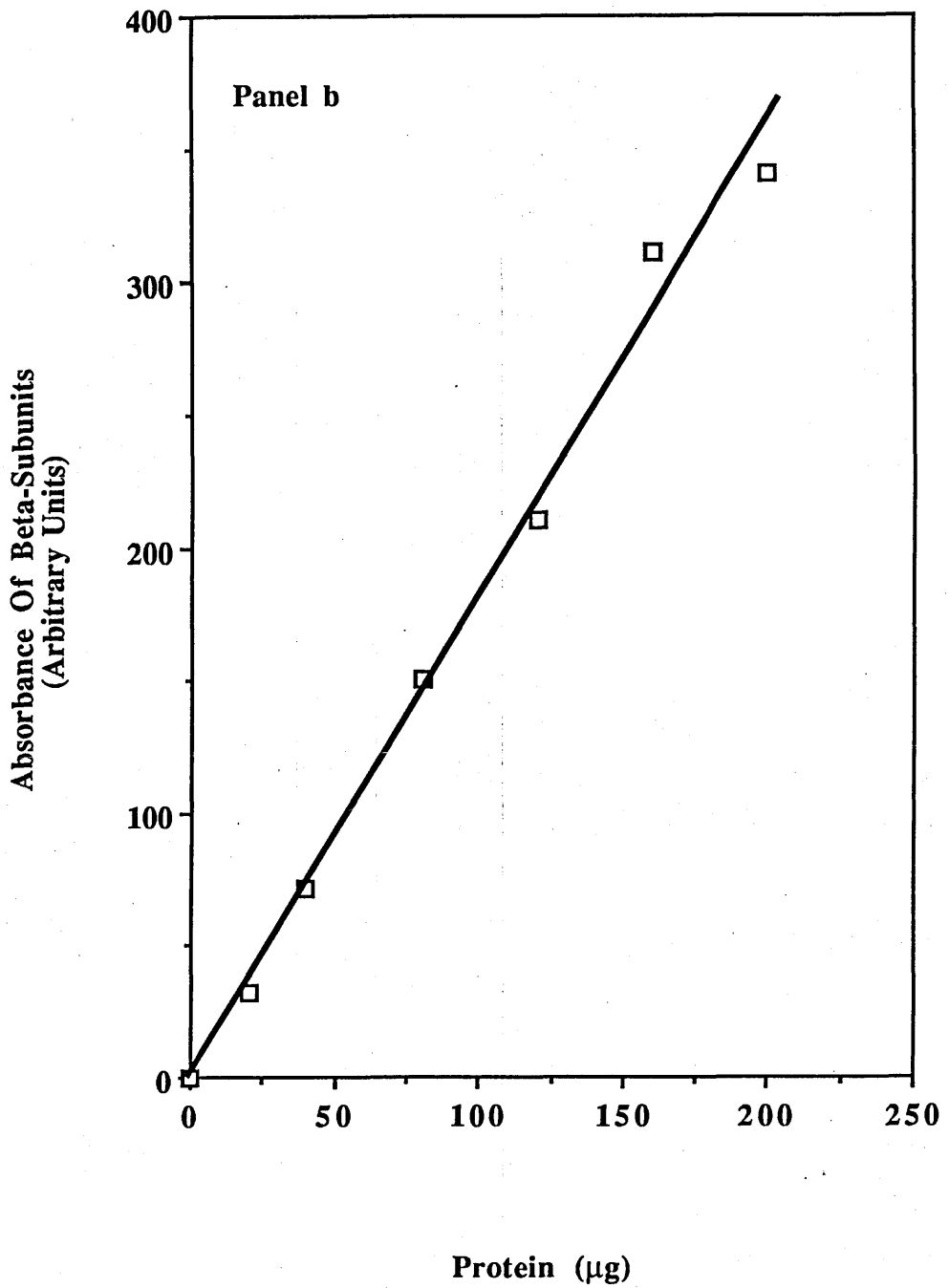

Panel b
Absorbance Of Beta-Subunits
(Arbitrary Units)
400
300
200
100
0
0
50
100
150
200
250
Protein (μg)

Figure 4.6:

Immunoblotting Of $G_s\alpha$ In Adipocyte Membranes From Lean And Diabetic (*db/db*) Animals

40 μg of adipocyte membrane protein from lean (lane 1) and diabetic (*db/db*) (lane 2) animals were prepared and subjected to SDS-PAGE under 'normal' conditions before transfer to nitrocellulose paper and immunoblotting using antiserum CS1 as the primary antiserum as described in Sections 2.8 and 2.9. The immunoblot shown is representative of three performed with different membrane preparations from lean and diabetic (*db/db*) animals.

Comparison of levels of $G_s\alpha$ in
control and db/db mouse adipocyte membranes

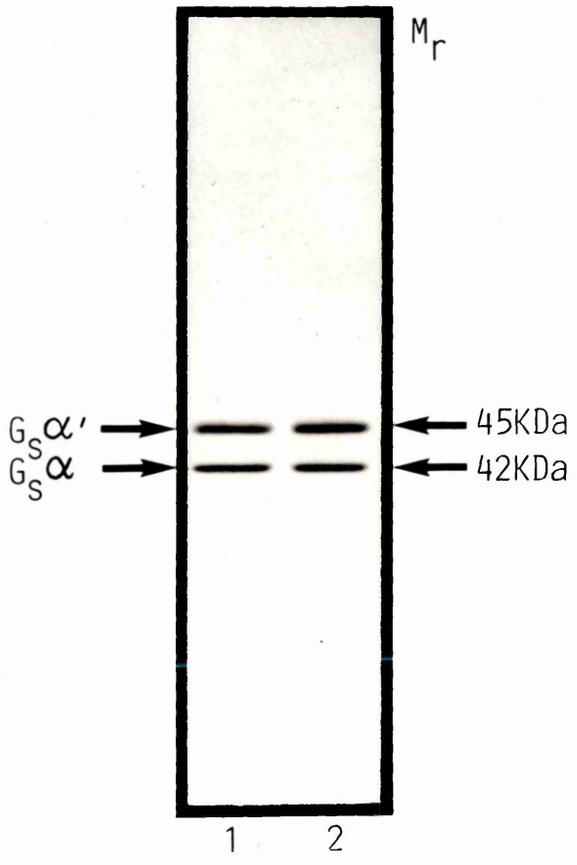


Figure 4.7:

Immunoblotting Of $G_i\alpha$-1 And $G_i\alpha$-2 In Adipocyte Membranes From Lean And Diabetic (*db/db*) Animals

70 μg of adipocyte membrane protein from lean (lane 1) and diabetic (*db/db*) (lane 2) animals were prepared and subjected to SDS PAGE under conditions allowing resolution of $G_i\alpha$-1 and $G_i\alpha$-2 before transfer to nitrocellulose paper and immunoblotting using antiserum SG1 as the primary antiserum as described in Sections 2.8.4 and 2.9. The immunoblot shown is representative of three performed with different membrane preparations from lean and diabetic (*db/db*) animals.

Comparison of levels of $G_i\alpha 1$ and 2
in lean and db/db adipocyte membranes

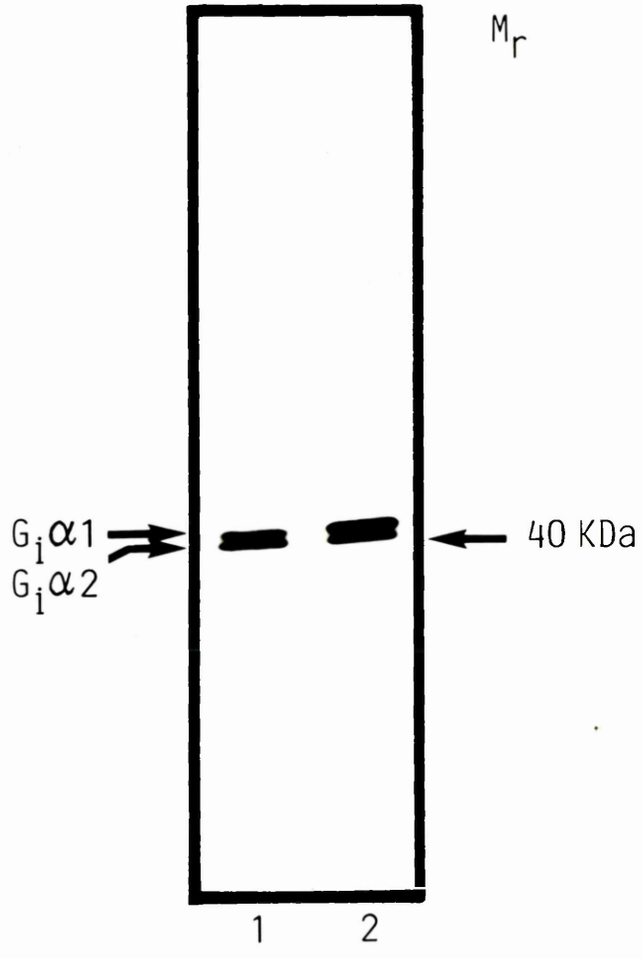


Figure 4.8:

Immunoblotting Of $G_i\alpha$–3 In Adipocyte Membranes From Lean And Diabetic (*db/db*) Animals

100 μg of adipocyte membrane protein from lean (lane 1) and diabetic (*db/db*) (lane 2) animals were prepared and subjected to SDS-PAGE under 'normal' conditions before transfer to nitrocellulose paper and immunoblotting using antiserum I3B as the primary antiserum as described in Sections 2.8 and 2.9. The immunoblot shown is representative of three performed with different membrane preparations from lean and diabetic (*db/db*) animals.

Comparison of levels of $G_i\alpha 3$ in
control and db/db mouse adipocyte membranes

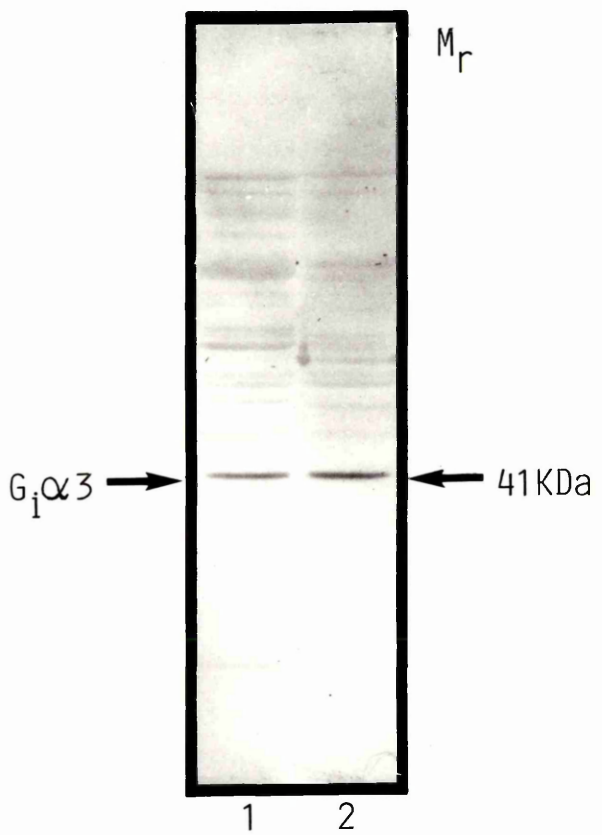


Figure 4.9:

Immunoblotting Of β-Subunits In Adipocyte Membranes From Lean And Diabetic (*db/db*) Animals

80 μg of adipocyte membrane protein from lean (lane 1) and diabetic (*db/db*) (lane 2) animals were prepared and subjected to SDS-PAGE under 'normal' conditions before transfer to nitrocellulose paper and immunoblotting using antiserum BN1 as the primary antiserum as described in Sections 2.8 and 2.9. The immunoblot shown is representative of three performed with different membrane preparations from lean and diabetic (*db/db*) animals.

Comparison of levels of β-subunit
in control and db/db mouse adipocyte membranes

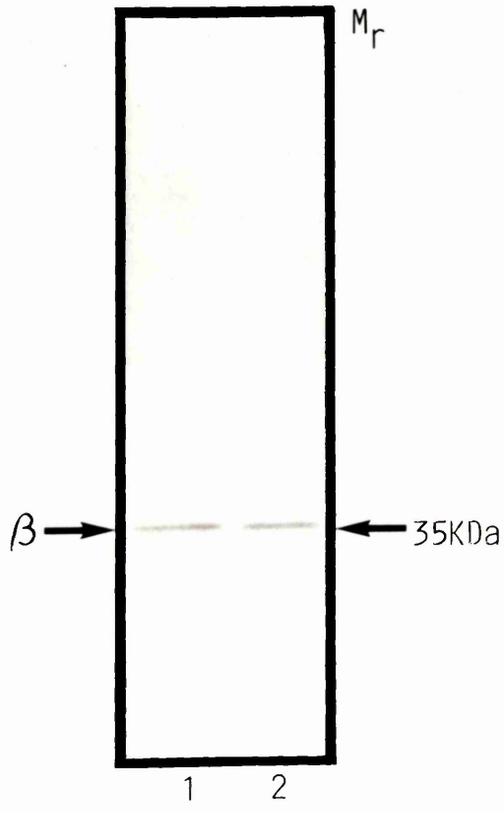


Table 4.2:

Comparative Levels Of G-Protein Subunits In Adipocyte Membranes From Lean And Diabetic (*db/db*) Animals

Adipocyte membranes from lean and diabetic (*db/db*) animals were prepared and subjected to SDS-PAGE before transfer to nitrocellulose and immunoblotting with the primary antisera described in Section 2.9.2 by the method outlined in Sections 2.8 and 2.9.1. Data express levels of G-protein components in diabetic (*db/db*) animals as a percentage of those found in lean animals. For each antiserum, a range of amounts of membrane protein from lean and diabetic (*db/db*) animals was loaded on a single gel to determine protein loadings which produced linear increases in absorption with increasing amounts of membrane protein applied when the blots were densiometrically scanned after visualisation with a peroxidase-conjugated second antibody. Subsequent comparative blots employed protein concentrations within these parameters.

For each G-protein subunit, data are expressed as MEAN ± S.D. for three immunoblots performed with different membrane preparations.

* denotes a significant difference between lean and diabetic animals, *i.e.* $p<0.001$.

Table 4.2:

G-Protein Subunit	Level As A Percentage Of That In Lean Membranes (100%)
$G_i\alpha$-1	192 ± 8*
$G_i\alpha$-2	115 ± 12
$G_i\alpha$-3	115 ± 10
β-subunit	105 ± 2
$G_s\alpha$ (42 kDa)	111 ± 7
$G_s\alpha$ (45 kDa)	108 ± 7

Figure 4.10:

Dose-Response Curves For PIA Inhibition Of Isoproterenol-Stimulated Adenylate Cyclase Activity In Adipocyte Membranes From Lean And Diabetic (*db/db*) Animals

Adipocyte membranes from lean (❑) and diabetic (*db/db*) (○) animals were assayed for adenylate cyclase activity for 30 minutes at 23°C in the presence of 100 μM GTP, 100 μM isoproterenol and increasing concentrations of PIA as described in Section 2.7.1. The cyclic AMP formed was measured by the competitive binding method also described in Section 2.7.1. The figure shows results pooled from three experiments performed on different membrane preparations and values are presented as MEAN ± S.D.

The activities obtained with isoproterenol and GTP alone are given in Table 4.3.

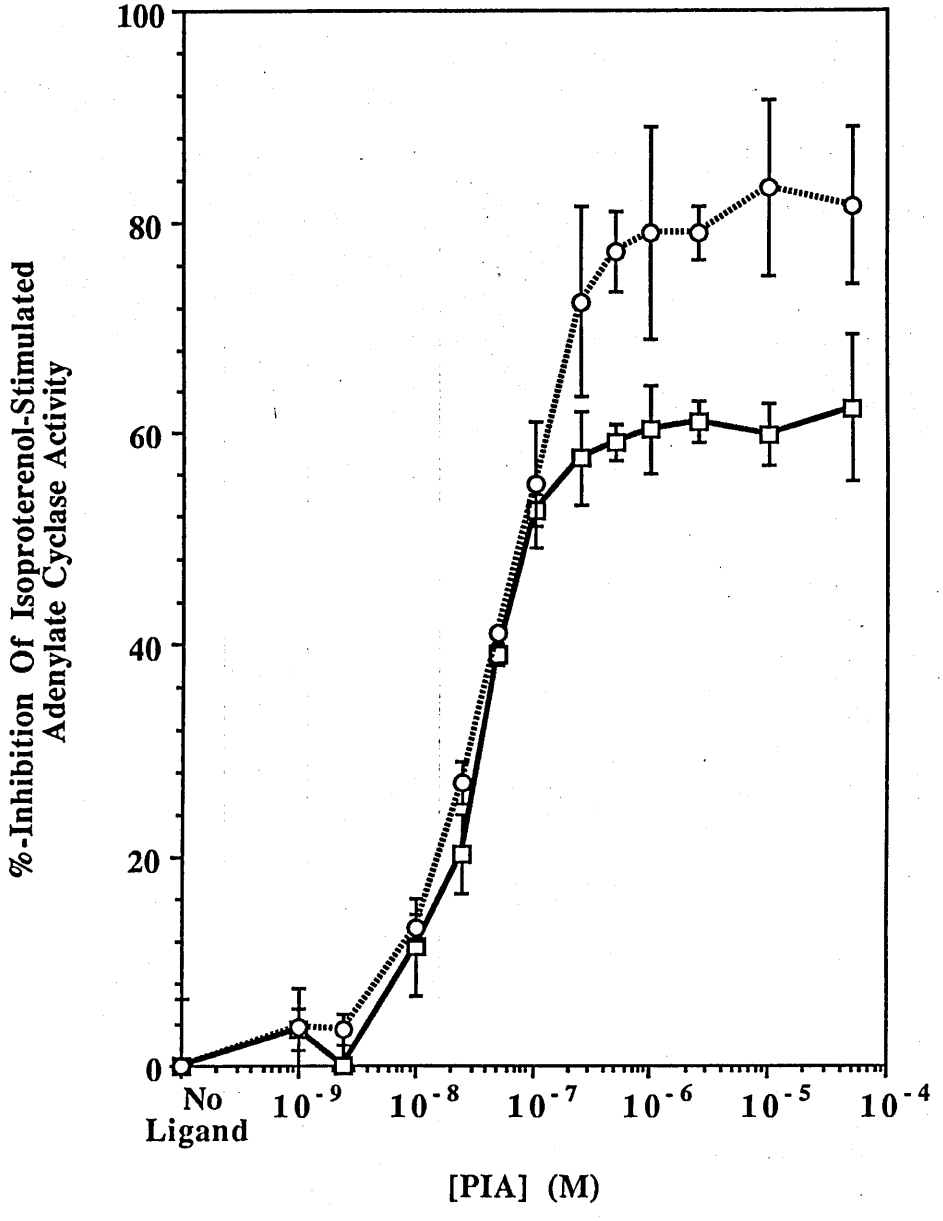

100
80
60
40
20
0
%-Inhibition Of Isoproterenol-Stimulated Adenylate Cyclase Activity
No Ligand
10-9
10-8
10-7
10-6
10-5
10-4
[PIA] (M)

Figure 4.11:

Dose-Response Curves For Prostaglandin E_1 Inhibition Of Isoproterenol-Stimulated Adenylate Cyclase Activity In Adipocyte Membranes From Lean And Diabetic (*db/db*) Animals

Adipocyte membranes from lean (❑) and diabetic (*db/db*) (○) animals were assayed for adenylate cyclase activity for 30 minutes at 23°C in the presence of 100 μM GTP, 100 μM isoproterenol and increasing concentrations of prostaglandin E_1 as described in Section 2.7.1. The cyclic AMP formed was measured by the competitive binding method also described in Section 2.7.1. The figure shows results pooled from three experiments performed on different membrane preparations and values are presented as MEAN ± S.D.

The activities obtained with isoproterenol and GTP alone are given in Table 4.3.

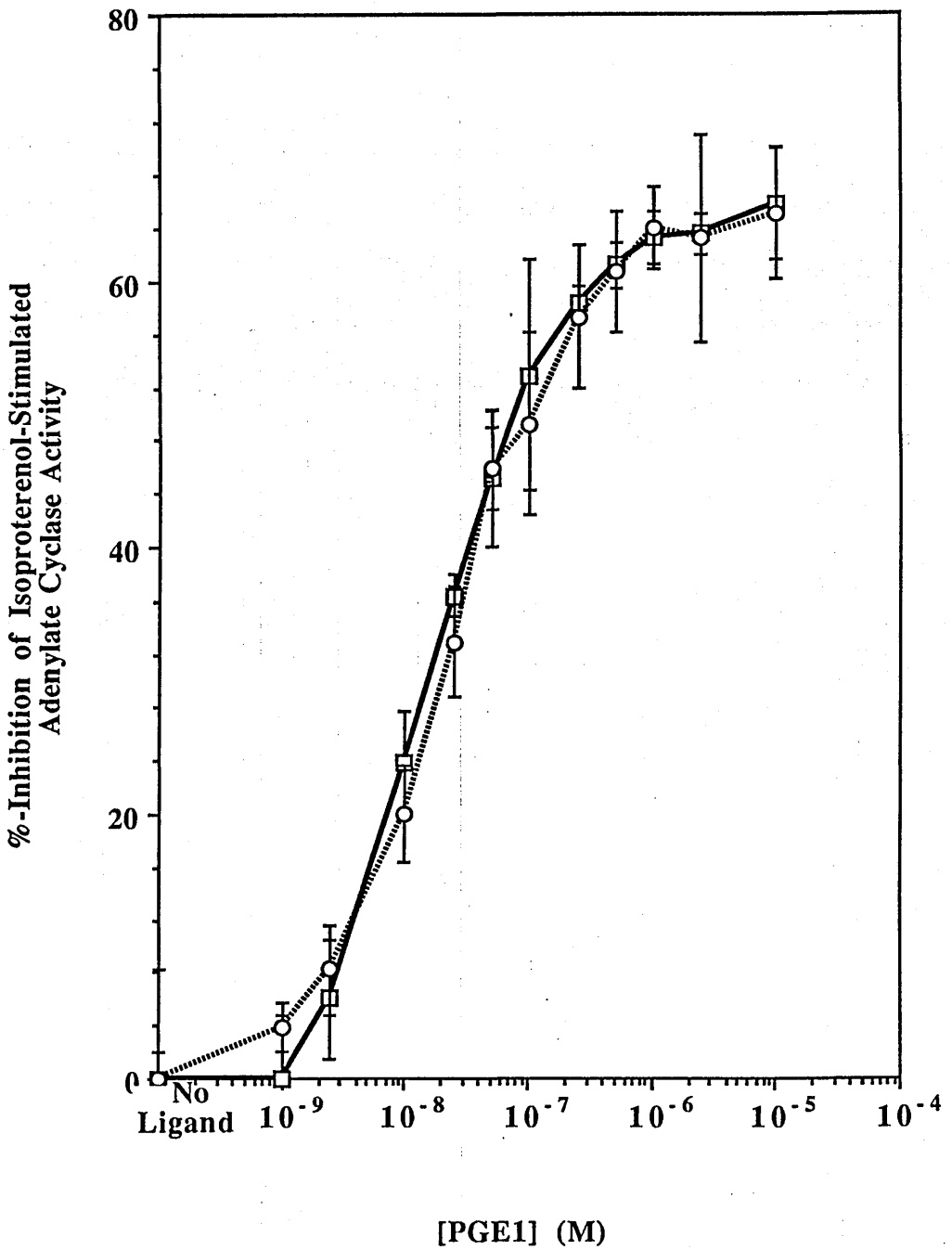

%-Inhibition of Isoproterenol-Stimulated Adenylate Cyclase Activity
80
60
40
20
0
No Ligand
10-9
10-8
10-7
10-6
10-5
10-4
[PGE1] (M)

Figure 4.12:

Dose-Response Curves For Nicotinic Acid Inhibition Of Isoproterenol-Stimulated Adenylate Cyclase Activity In Adipocyte Membranes From Lean And Diabetic (*db/db*) Animals

Adipocyte membranes from lean (❑) and diabetic (*db/db*) (○) animals were assayed for adenylate cyclase activity for 30 minutes at 23°C in the presence of 100 μM GTP, 100 μM isoproterenol and increasing concentrations of nicotinic acid as described in Section 2.7.1. The cyclic AMP formed was measured by the competitive binding method also described in Section 2.7.1. The figure shows results pooled from three experiments performed on different membrane preparations and values are presented as MEAN ± S.D.

The activities obtained with isoproterenol and GTP alone are given in Table 4.3.

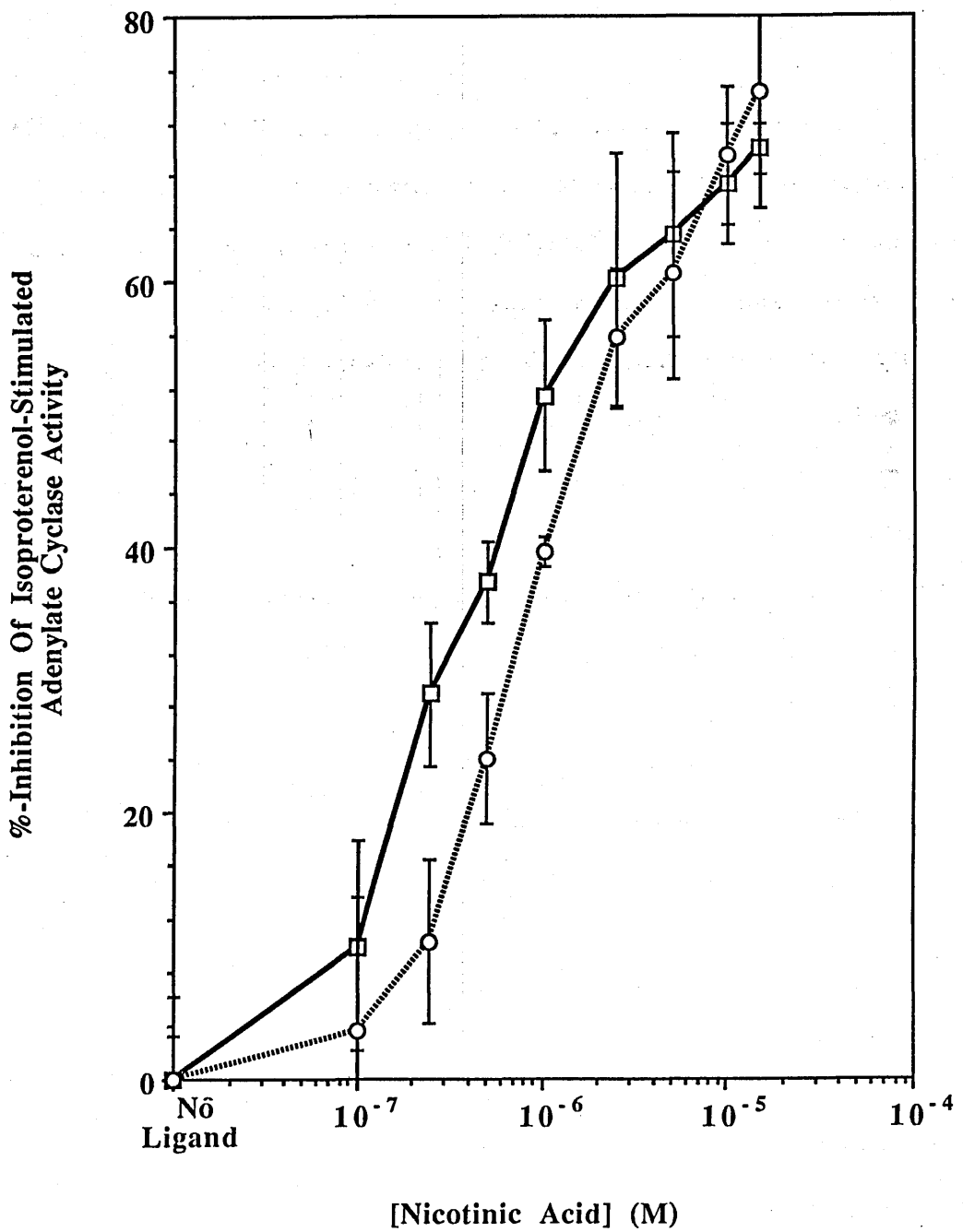

%-Inhibition Of Isoproterenol-Stimulated Adenylate Cyclase Activity
80
60
40
20
0
Nô Ligand
10-7
10-6
10-5
10-4
[Nicotinic Acid] (M)

Table 4.3:

Receptor-Mediated Inhibition Of Adenylate Cyclase Activity In Adipocyte Membranes From Lean And Diabetic (*db/db*) Animals

Adipocyte membranes from lean and diabetic (*db/db*) animals were assayed for adenylate cyclase activity for 30 minutes at 23°C in the presence of 100 μM GTP, 100 μM isoproterenol and either 10 μM PIA, 10 μM prostaglandin E_1 or 100 μM nicotinic acid as described in Section 2.7.1. Data are expressed as %-inhibition of the activity observed in the absence of an inhibitory ligand but with 100 μM isoproterenol and 100 μM GTP present. For each ligand, results were pooled from three experiments performed on different membrane preparations and values are presented as MEAN ± S.D. IC_{50} is the ligand concentration at which half-maximal inhibition was achieved.

Specific activities obtained in the presence of 100 μM isoproterenol and 100 μM GTP were 275 ± 31 and 97 ± 12 pmol/min/mg protein for lean and diabetic animals respectively (MEAN ± S.D. for nine experiments).

* denotes a significant difference between lean and diabetic animals, *i.e* $p<0.005$.

Table 4.3:

	Lean		Diabetic (*db/db*)	
Ligand	Inhibition (%)	IC_{50} (nM)	Inhibition (%)	IC_{50} (nM)
PIA	60 ± 3	46 ± 5	83 ± 9*	25 ± 7
Prostaglandin E_1	66 ± 5	16 ± 3	65 ± 7	18 ± 3
Nicotinic Acid	74 ± 9	400 ± 90	69 ± 3	950 ± 120*

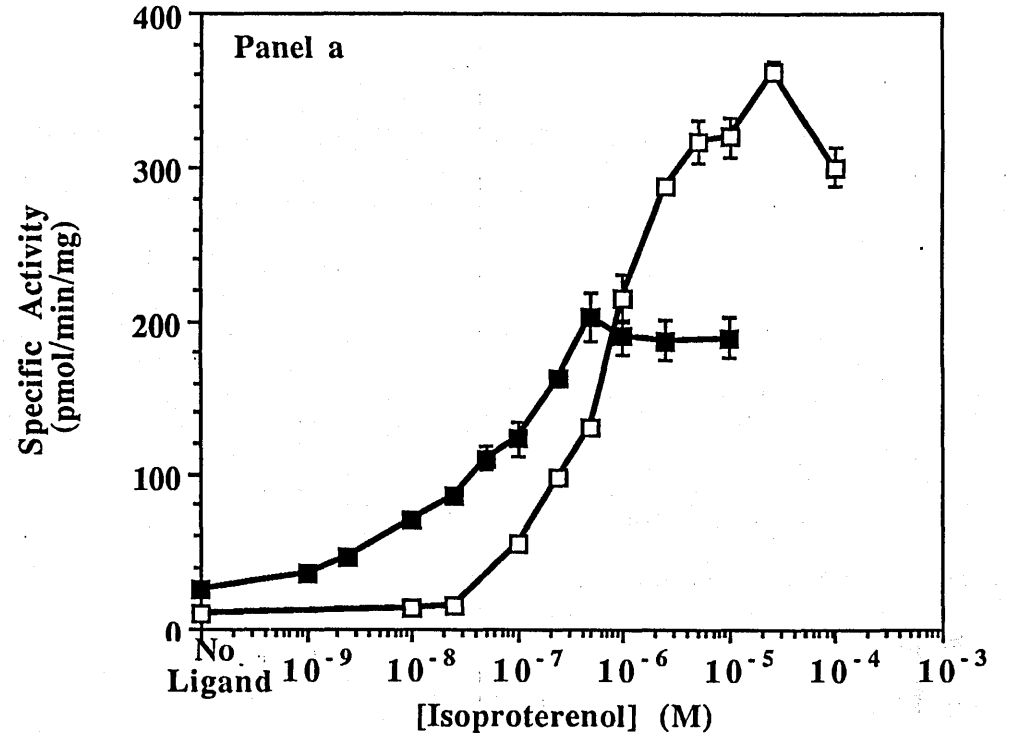
Figure 4.13:

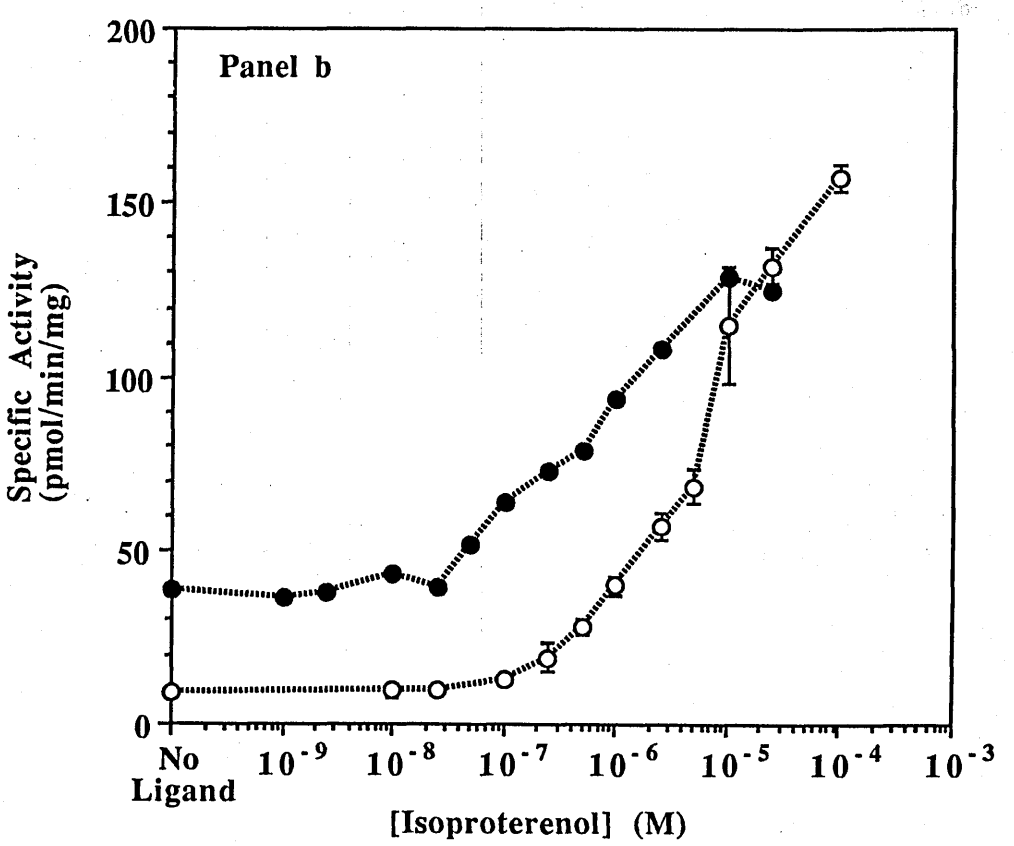
Effect Of Replacing GTP With Gpp(NH)p On Isoproterenol Stimulation Of Adenylate Cyclase Activity In Adipocyte Membranes From Lean And Diabetic (*db/db*) Animals

Adipocyte membranes from lean (□, ■) and diabetic (*db/db*) (○, ●) animals were assayed for adenylate cyclase activity for 30 minutes at 30°C as described in Section 2.7.1.with increasing concentrations of isoproterenol in the presence of either 100 μM GTP (open symbols) or 100 μM Gpp(NH)p (closed symbols). The cyclic AMP formed was measured by the competitive binding method also described in Section 2.7.1.

The experiments show the effects of the different nucleotides on the responses to isoproterenol in membranes from lean (*panel a*) and diabetic (*panel b*) animals.

The dose-response curves are shown for an experiment representative of three performed with different membrane preparations from lean and diabetic (*db/db*) animals which gave essentially identical results. In this experiment, the activities obtained in the presence of 100 μM GTP were 10.0 ± 0.3 (lean) and 7.2 ± 1.1 (diabetic) pmol/min/mg. In the presence of 100 μM Gpp(NH)p, the specific activities obtained were 30.8 ± 2.2 (lean) and 45.5 ± 1.0 (diabetic) pmol/min/mg.


Panel a
Specific Activity (pmol/min/mg)
400
300
200
100
0
No Ligand
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
[Isoproterenol] (M)


Panel b
Specific Activity (pmol/min/mg)
200
150
100
50
0
No Ligand
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
[Isoproterenol] (M)

4.2 RESULTS & DISCUSSION

As previously discussed, an impairment in the responsiveness of adenylate cyclase to stimulation by catecholamines is a characteristic property of adipocyte membranes prepared from genetically diabetic (*db/db*) mice (Levilliers *et al.*, 1978; Bégin-Heick & Coleman, 1988). This was shown to be true for adipocyte membranes prepared from the diabetic animals used in these studies (Figure 4.1 and Table 4.1). However, one important difference from the findings of other groups was the observed reduction in sensitivity of adenylate cyclase to stimulation by isoproterenol in membranes from diabetic animals (Figure 4.1 and Table 4.1). When similar dose-response curves were performed using other lipolytic hormones, it was found that the maximal stimulation elicited by either glucagon or secretin was also reduced by some 50% in membranes from diabetic animals compared with leans, although membranes from diabetic animals were slightly more sensitive to activation by these ligands (Table 4.1). In contrast, neither maximal stimulation of adenylate cyclase by TSH nor the EC_{50} value for stimulation were markedly different in membranes from diabetic animals compared with those from lean animals (Table 4.1).

There were several potential explanations for the observed reduction in hormone-stimulated adenylate cyclase activity observed in membranes from diabetic animals. For example, a reduced activity of the common catalytic unit to which the receptors are coupled could account for the reduced response in diabetic animals. However, both basal and forskolin-stimulated activities were similar in membranes from lean and diabetic samples (Table 4.1). Coupling of the catalytic unit with G_s was also unaffected by the diabetic state as NaF-stimulated activities were the same in membranes from lean and diabetic animals (Table 4.1).

Altered expression or functioning of any of the G-protein subunits controlling adenylate cyclase activity could have potentially accounted for the observed reduction in responsiveness. Therefore, comparative immunoblotting

experiments were performed. Preliminary experiments performed for each antiserum determined which range of protein loadings produced directly proportional increases in absorption of the second antibody-substrate complex as measured by densiometric scanning (Figures 4.2, 4.3, 4.4 and 4.5). Subsequent comparisons employed protein concentrations which produced a signal within this linear range. Comparative immunoblotting of $G_s\alpha$-subunits using antiserum CS1 showed there to be no alterations in the expression of either the 42 kDa or 45 kDa forms of the protein expressed in membranes from lean and diabetic animals (Figure 4.6 and Table 4.2), confirming the lack of any defect in G_s.C coupling as implied by the similar responses of membranes from lean and diabetic animals to NaF (Table 4.1). Using electrophoretic conditions which allowed the resolution of $G_i\alpha$-1 and $G_i\alpha$-2 before detection with antiserum SG1, a two-fold increase in the relative expression of $G_i\alpha$-1 was observed in the absence of any significant change in the expression of $G_i\alpha$-2 between membranes from lean and diabetic animals (Figure 4.7 and Table 4.2). Similarly, $G_i\alpha$-3 was expressed at similar levels in membranes from both lean and diabetic animals as assessed using antiserum I3B (Figure 4.8 and Table 4.2). Unlike the situation for liver plasma membranes from diabetic (*db/db*) animals, there was no alteration in expression of β-subunits between adipocyte membranes from lean and diabetic animals (Figure 4.9 and Table 4.2). However, since the BN1 antiserum recognizes β_1- and β_2-subunits, which are both expressed in adipocytes (Rapiejko *et al.*, 1989), the possibility that there were alterations in the ratio of expression of both these forms without any change in the total complement of β-subunits cannot be discounted.

As discussed in Section 1.9.3, the mechanisms by which G_i inhibits adenylate cyclase activity have not yet been proven unequivocally. In adipocytes, the identity of the major inhibitory species has yet to be determined. Thus it was not initially possible to discount the presence of an enhanced tonic inhibitory effect of G_i in diabetic animals, due to the elevated expression of $G_i\alpha$-1 (Figure 4.7 and

Table 4.2), resulting in the observed attenuation of hormone-stimulated adenylate cyclase activity (Figure 4.1 and Table 4.1). However, studies carried out by Bégin-Heick & Coleman (1988) suggested that the tonic inhibitory effect of GTP on forskolin-stimulated adenylate cyclase activity in adipocyte membranes from diabetic animals was of a similar magnitude to that found in membranes from lean animals. Nevertheless it was also important to determine whether the increased expression of $G_i\alpha$-1 had any affect on receptor-dependent inhibitory phenomena. This was tested using the classical adipocyte anti-lipolytic agents PIA, (the A_1 adenosine receptor agonist) prostaglandin E_1 and nicotinic acid, which inhibit adenylate cyclase activity *via* activation of G_i (Londos *et al.*, 1978). Maximal inhibition of 100 μM isoproterenol-stimulated adenylate cyclase activity by PIA was slightly enhanced in membranes from diabetic animals, and diabetic animals were also slightly more sensitive to inhibition by this ligand (Figure 4.10 and Table 4.3). In contrast, the maximal inhibition elicited by either PGE_1 or nicotinic acid was similar between membranes from lean and diabetic animals, although membranes from diabetic animals were slightly less sensitive to inhibition by nicotinic acid (Figures 4.11, 4.12 and Table 4.3). The fact that the maximal inhibitions elicited by PGE_1 and nicotinic acid were unaffected by the diabetic state demonstrated that the increased expression of $G_i\alpha$-1 in adipocyte membranes from diabetic animals had little affect on inhibitory processes, as any potentiation of inhibition might be expected to alter each inhibitory process to some degree. That PIA-mediated inhibition was slightly enhanced might have reflected an increased abundance or activity of the adenosine A_1 receptor: binding studies with [^{3}H]PIA would have tested these proposals. However we cannot discount the possibility that the A_1 adenosine receptor was preferentially affected by the increase in $G_i\alpha$-1 relative to the other two inhibitory receptors. In either case, enhanced adenosine receptor-mediated inhibition might be expected to exacerbate the reductions in cyclic AMP levels and lipolysis caused by the defect in hormone-stimulated adenylate cyclase activity (Figure 4.1 and Table 4.1) although the latter defect would be expected to

make a greater contribution to the observed triglyceride accumulation.

A basic preliminary study was undertaken to determine whether the coupling between the β-adrenergic receptor and G_s was defective. This was achieved by carrying out isoproterenol dose-response experiments using saturating concentrations of either GTP or GTPγS as the guanine nucleotide, and comparing the nature of the altered kinetics when GTPγS replaces GTP in the dose-response curves to isoproterenol for membranes from lean and diabetic animals. In adipocyte membranes from lean and obese Zucker rats, the responses to isoproterenol, when GTP was replaced with GTPγS, were markedly different (Strassheim *et al.*, 1991b). In the presence of GTP, there was a marked reduction in the maximal specific activity (V_{max}) attained by obese animals compared with leans with no difference in the EC_{50} value with which isoproterenol could stimulate adenylate cyclase. When GTPγS replaced GTP in the assays, the V_{max} values obtained with lean and obese animals were similar but membranes from obese animals became less sensitive to stimulation by isoproterenol. Hence the inclusion of GTPγS caused a greater increase in sensitivity to isoproterenol in membranes from lean animals than obese animals. In contrast to those results, a similar increase in sensitivity to isoproterenol was noted in membranes from lean and diabetic animals when GTPγS replaced GTP in the isoproterenol dose-response experiments (Figure 4.13). Therefore any defect in coupling between the β-adrenergic receptor and G_s in adipocyte membranes from diabetic (*db/db*) animals was distinct from that observed in membranes from obese Zucker rats (Strassheim *et al.* 1991b). Of course, it is possible that that the reduced stimulation of adenylate cyclase activity by isoproterenol was due to the 70% reduction in the number of β-adrenergic receptor binding sites in membranes from diabetic animals compared with lean animals, as determined by receptor binding experiments using the β-adrenergic receptor antagonist [^{125}I]iodocyanopindolol (Strassheim *et al.*, 1991a). However, work carried out by Tolkovsky & Levitzki (1978) on the β-adrenergic receptor

mediated-stimulation of turkey erythrocyte membrane adenylate cyclase activity has suggested that a successive reduction in receptor number may only reduce the rate at which V_{max} is attained without affecting its magnitude. Hence, if this is correct, the observed reduction in receptor number in membranes from diabetic animals would merely account for the reduced sensitivity to stimulation by isoproterenol without accounting for the reduced V_{max}. This might be taken to imply the existence of a separate defect(s) in hormone-stimulated adenylate cyclase activity. One might expect that any potential defect is at the level of receptor modification since the response to TSH in membranes from diabetic animals was identical to that in membranes from lean animals (Table 4.1). In support of this idea was the finding that stimulation of adenylate cyclase activity by a saturating concentration of the wasp venom mastoparan, which is thought to activate G-proteins by a similar mechanism to that employed by G-protein-linked receptors (Hagashijima *et al.*, 1988), was similar in membranes from lean and diabetic animals (Table 4.1). Nevertheless the possibility that a defect at the level of G_s may exist which results in less efficient coupling between some, but not all, R_s receptors and G_s cannot be discounted. For instance, the TSH receptor may already have been poorly coupled to G_s in control membranes under the assay conditions employed such that any abblated coupling in membranes from diabetic animals was not detectable.

4.3 CONCLUSIONS

Several rodent model systems of diabetes mellitus and obesity are associated with a reduced ability of lipolytic hormones to mobilise triglyceride stores present in adipose tissue. These include the Zucker rat, obese (*ob/ob*) mouse, the NZO mouse and the high fat diet-fed rat. In all of these systems an impaired lipolytic response is associated with a reduced ability of catecholamines to stimulate adenylate cyclase activity in isolated adipocyte membranes. Such characteristics are also found in the genetically diabetic (*db/db*) mouse (see Section 1.11).

As well as exhibiting a reduced responsiveness and sensitivity to stimulation by isoproterenol, a reduced responsiveness was also observed for adenylate cyclase stimulation by two other lipolytic hormones (glucagon and secretin) in adipocyte membranes from diabetic animals. However, stimulation by TSH was unaffected by the diabetic state. This contrasts with the kinetics of adenylate cyclase stimulation in adipocyte membranes prepared from obese Zucker rats, where stimulation by all G_s-linked hormones was severely attenuated (Strassheim *et al.*, 1991b). Like the diabetic mouse, adipocyte membranes from the genetically obese mouse also exhibit a drastically reduced response to stimulation of adenylate cyclase by isoproterenol but the responsiveness to other G_s-linked receptors has not been tested. However, one important difference observed for the diabetic (*db/db*) mice, compared to both the obese mouse and Zucker rat systems, was the presence of a functional G_i activity in diabetic mouse adipocyte membranes, both with respect to GTP-dependent and receptor-dependent inhibition of isoproterenol-stimulated adenylate cyclase activity. In this respect, adipose tissue from diabetic animals resembles the situation in liver membranes which also exhibit guanine nucleotide-dependent inhibitory effects, albeit slightly reduced, in contrast to hepatocyte membranes from the obese Zucker rat and the Type I diabetic rat, where 'tonic' inhibitory functioning of G_i is abolished (Houslay *et al.*, 1989; Bushfield *et al.*, 1990c).

An important finding in the course of these studies on insulin-resistant animals has been the altered expression of specific G-protein subunits. Adipose tissue from the genetically diabetic mouse contained elevated levels of $G_i\alpha$-1 subunits compared with lean controls, but this had very little effect on inhibitory phenomena. In adipocyte membranes prepared from the obese Zucker rat, there were large reductions in expression of both $G_i\alpha$-1 and $G_i\alpha$-3 with no alteration in levels of $G_i\alpha$-2: as with the diabetic mouse system, receptor-dependent inhibition of adenylate cyclase activity was largely unaffected by these changes. Also, the lack of any change in expression of $G_i\alpha$-2, $G_i\alpha$-3 or β-subunits, unlike liver

membranes prepared from the same animals (see Chapter 3), indicates that alterations in the regulation of G-protein subunit expression caused by the diabetes (*db*) gene are tissue-specific.

The mechanisms by which the reductions in reponsiveness to isoproterenol, glucagon and secretin arose are unknown. The large reduction in β-adrenergic receptor number could potentially account for the reduction in responsiveness and sensitivity observed for isoproterenol-stimulated adenylate cyclase activity. However, the assumption of a Collision Coupling model of adenylate cyclase activation by receptors would suggest the existence of a separate defect to account for the reduced activity. Nevertheless, preliminary studies on the effect of replacing GTP with a non-hydolysable GTP analogue Gpp(NH)p, when performing isoproterenol dose-resonse experiments, indicated that if there was a defect in the coupling between the β-adrenergic receptor and G_s, it was distinct from that observed in membranes from obese Zucker rats and the obese mouse (Strassheim *et al.*, 1991b; Bégin-Heick, 1980). Comprehensive binding studies are therefore necessary to determine whether the observed 70% reduction in β-adrenergic receptor number is sufficient to cause the drastic reduction in maximal stimulation by isoproterenol, or whether another defect is responsible. This could be achieved by measuring the effect of successively reducing receptor number, either by using irreversible antagonists or trypsin-treating intact adipocytes, on adenylate cyclase activity in normal adipocyte membranes and correlating changes with reductions in receptor number. If inactivation of 70% of the total number of β-adrenergic receptors produced the same response as that observed in membranes from diabetic animals, then the reduction in receptor number found in membranes from diabetic animals would presumably account for the attenuated response to β-adrenergic receptor agonists. However, if a different response was obtained to that observed in membranes from diabetic animals, then a separate defect would be implicated.

One would also need to determine whether the pharmacological properties

of β-adrenergic receptor-mediated stimulation of adenylate cyclase were the same between membranes from lean and diabetic animals. It is theoretically possible that another β-adrenergic receptor subtype expressed in adipocytes becomes coupled to adenylate cyclase in diabetic animals due to a loss in β_1 receptors (mainly responsible for catecholamine stimulation of adenylate cyclase in fat, as recently confirmed by Bahouth & Malbon (1988)), and that these subtypes do not couple to G_s as efficiently as β_1. Thorough pharmacological studies would be necessary to test this hypothesis.

Finally, it can be concluded that the gross effects of the diabetic (*db/db*) syndrome on reducing the stimulation of adenylate cyclase activity in adipocyte membranes probably contribute greatly to the attenuated lipolytic response and the concomitant accumulation of triglyceride. This state would be compounded by the slightly enhanced adenosine receptor-mediated inhibition of cyclic AMP production and the enhanced lipogenic effect of insulin in adipose tissue from genetically diabetic animals (Kupiecki & Adams, 1974). However the mechanisms by which these changes arise, and whether they are a cause or a consequence of the development of obesity, remain unclear.

CHAPTER 5

The Regulation Of Adenylate Cyclase Activity In Adipocyte Membranes From Lean And Obese CBA/Ca Mice

5.1 INTRODUCTION

As with all the genetic models of Type II diabetes mellitus, the CBA/Ca mouse is characterised by severe obesity. However, to our knowledge, no studies have been carried out to determine whether any defect in hormone-stimulated lipolysis could be explained by perturbed regulation of adenylate cyclase activity in adipocyte membranes prepared from these obese animals. Considering the nature of the alterations in the regulation of adenylate cyclase activity seen in adipocyte membranes from other obese rodents, as discussed earlier, it was considered important to determine whether similar defects were observed in the CBA/Ca mouse. In particular, I was particularly interested in finding out whether a reduced stimulation of adenylate cyclase activity occurred *via* any of the classical lipolytic hormone receptors, as has been conclusively shown for adipocyte membranes from the obese (*fa/fa*) Zucker rat, the obese (*ob/ob*) mouse and the diabetic (*db/db*) mouse. In these three models of obesity, a resistance to the action of G_s-linked hormones appears to be a major factor contributing to the abnormal triglyceride accumulation (see Section 1.11). It was also necessary to examine the inhibitory regulation of adenylate cyclase activity, as one could envisage a scenario whereby enhanced inhibition of adenylate cyclase activity, and the ensuing reduction in cyclic AMP accumulation, could lead to reduced rates of lipolysis and a net accumulation of triglyceride. With regard to this point, altered receptor-dependent and -independent inhibitory phenomena have been noted in other models of diabetes mellitus as well as hypothyroid states (see Section 1.11).

Figure 5.1:

Dose-Response Curves For Isoproterenol Stimulation Of Adenylate Cyclase Activity In Adipocyte Membranes From Lean And Obese CBA/Ca Animals

Adipocyte membranes from lean (❑) and obese (O) CBA/Ca animals were assayed for adenylate cyclase activity for 30 minutes at 30°C in the presence of 100 μM GTP and increasing concentrations of isoproterenol as described in Section 2.7.1. The [^{32}P]cyclic AMP formed was measured by the double column method described in Section 2.7.2. The figure shows results pooled from three experiments performed on different membrane preparations and values are presented as MEAN ± S.D.

The specific activities obtained in the presence of 100 μM GTP alone are given in Table 5.2.

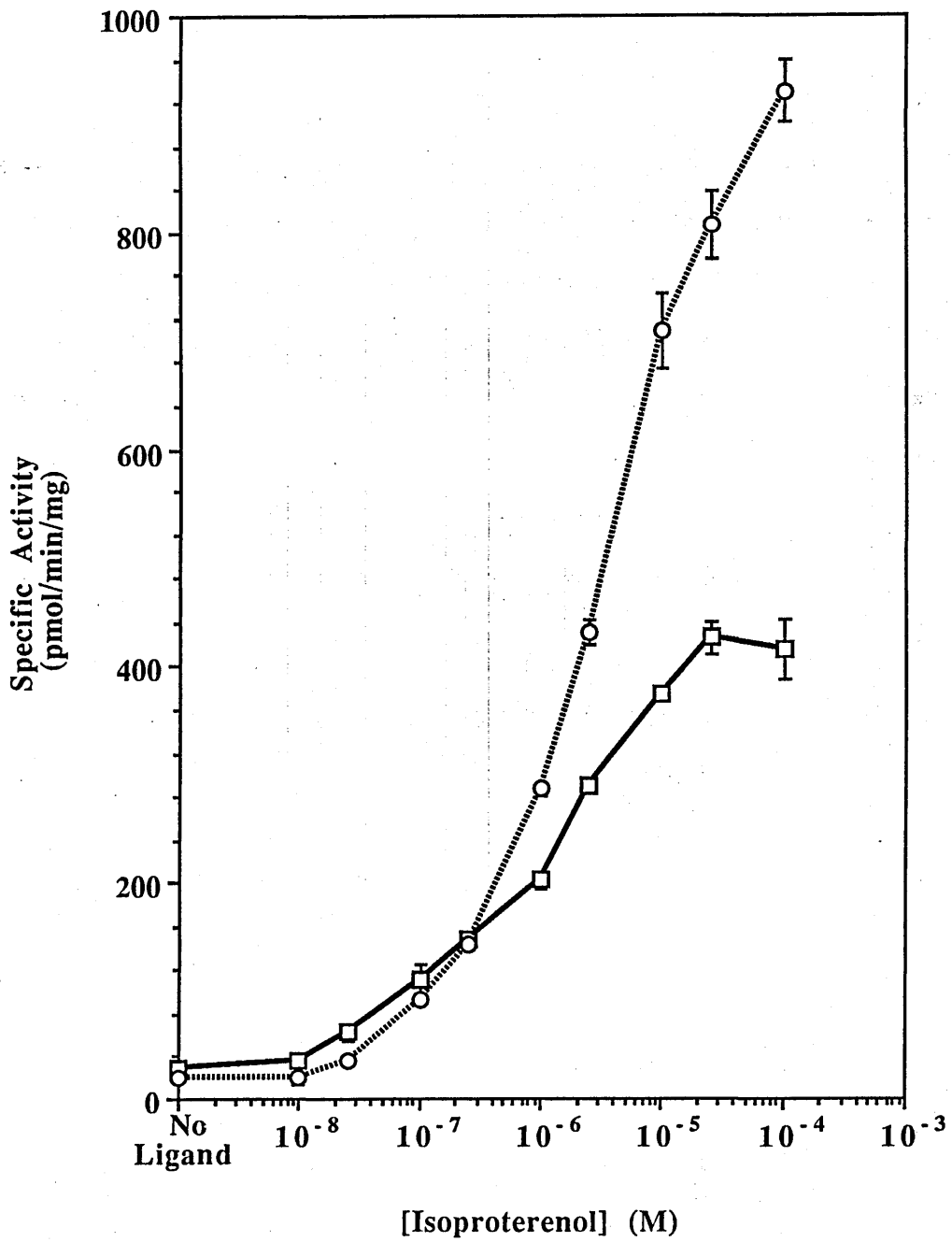

1000
800
600
400
200
0
Specific Activity
(pmol/min/mg)
No
Ligand
10-8
10-7
10-6
10-5
10-4
10-3
[Isoproterenol] (M)

Figure 5.2:

Dose-Response Curves For Glucagon Stimulation Of Adenylate Cyclase Activity In Adipocyte Membranes From Lean And Obese CBA/Ca Animals

Adipocyte membranes from lean (❑) and obese (O) CBA/Ca animals were assayed for adenylate cyclase activity for 30 minutes at 30°C in the presence of 100 μM GTP and increasing concentrations of glucagon as described in Section 2.7.1. The [^{32}P]cyclic AMP formed was measured by the double column method described in Section 2.7.2. The figure shows results pooled from three experiments performed on different membrane preparations and values are presented as MEAN ± S.D.

The specific activities obtained in the presence of 100 μM GTP alone are given in Table 5.2.

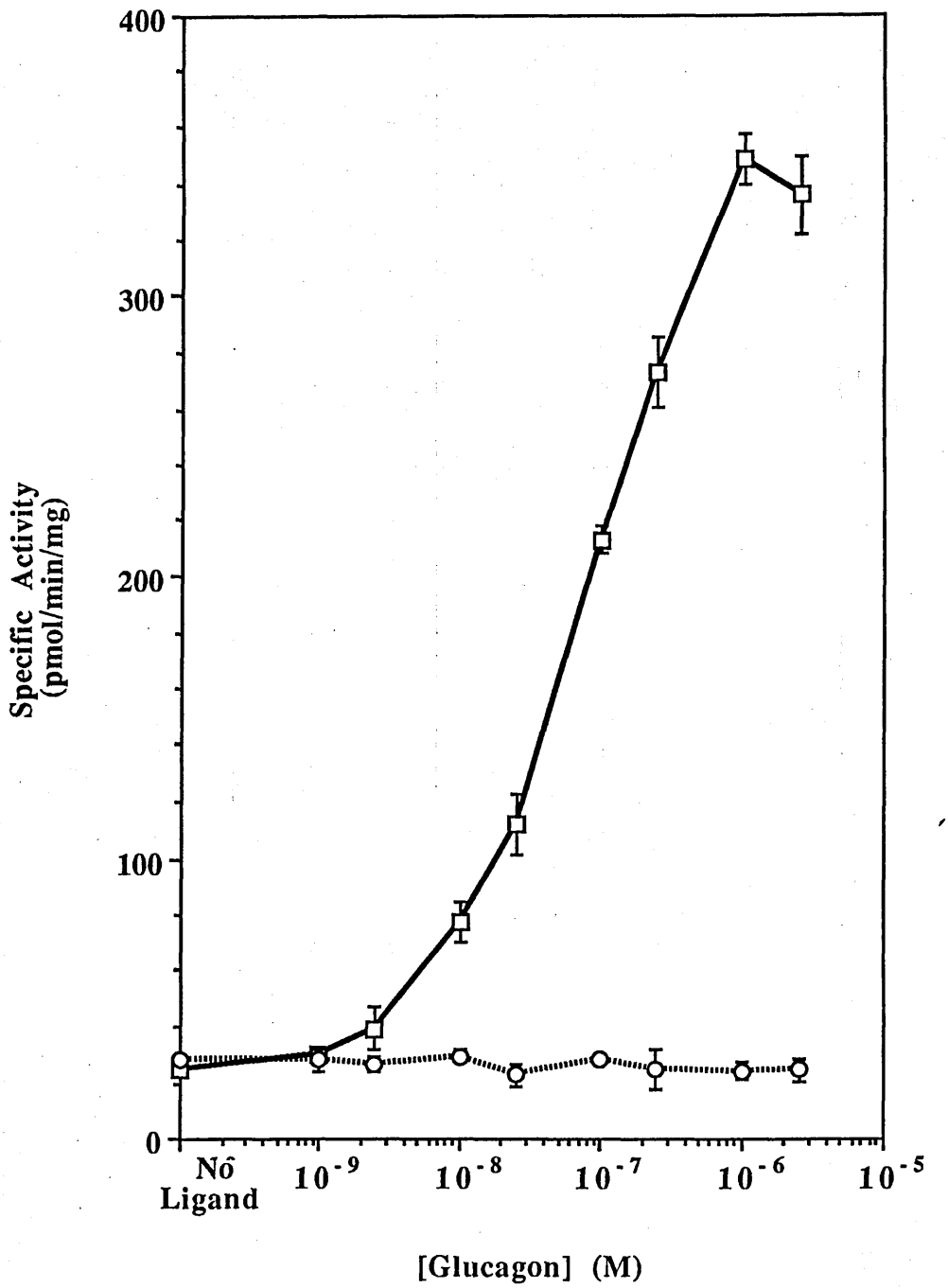

400
300
200
100
0
Specific Activity
(pmol/min/mg)
No
Ligand
10-9
10-8
10-7
10-6
10-5
[Glucagon] (M)

Figure 5.3:

Dose-Response Curves For Secretin Stimulation Of Adenylate Cyclase Activity In Adipocyte Membranes From Lean And Obese CBA/Ca Animals

Adipocyte membranes from lean (❑) and obese (O) CBA/Ca animals were assayed for adenylate cyclase activity for 30 minutes at 30°C in the presence of 100 μM GTP and increasing concentrations of secretin as described in Section 2.7.1. The [^{32}P]cyclic AMP formed was measured by the double column method described in Section 2.7.2. The figure shows results pooled from three experiments performed on different membrane preparations and values are presented as MEAN ± S.D.

The specific activities obtained in the presence of 100 μM GTP alone are given in Table 5.2.

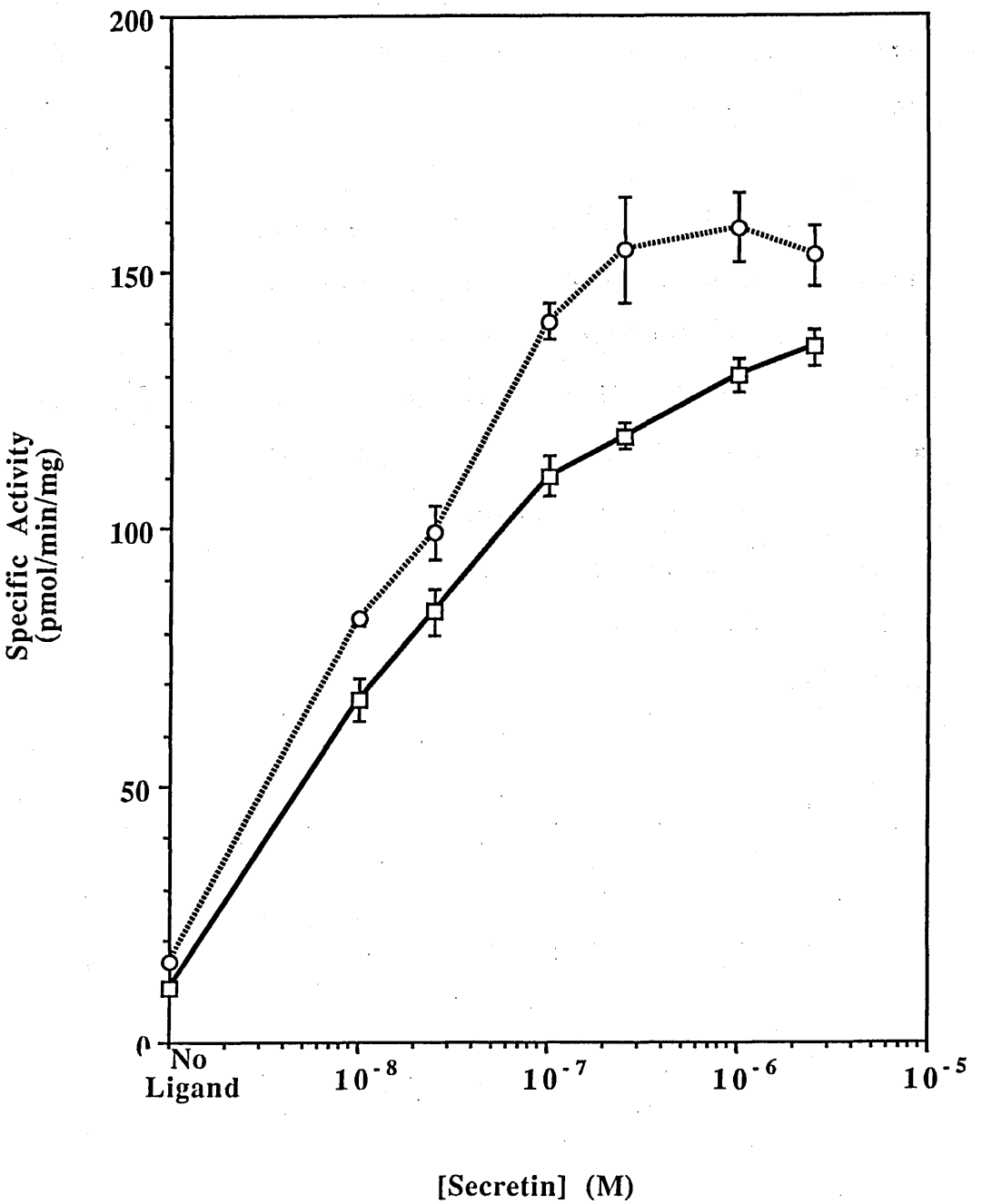

200
150
100
50
0
Specific Activity
(pmol/min/mg)
No
Ligand
10-8
10-7
10-6
10-5
[Secretin] (M)

Table 5.1:

Hormonal Stimulation Of Adenylate Cyclase Activity In Adipocyte Membranes From Lean And Obese CBA/Ca Mice

Adipocyte membranes from lean and obese CBA/Ca animals were assayed for adenylate cyclase activity for 30 minutes at 30°C in the presence of either 100 μM GTP alone or with 10 μM glucagon, 100 μM isoproterenol or 2.5 μM secretin as described in Section 2.7.1. The [^{32}P]cyclic AMP formed was measured by the double column method described in Section 2.7.2. Specific activities are given as pmol/min/mg of protein and expressed as MEAN ± S.D. for three experiments using different membrane preparations.

EC_{50} is the hormone concentration at which half-maximal activation was achieved. n.a.=not applicable. *denotes significant differences between samples from lean and obese animals *i.e.* $p<0.005$.

Table 5.1:

		Lean		Obese	
	Ligand	Spec. Ac.	EC_{50} (μM)	Spec. Ac.	EC_{50} (μM)
	100 μM GTP	24.7±2.1	n.a.	28.3±1.9	n.a.
100 μM GTP +	Isoproterenol	414.3±27.6	0.9±0.2	930.3±30.0*	2.5±0.3
100 μM GTP +	Glucagon	335.7±13.5	0.095±0.012	24.7 ± 5.0*	n.a.*
100 μM GTP +	Secretin	140.4± 5.6	0.011±0.05	151.1±6.0	0.009±0.007

Table 5.2:

Non-Hormonal Stimulation Of Adenylate Cyclase Activity In Adipocyte Membranes From Lean And Obese CBA/Ca Mice

Adipocyte membranes prepared from lean and obese CBA/Ca animals were assayed for adenylate cyclase activity in the absence (basal) and in the presence of the ligands mentioned below for 30 minutes at 30°C as described in Sections 2.7.1. The [^{32}P]cyclic AMP formed was measured by the double column method described in Section 2.7.2. Specific activities are in pmol/min/mg protein and data are expressed as MEAN ± S.D. for three experiments using different membrane preparations. *denotes significant differences between samples from lean and obese animals *i.e.* $p<0.005$.

(Fold activations above basal activity are given in parentheses).

Ligand	Lean	Obese
Basal	17.6 ± 5.3	20.3 ± 3.2
100 μM GTP	24.7 ± 2.1	28.3 ± 1.9
10 mM NaF	237 ± 20 (13.5)	284 ± 15 (14.0)
5 mM $MnCl_2$	215 ± 13 (12.2)	364 ± 21 (17.9)*
100 μM Forskolin	1381 ± 110 (78.5)	2600 ± 115 (128.1)*

Figure 5.4:

Dose-Response Curves For PIA Inhibition Of Forskolin-Stimulated Adenylate Cyclase Activity In Adipocyte Membranes From Lean And Obese CBA/Ca Animals

Adipocyte membranes from lean (❑) and obese (O) CBA/Ca animals were assayed for adenylate cyclase activity for 30 minutes at 23°C in the presence of 100 μM GTP, 100 μM forskolin and increasing concentrations of PIA as described in Section 2.7.1. The cyclic AMP formed was measured by the competitive binding method also described in Section 2.7.1. The figure shows results pooled from three experiments performed on different membrane preparations and values are presented as MEAN ± S.D.

The specific activities obtained with 100 μM forskolin and 100 μM GTP are given in Table 5.3.

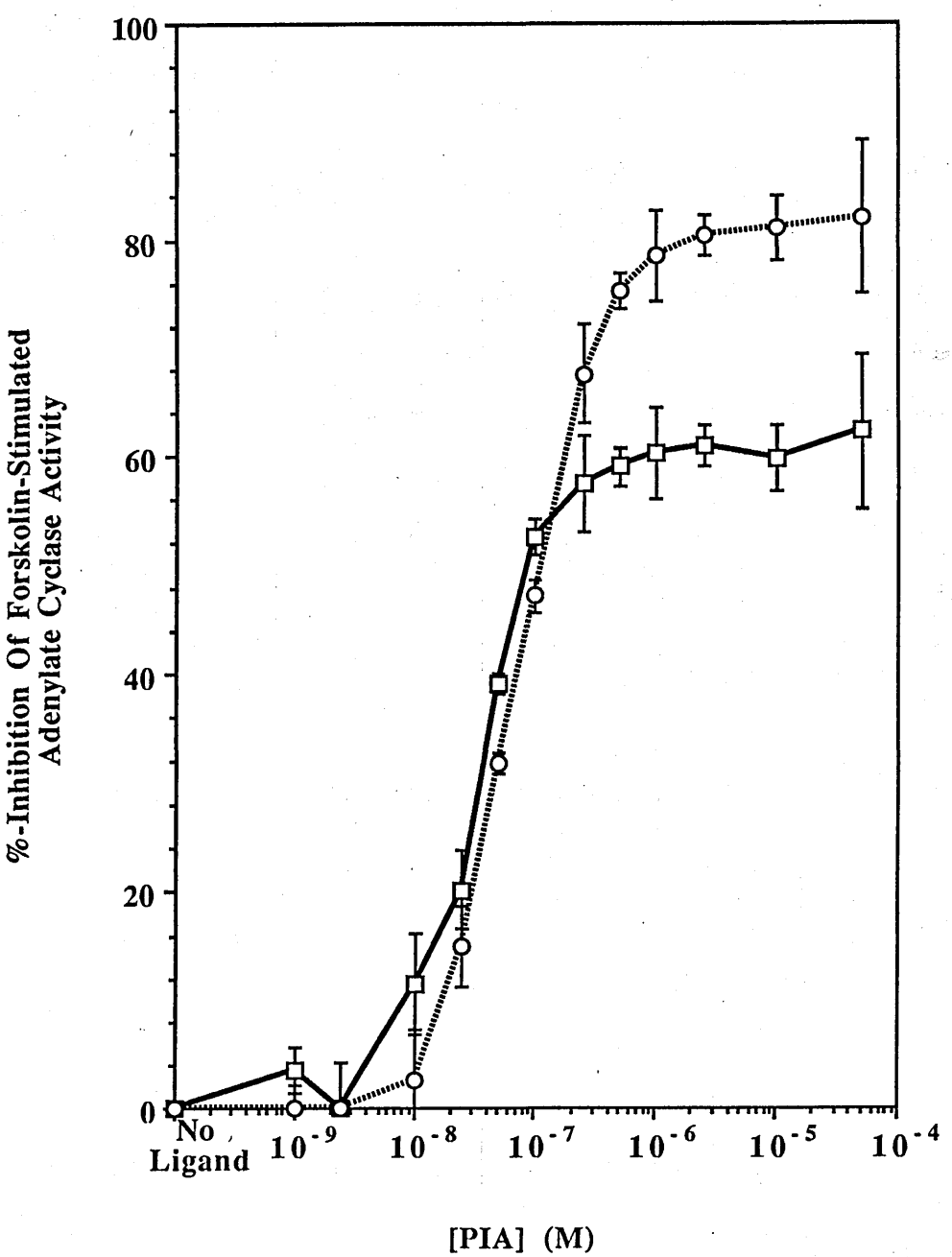

100
80
60
40
20
0
%-Inhibition Of Forskolin-Stimulated Adenylate Cyclase Activity
No Ligand
10-9
10-8
10-7
10-6
10-5
10-4
[PIA] (M)

Figure 5.5:

Dose-Response Curves For Prostaglandin E_1 Inhibition Of Forskolin-Stimulated Adenylate Cyclase Activity In Adipocyte Membranes From Lean And Obese CBA/Ca Animals

Adipocyte membranes from lean (❑) and obese (O) CBA/Ca animals were assayed for adenylate cyclase activity for 30 minutes at 23°C in the presence of 100 μM GTP, 100 μM forskolin and increasing concentrations of prostaglandin E_1 as described in Section 2.7.1. The cyclic AMP formed was measured by the competitive binding method also described in Section 2.7.1. The figure shows results pooled from three experiments performed on different membrane preparations and values are presented as MEAN ± S.D.

The specific activities obtained with 100 μM forskolin and 100 μM GTP are given in Table 5.3.

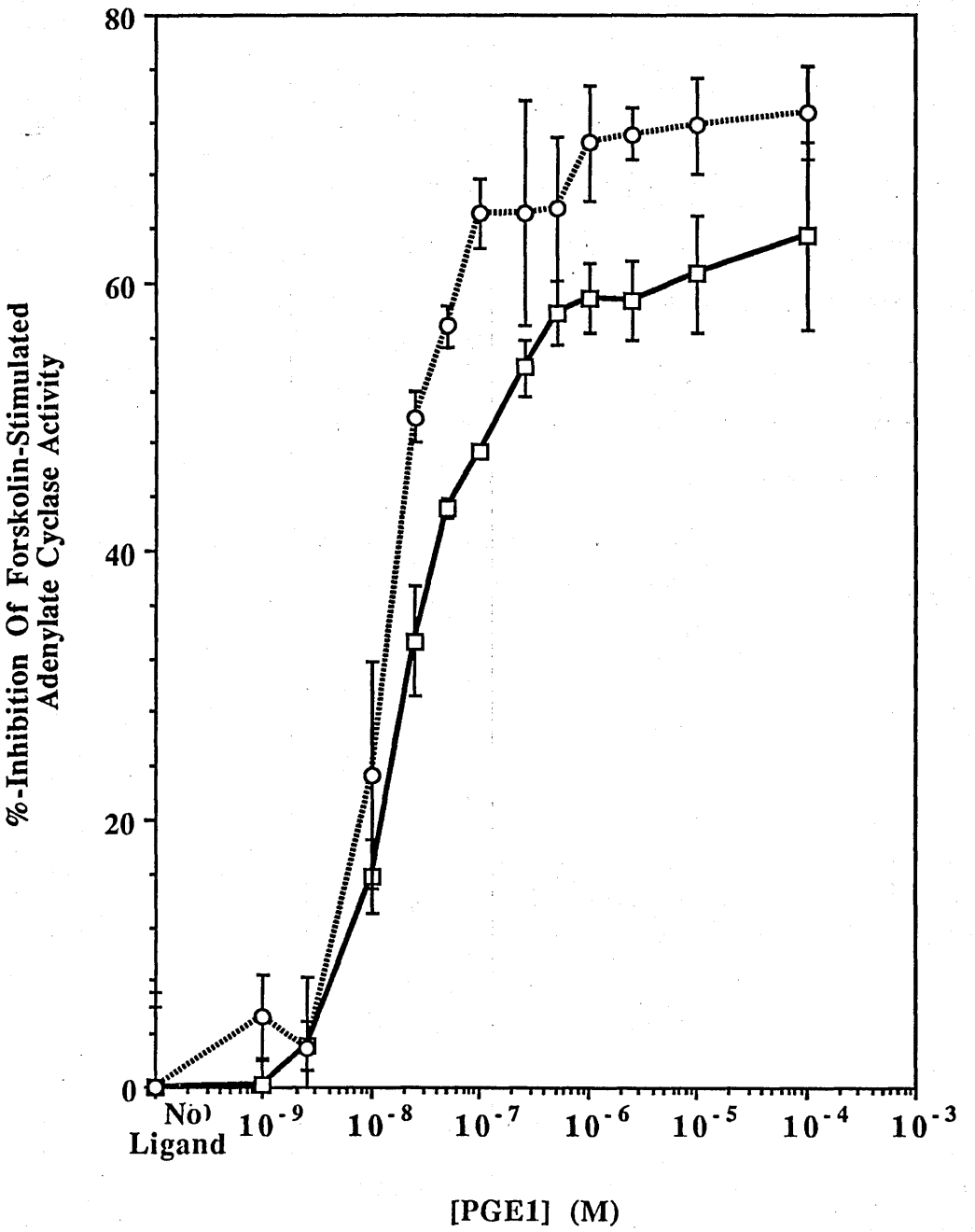

%-Inhibition Of Forskolin-Stimulated Adenylate Cyclase Activity
80
60
40
20
0
No Ligand
10-9
10-8
10-7
10-6
10-5
10-4
10-3
[PGE1] (M)

Figure 5.6:

Dose-Response Curves For Nicotinic Acid Inhibition Of Forskolin-Stimulated Adenylate Cyclase Activity In Adipocyte Membranes From Lean And Obese CBA/Ca Animals

Adipocyte membranes from lean (❑) and obese (O) CBA/Ca animals were assayed for adenylate cyclase activity for 30 minutes at 23°C in the presence of 100 μM GTP, 100 μM forskolin and increasing concentrations of nicotinic acid as described in Section 2.7.1. The cyclic AMP formed was measured by the competitive binding method also described in Section 2.7.1. The figure shows results pooled from three experiments performed on different membrane preparations and values are presented as MEAN ± S.D.

The specific activities obtained with 100 μM forskolin and 100 μM GTP are given in Table 5.3.

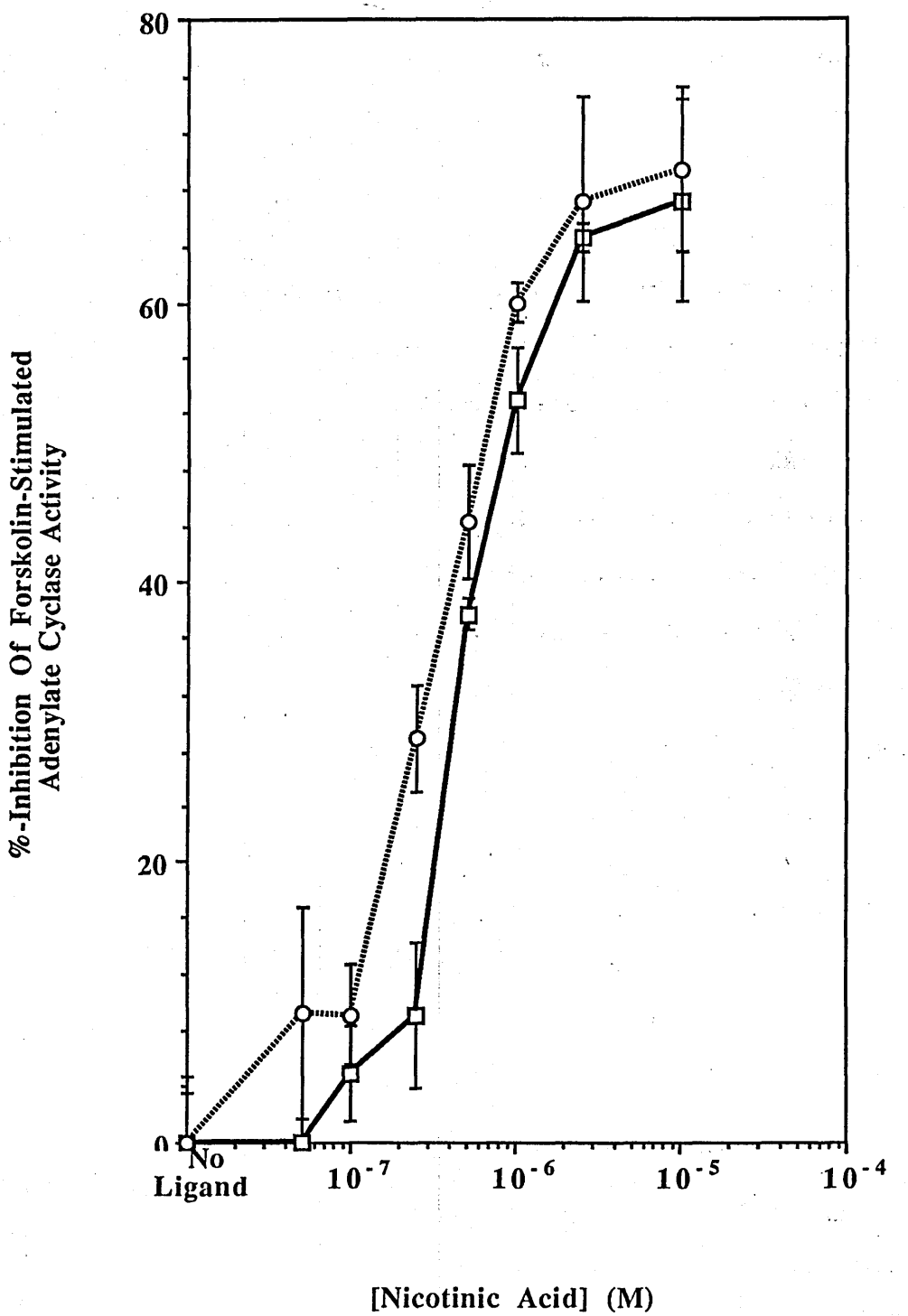

%-Inhibition Of Forskolin-Stimulated Adenylate Cyclase Activity
80
60
40
20
0
No Ligand
10-7
10-6
10-5
10-4
[Nicotinic Acid] (M)

Table 5.3:

Receptor-Mediated Inhibition Of Adenylate Cyclase Activity In Adipocyte Membranes From Lean And Obese CBA/Ca Animals

Adipocyte membranes from lean and obese CBA/Ca animals were assayed for adenylate cyclase activity for 30 minutes at 23°C in the presence of 100 μM GTP, 100 μM forskolin and either 10 μM PIA, 10 μM prostaglandin E_1 or 100 μM nicotinic acid as described in Section 2.7.1 and the legends to Figures 5.4, 5.5 and 5.6. Data are expressed as %-inhibition of the activity observed in the absence of an inhibitory ligand but with 100 μM forskolin and 100 μM GTP present. For each ligand, results are pooled from three experiments performed on different membrane preparations and values are presented as MEAN ± S.D. IC_{50} is the ligand concentration at which half-maximal inhibition was achieved.

Specific activities obtained in the presence of 100 μM forskolin and 100 μM GTP were 952.2 ± 81.3 and 2177 ± 226.6 pmol/min/mg protein for samples from lean and obese animals respectively (MEAN ± S.D. for nine experiments).

*denotes a significant difference between lean and obese animals, *i.e.* $p<0.005$.

Table 5.3:

	Lean		Obese	
Ligand	Inhibition (%)	IC_{50} (nM)	Inhibition (%)	IC_{50} (nM)
PIA	62 ± 8	30 ± 4	81 ± 6*	40 ± 5
Prostaglandin E_1	61 ± 7	15 ± 9	72 ± 5	10 ± 5
Nicotinic Acid	65 ± 8	450 ± 60	69 ± 6	150 ± 70*

Figure 5.7:

Dose-Response Curves For Gpp(NH)p Inhibition Of Forskolin-Stimulated Adenylate Cyclase Activity In Adipocyte Membranes From Lean And Obese CBA/Ca Animals

Adipocyte membranes from lean (❑) and obese (O) CBA/Ca animals were assayed for adenylate cyclase activity for 30 minutes at 23°C in the presence of 100 μM forskolin and increasing concentrations of Gpp(NH)p as described in Section 2.7.1. The cyclic AMP formed was measured by the competitive binding method also described in Section 2.7.1. The figure shows results pooled from three experiments performed on different membrane preparations and values are presented as MEAN ± S.D. Basal activities were 10.1 ± 2.2 (lean animals) and 9.0 ± 18 (obese animals) pmol/min/mg. The specific activities obtained in the presence of 100 μM forskolin alone were 981 ± 32 (lean animals) and 2251 ± 53 (obese animals) pmol/min/mg.

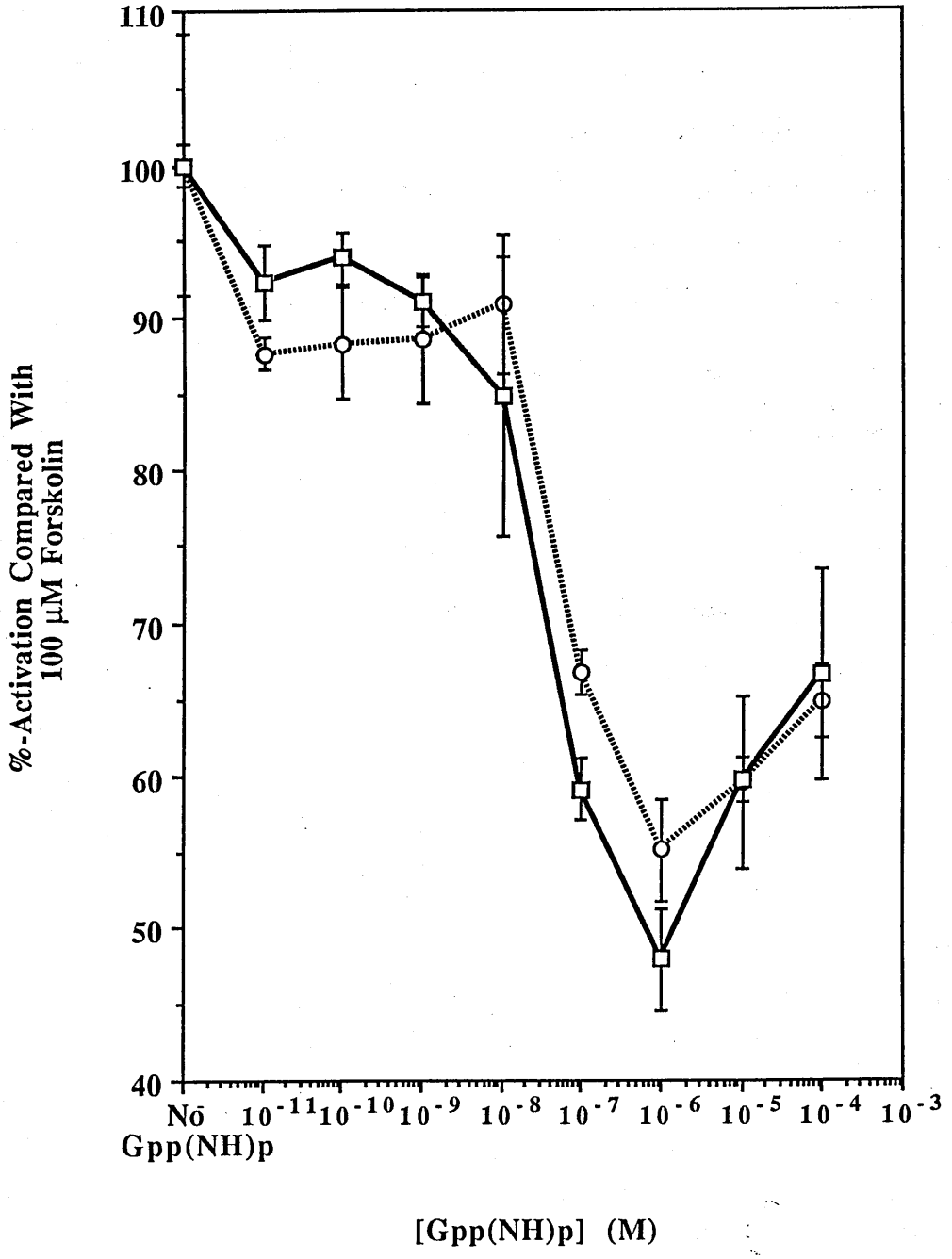
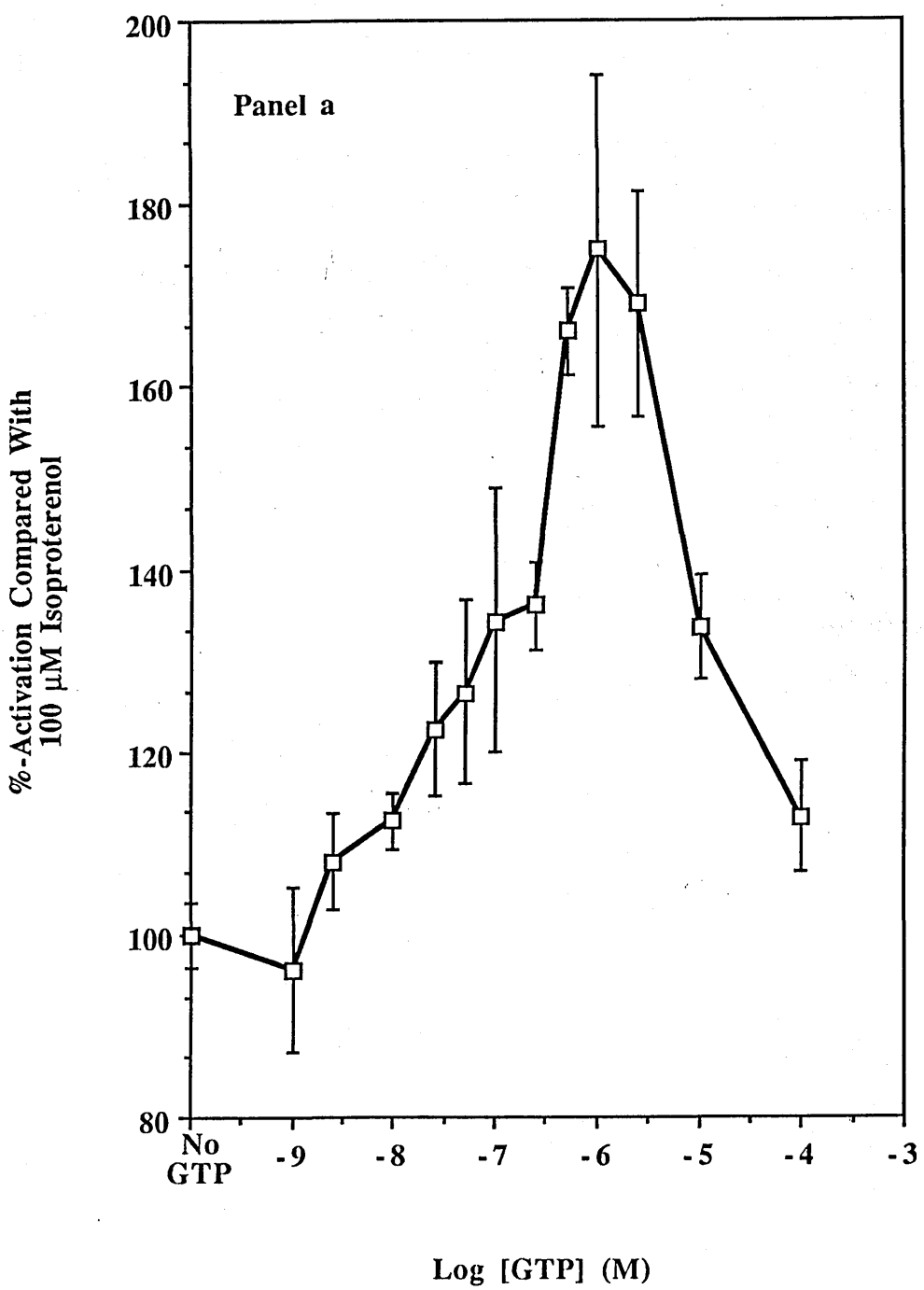

110
100
90
80
70
60
50
40
%-Activation Compared With
100 μM Forskolin
No Gpp(NH)p
10-11
10-10
10-9
10-8
10-7
10-6
10-5
10-4
10-3
[Gpp(NH)p] (M)

Figure 5.8:

Dose-Response Curves For GTP-Dependent Inhibition Of Isoproterenol-Stimulated Adenylate Cyclase Activity In Adipocyte Membranes From Lean And Obese CBA/Ca Animals

Adipocyte membranes from lean (*panel a*, ❑) and obese (*panel b*, ○) CBA/Ca animals were assayed for adenylate cyclase activity for 30 minutes at 23°C in the presence of 100 μM isoproterenol and increasing concentrations of GTP as described in Section 2.7.1. The cyclic AMP formed was measured by the competitive binding method also described in Section 2.7.1. The figure shows results from one experiment representative of three performed on different membrane preparations.

For this experiment, the specific activities obtained with 100 μM isoproterenol alone were 342.2 ± 8.4 (lean) and 453.2 ± 40.0 (obese) pmol/min/mg.


Panel a
%-Activation Compared With
100 µM Isoproterenol
200
180
160
140
120
100
80
No
GTP
-9
-8
-7
-6
-5
-4
-3
Log [GTP] (M)

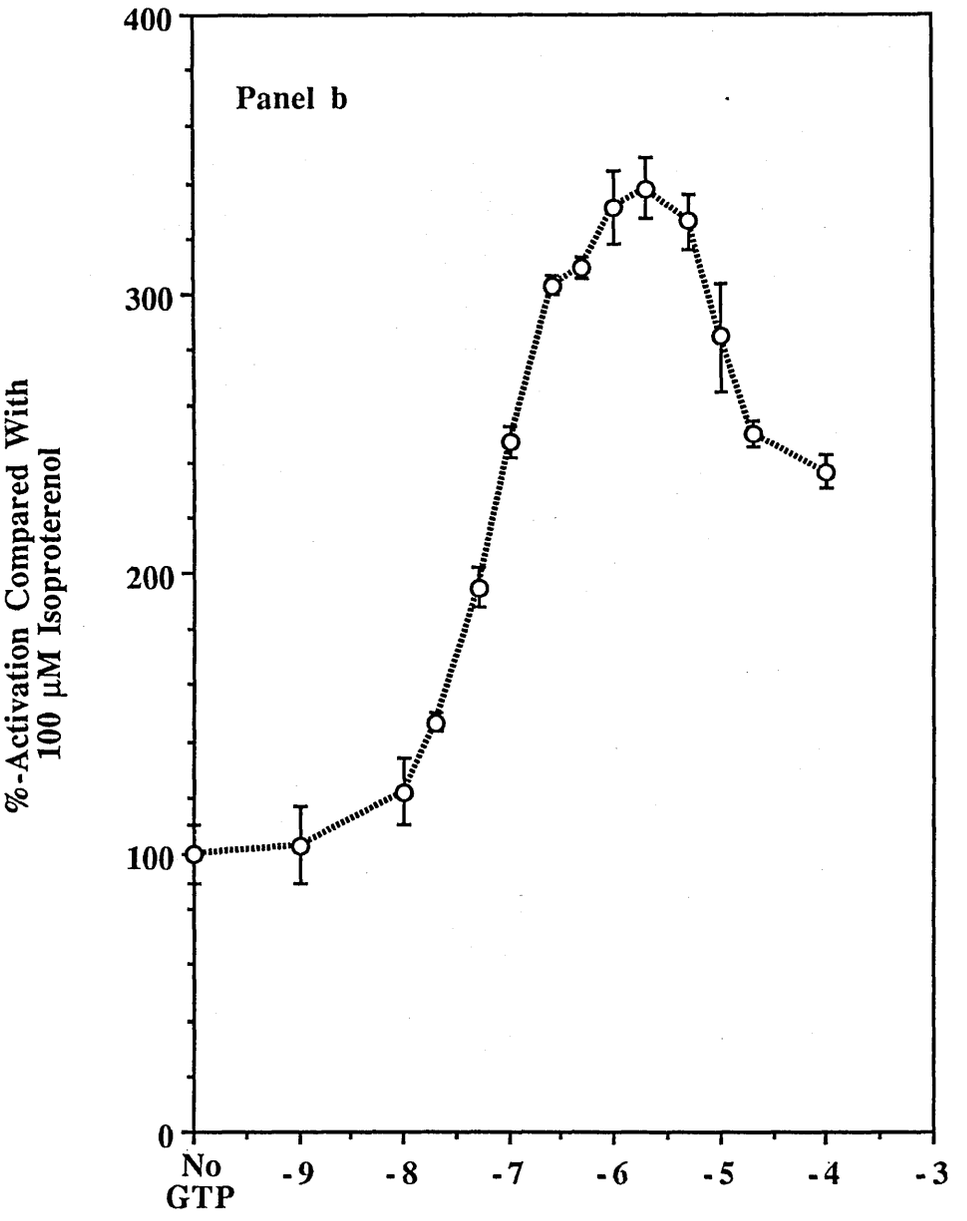

Panel b
400
300
200
100
0
%-Activation Compared With
100 µM Isoproterenol
No
GTP
-9
-8
-7
-6
-5
-4
-3
Log [GTP] (M)

Figure 5.9:

Immunoblotting Of $G_i\alpha$-1 And $G_i\alpha$-2 In Adipocyte Membranes From Lean And Obese CBA/Ca Animals

30 μg of adipocyte membrane protein from lean (lane 1) and obese (lane 2) CBA/Ca animals were prepared and subjected to SDS-PAGE under conditions allowing the resolution of $G_i\alpha$-1 and $G_i\alpha$-2 before transfer to nitrocellulose paper and immunoblotting using antiserum SG1 as the primary antiserum as described in the Sections 2.8.4 and 2.9. The immunoblot shown is representative of three performed with different membrane preparations from lean and obese CBA/Ca animals.

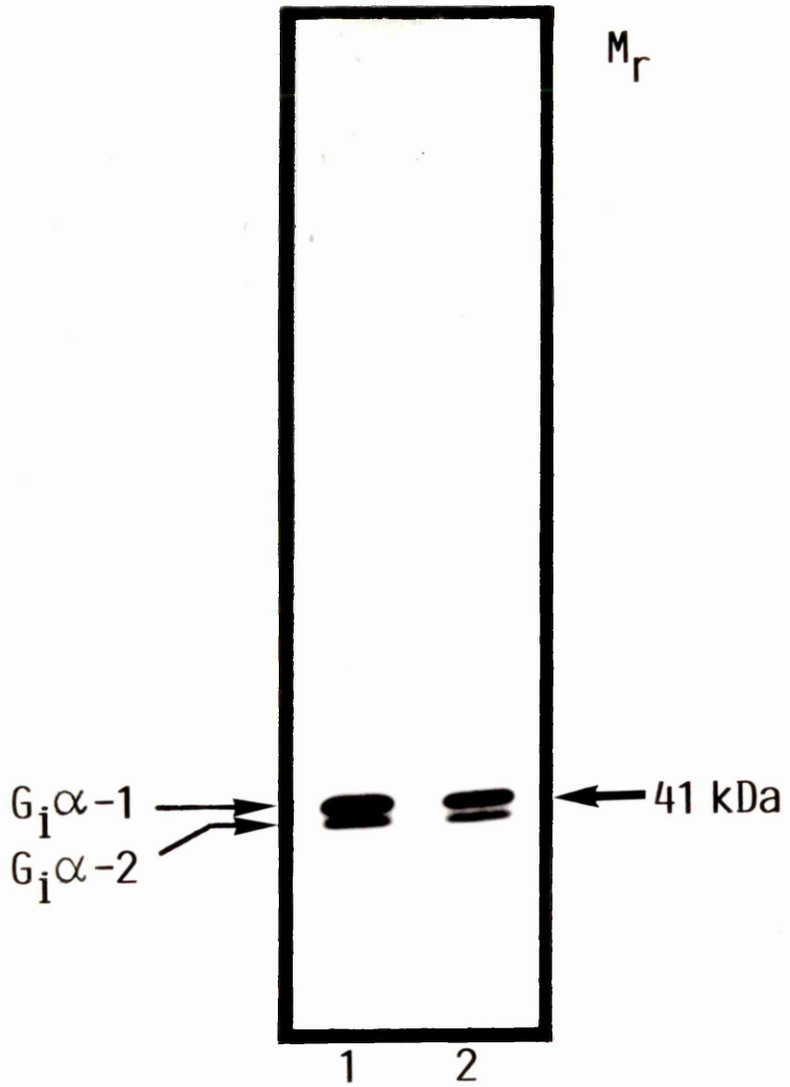

M_r
$G_i\alpha$-1
$G_i\alpha$-2
41 kDa
1
2

Figure 5.10:

Immunoblotting Of $G_i\alpha$-3 In Adipocyte Membranes From Lean And Obese CBA/Ca Animals

100 µg of adipocyte membrane protein from lean (lane 1) and obese (lane 2) CBA/Ca animals were prepared and subjected to SDS-PAGE under 'normal' conditions before transfer to nitrocellulose paper and immunoblotting using antiserum I3B as the primary antiserum as described in Sections 2.8 and 2.9. The immunoblot shown is representative of three performed with different membrane preparations from lean and obese CBA/Ca animals.

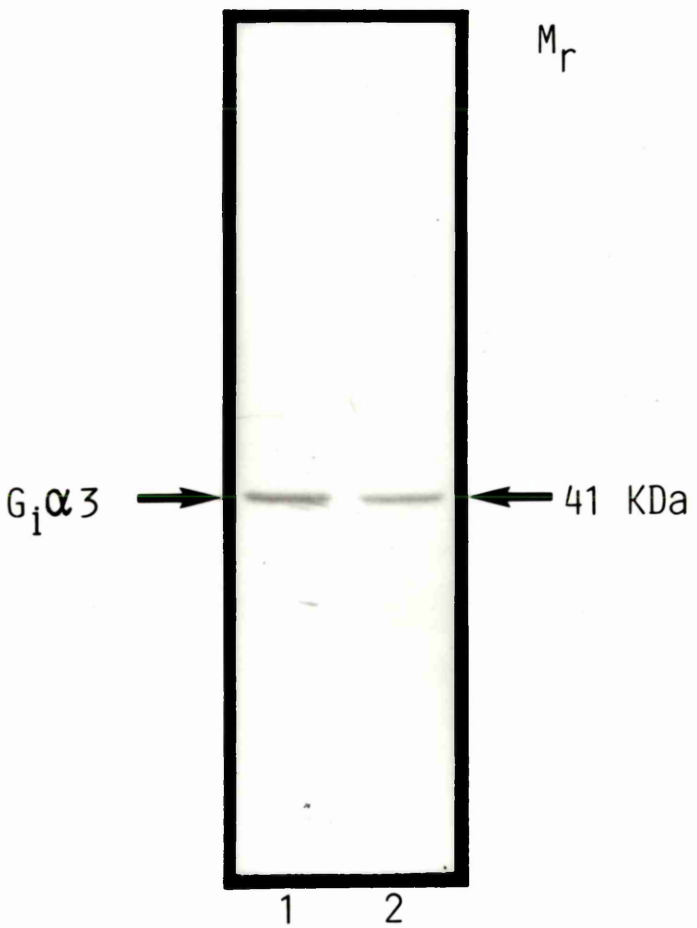

M_r
$G_i\alpha 3$
41 KDa
1
2

Figure 5.11:

Immunoblotting Of $G_s\alpha$ In Adipocyte Membranes From Lean And Obese CBA/Ca Animals

40 μg of adipocyte membrane protein from lean (lane 1) and obese (lane 2) CBA/Ca animals were prepared and subjected to SDS-PAGE under 'normal' conditions before transfer to nitrocellulose paper and immunoblotting using antiserum CS1 as the primary antiserum as described in Sections 2.8 and 2.9. The immunoblot shown is representative of three performed with different membrane preparations from lean and obese CBA/Ca animals.

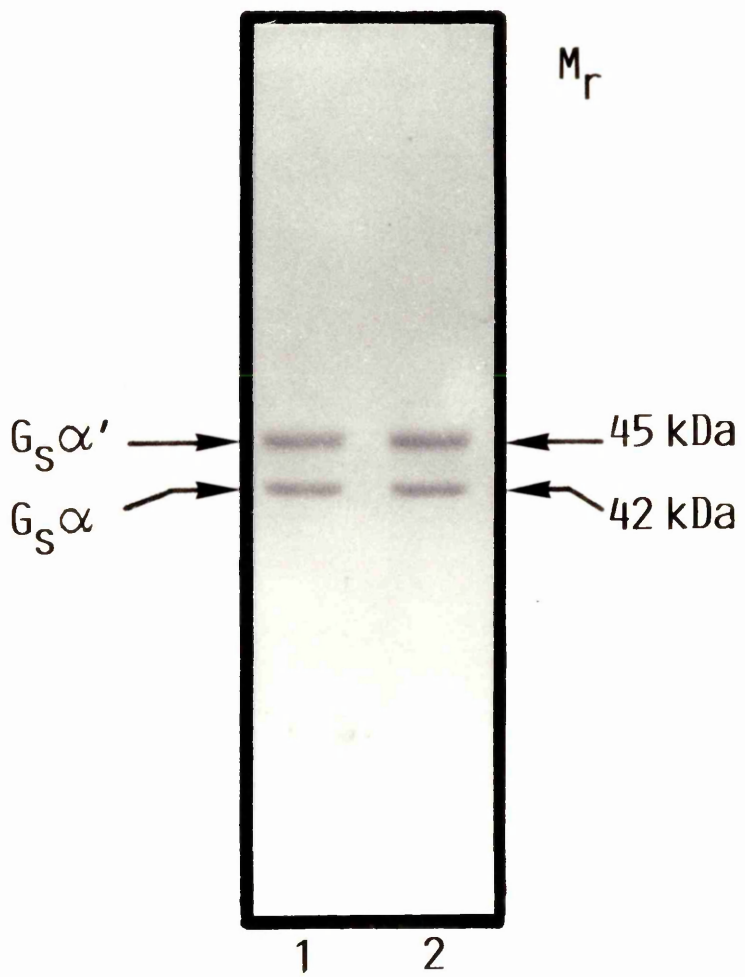

M_r
$G_s\alpha'$
$G_s\alpha$
45 kDa
42 kDa
1
2

Figure 5.12:

Immunoblotting Of β-Subunits In Adipocyte Membranes From Lean And Obese CBA/Ca Animals

70 μg of adipocyte membrane protein from lean (lane 1) and obese (lane 2) CBA/Ca animals were prepared and subjected to SDS-PAGE under 'normal' conditions before transfer to nitrocellulose paper and immunoblotting using antiserum BN1 as the primary antiserum as described in Sections 2.8 and 2.9. The immunoblot shown is representative of three performed with different membrane preparations from lean and obese CBA/Ca animals.

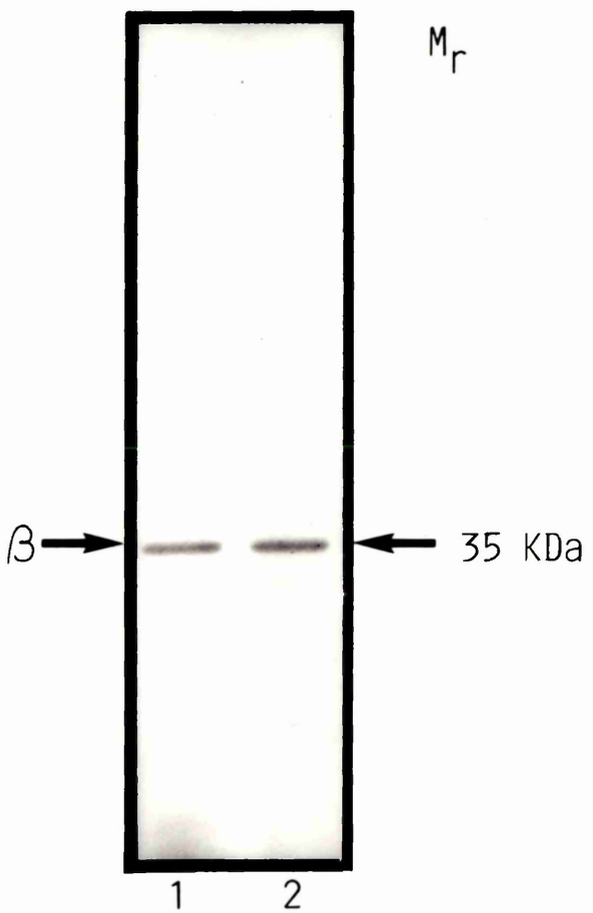

M_r
β
35 KDa
1
2

Table 5.4:

Comparative Levels Of G-Protein Subunits In Adipocyte Membranes From Lean And Obese CBA/Ca Animals

Adipocyte membranes from lean and obese CBA/Ca animals were prepared and subjected to SDS-PAGE before transfer to nitrocellulose and immunoblotting with the primary antisera described in Section 2.9.2 by the method outlined in Sections 2.8 and 2.9.1. Data express levels of G-protein components in membranes from obese animals as a percentage of those found in membranes from lean animals. For each antiserum, a range of amounts of adipocyte membrane protein from lean and obese animals was loaded on a single gel to determine protein loadings which produced linear increases in absorption with increasing amounts of membrane protein applied when the blots were densiometrically scanned after visualisation with a peroxidase-conjugated second antibody. Subsequent comparative blots employed protein concentrations within these parameters.

For each G-protein subunit, data are expressed as MEAN ± S.D. for three immunoblots performed with different membrane preparations.

* denotes a significant difference between lean and diabetic animals, *i.e.* $p<0.005$.

Table 5.4:

G-Protein Subunit	Level As A Percentage Of That In Lean Animals (100%)
$G_i\alpha$-1	49 ± 10*
$G_i\alpha$-2	76 ± 8*
$G_i\alpha$-3	54 ± 9*
β-subunit	111 ± 9
$G_s\alpha$ (42 kDa)	107 ± 12
$G_s\alpha$ (45 kDa)	115 ± 13

5.2 RESULTS & DISCUSSION

Since a marked attenuation in hormone-stimulated adenylate cyclase activity is a characteristic feature of adipocytes from several models of obesity associated with insulin resistance, the responses to three G_s-linked hormones were examined in adipocyte membranes prepared from lean and obese CBA/Ca mice. In the presence of 100 μM GTP, the stimulation of adenylate cyclase activity elicited by increasing concentrations of secretin was similar in membranes from lean and obese animals (Figure 5.3, Table 5.1) as were the EC_{50} values for stimulation by this ligand (Figure 5.3, Table 5.1). In contrast, the maximal stimulatory response to the β-adrenergic receptor agonist isoproterenol was markedly greater in membranes from obese animals compared with lean animals, although the EC_{50} values obtained for stimulation were similar (Figure 5.1, Table 5.1). In this respect, the regulation of adenylate cyclase activity in adipocyte membranes from obese CBA/Ca animals was distinct from that of other rodent models of obesity in which the stimulatory responses to β-adrenergic receptor agonists have been shown to be severely attenuated. However, under conditions where membranes from lean CBA/Ca mice responded potently to increasing concentrations of glucagon in the presence of 100 μM GTP, no stimulation above basal activity was observed for membranes from obese animals (Figure 5.2, Table 5.1). Considering the heterogenous nature of the changes in responsiveness found in membranes from obese animals it seemed most likely that these changes arose from changes in either receptor number or functioning as opposed to a G-protein defect, as the latter might be expected to affect all stimulatory processes to the same extent. Nevertheless, it is possible that post-receptor defects could either exacerbate or counteract any affect of altered receptor functioning. Hence it was decided to assess any potential contribution of the functioning of the catalytic unit of adenylate cyclase and of G_s to the observed alterations in the responses to G_s-linked hormones in obese animals (Table 5.2). Although basal adenylate cyclase activity, *i.e.* the activity observed in the absence

of any ligand, was the same in membranes from lean and obese animals, direct stimulation of the catalytic unit, achieved using either 100 μM forskolin or 5 mM $MnCl_2$, resulted in a greater stimulation of activity in membranes from obese animals compared with lean animals (Table 5.2). If adenylate cyclase activity assayed in the presence of either of these two ligands is taken as an index of the functioning of the catalytic unit of adenylate cyclase itself, then there there is clearly a discrepancy between these observations and those showing that basal activities are similar between membranes from lean and obese animals. Potential explanations for this discrepancy could relate to the mechanisms by which $MnCl_2$ and forskolin activate adenylate cyclase. These are likely to involve interactions and unmask differences other than those resulting from a 'pure' interaction with the catalytic unit alone. Some of these, of course, are not relevant *in vivo* where C would not encounter these ligands. Thus, Mn^{2+} ions have two effects on the adenylate cyclase system; firstly, they uncouple the catalytic unit from G-protein control, thereby removing input to the system from both G_s and G_i, and, secondly, they stimulate the catalytic unit by binding to its allosteric Mg^{2+}-binding site (Limbird *et al.*, 1979). Forskolin also binds directly to the catalytic unit, although some studies have suggested that it also affects regulation of C by G_s (Seamon & Daly, 1981). The stimulation of adenylate cyclase activity by 10 mM NaF was similar in membranes from obese animals compared with lean animals, as was the specific activity attained with 100 μM GTP (Table 5.2). Therefore, the observation that the coupling between the catalytic unit of adenylate cyclase and G_s appeared to be unaltered in membranes from obese animals compared with those from lean animals seemed to confirm the hypothesis that the heterogeneous changes in hormonal stimulation of adenylate cyclase observed in membranes from obese animals compared with lean animals were due to specific lesions at the level of individual receptor number and functioning. However, although the coupling between G_s and the catalytic unit of adenylate cyclase was unaffected, it was theoretically possible that a reduced functioning of G_i could account for the enhanced stimulation of

adenylate cyclase activity seen in response to increasing concentrations of isoproterenol in membranes from obese animals.

Therefore, studies on the inhibitory regulation of adenylate cyclase activity in membranes from lean and obese CBA/Ca mice were undertaken for two reasons; firstly, it was possible that enhanced inhibition of adenylate cyclase activity could lower intracellular cyclic AMP levels, resulting in a reduced rate of lipolysis and a net increase in triglyceride synthesis. Secondly, as discussed in Section 1.11 and Chapter 4, altered inhibitory regulation of adenylate cyclase activity is a common feature of several rodent models of insulin resistance. Therefore it was important to ascertain whether similar aberrations in G_i function were present in this system as it has been proposed that altered functioning of this protein is a characteristic feature of insulin-resistant states (Bushfield *et al.*, 1990d): as stated above, such an attenuated functioning of G_i could potentially have accounted for the enhanced β-adrenergic receptor-mediated stimulation of adenylate cyclase activity observed in adipocyte membranes from obese animals compared with those from lean animals.

The ability of the non-hydrolysable GTP analogue Gpp(NH)p to inhibit forskolin-stimulated adenylate cyclase activity has been used by several groups investigating liver (Gawler *et al.*, 1987) and adipocyte (Strassheim *et al.*, 1990; Bégin-Heick, 1985) membranes to assess G_i functioning in the absence of inhibitory receptor agonists. Potent inhibitory effects of increasing concentrations of Gpp(NH)p were observed for adipocyte membranes from both lean and obese animals (Figure 5.7) with both sets of membranes producing similar maximal inhibitions of 52.1 ± 3.4% (lean animals) and 45.0 ± 3.4% (obese animals) (data presented as MEAN ± S.D. for three separate experiments performed on different membrane preparations) at a Gpp(NH)p concentration of 1 μM. The presence of a 'tonic' inhibitory effect of G_i in adipocyte membranes from lean and obese animals was also tested by using the ability of high concentrations of GTP to inhibit isoproterenol-stimulated adenylate cyclase activity (Cooper *et al.*, 1978; Murayama

& Ui, 1983). Membranes from lean and obese animals both exhibited classical biphasic responses to increasing concentrations of GTP, with G_s being preferentially activated at low GTP concentrations before G_i became activated at higher concentrations (Figure 5.8, *panels a* and *b*). Other groups have shown that the inhibitory phase of this response represents G_i functioning as it can be abolished by pertussis toxin treatment to produce a simple monophasic stimulatory response to GTP (Murayama & Ui, 1983). Nevertheless, whereas in lean animals a maximally effective low concentration of GTP (1 μM) produced a 76 ± 20% increase in adenylate cyclase activity over the control value (Figure 5.8, *panel a*) an increase of 338 ± 5% over control was observed in membranes from obese animals (Figure 5.8, *panel b*). Also, a GTP concentration of 100 μM produced an 80% inhibition of the activity seen at 1 μM in membranes from lean animals; this contrasts with the response observed for membranes from obese animals, where only a 30% inhibitory effect was observed comparing the activities at the same GTP concentrations (Figure 5.8, *panels a* and *b*). These differences are unlikely to have been due to any enhanced functioning of G_s as GTP- and NaF-stimulated adenylate cyclase activities were similar between membranes from lean and obese animals (Table 5.2). Also, there was no alteration in the expression of the two $G_s\alpha$-subunit isoforms present in adipocyte membranes from lean and obese animals (Figure 5.11 and Table 5.4). However, it is possible that the greater stimulation of adenylate cyclase activity observed at low concentrations of GTP in membranes from obese animals compared with lean animals merely reflects the enhanced stimulatory response to isoproterenol (Figure 5.1 and Table 5.1). Nevertheless, the simplest conclusion from these experiments is that the 'tonic' GTP-dependent inhibitory functioning of G_i is severely attenuated in membranes from obese animals, resulting in a potentiation of the stimulation elicited at low GTP concentrations and a dimunition of the inhibition caused at higher GTP concentrations.

Receptor-dependent inhibition of forskolin-stimulated adenylate cyclase

activity was also measured for membranes from lean and obese animals. Whereas the maximal inhibition of adenylate cyclase activity elicited by either PGE_1 or nicotinic acid was similar in membranes prepared from both lean and obese animals (Figures 5.5 and 5.6; Table 5.3), the maximal inhibitory effect of the non-metabolisable A_1 adenosine receptor agonist PIA was approximately 20% greater in membranes from obese animals (Figure 5.4, Table 5.3). Membranes from lean and obese animals exhibited very similar IC_{50} values for inhibition by PIA and PGE_1, although membranes from obese animals were slightly more sensitive to inhibition by nicotinic acid (Figures 5.4, 5.5 and 5.6; Table 5.3). The enhanced maximal inhibition attained by PIA may have been due to altered A_1 adenosine receptor functioning or expression since the maximal inhibition achieved by PGE_1 and nicotinic acid were similar between lean and obese animals (Table 5.3). Also, if enhanced inhibition was due to a more efficient functioning of the common step in the inhibitory process, *i.e.* G_i, one might have expected a greater tonic inhibitory effect of G_i to have been present in membranes from obese animals compared with lean animals, but this was obviously not the case as determined by both Gpp(NH)p-dependent and GTP-dependent inhibitory experiments (Figures 5.7 and 5.8). However, the possibility that the A_1 adenosine receptor was preferentially affected by an alteration in G_i function, compared with the other two G_i-linked hormones, cannot be discounted.

Adipocyte membranes from rodent models of pathological states associated with obesity, including hypothyroidism (Milligan & Saggerson, 1990) and diabetes mellitus (Bégin-Heick, 1990), are associated with changes in the expression of various G-protein subunits (see Section 1.11). Hence comparative immunoblotting studies were performed to assess the relative expression of various G-protein subunits in adipocyte membranes prepared from lean and obese CBA/Ca mice. Since the identity of the G_i protein which mediates the inhibition of adipocyte adenylate cyclase activity is unknown, comparative immunoblotting studies were performed to measure the relative levels of each of the three $G_i\alpha$-subunits

expressed in adipocyte membranes (Mitchell *et al.*, 1989). SDS-PAGE of adipocyte membranes under conditions which allow the resolution of $G_i\alpha$-1 and $G_i\alpha$-2 followed by immunoblotting with antiserum SG1 identifies two bands which correspond to each of these proteins (Mitchell *et al.*, 1989). Similar experiments performed with adipocyte membranes prepared from lean and obese CBA/Ca mice demonstrated that the levels of expression of $G_i\alpha$-1 and $G_i\alpha$-2 in membranes from obese animals were reduced by approximately 50% and 30% respectively compared with membranes from lean animals (Figure 5.9 and Table 5.4). The expression of $G_i\alpha$-3 was also reduced by some 50% in membranes from obese animals compared with lean animals, as determined using antiserum I3B (Figure 5.10 and Table 5.4). In contrast, the levels of the two forms of $G_s\alpha$-subunit recognised by antiserum CS1 were unaffected by the obese state (Figure 5.11 and Table 5.4), as was the level of the total pool of β-subunits present in adipocyte membranes, assessed using antiserum BN1 (Figure 5.12 and Table 5.4). However, since the single band recognised by antiserum BN1 represents a mixture of β_1- and β_2-subunits, the possibility that an increase in one form was associated with a decrease in the other such that the total amount of β-subunits remained unaltered cannot be discounted.

5.3 CONCLUSIONS

The elevation of intracellular cyclic AMP concentrations stimulates lipolysis in adipocytes *via* activation of cyclic AMP-dependent protein kinase and the subsequent phosphorylation and activation of hormone-sensitive lipase (Allen *et al.*, 1986). Therefore this process is intimately associated with changes in adenylate cyclase activity. In several rodent models of obesity, profound alterations in lipolytic hormone-stimulated adenylate cyclase activity have been observed, including the high-fat diet-fed rat (Gorman *et al.*, 1973), the genetically diabetic (*db/db*) mouse (Bégin-Heick & Coleman, 1988), the obese (*ob/ob*) mouse (Bégin-Heick, 1985) and the genetically obese (*fa/fa*) rat (Vannucci *et al.*, 1990).

Since defects in the regulation of adipocyte adenylate cyclase activity seem to characterise obese states, and may contribute to the abnormal accummulation of triglyceride, the regulation of adenylate cyclase activity in adipocyte membranes prepared from genetically obese CBA/Ca mice was studied to determine whether similar changes occurred to those observed in the other models of obesity mentioned above.

Isoproterenol and glucagon were potent stimulators of adenylate cyclase activity in adipocyte membranes from lean mice. Adipocyte membranes from genetically obese (*fa/fa*) Zucker rats (Strassheim *et al.*, 1991b), genetically diabetic (*db/db*) mice (Chapter 4) and high fat diet-fed rats (Gorman *et al.*, 1973) exhibit severely attenuated responses to maximally effective concentrations of both these ligands compared with membranes from lean animals. Indeed, a reduced catecholamine-stimulated lipolytic response is a universal feature of rodent models of obesity associated with either hypothyroidism (Debons & Schwartz, 1961) or Type II diabetes mellitus (Bégin-Heick & Heick, 1977; Levilliers *et al.*, 1978; Vannucci *et al.*, 1990). However, membranes from genetically obese CBA/Ca mice actually exhibited an enhanced maximal response to isoproterenol stimulation, whilst the response to glucagon was completely abolished in membranes from obese animals. The responses of both sets of membranes to increasing concentrations of secretin were similar, indicating that the alterations in the responses to isoproterenol and glucagon may have been receptor-specific.

Another possible explanation for the enhanced response to isoproterenol in membranes from obese animals could have been the increase in the activity of the catalytic unit (C), which was determined by measuring the stimulations elicited by maximally effective concentrations of forskolin and $MnCl_2$. However basal, GTP- and NaF-stimulated adenylate cyclase activities were similar between samples from lean and diabetic animals. There are two potential explanations for this discrepancy: firstly, both forskolin and $MnCl_2$ are potent stimulators of C which are not

encountered by the protein *in vivo*. Hence, any conformational change and concomitant increase in the activity of C which these ligands might unmask may not occur normally, *i.e.* under basal conditions or when C is activated by G_s, and this may explain why basal and G_s-stimulated activities are unaltered. Alternatively, it is possible that whilst maximally effective concentrations of $MnCl_2$ and forskolin bind to and activate the total complement of C present in adipocyte membranes, GTP and NaF are only capable of activating that proportion of C which is coupled to G_s, thus accounting for the lack of any affect of the increased activity of C on G_s-stimulated activity.

Since GTP- and NaF-stimulated activities were similar between membranes from lean and obese animals, the activation of C elicited by G_s.C coupling appeared to be normal. This was consistent with the lack of any change in expression of the two forms of $G_s\alpha$-subunits between adipocyte membranes from lean and obese animals. Hence the alterations in hormonal stimulation observed may have resulted from either altered receptor numbers or inefficient coupling between receptors and G_s. Glucagon receptor binding studies are necessary to address the reason for the abolition of a glucagon response in membranes from obese animals. A large reduction in the number of glucagon receptors may have accounted for the reduction, but as explained in Chapters 1 and 4, activated receptors behave kinetically as catalysts, being capable of activating several G_s molecules in their lifespan. One consequence of this mechanism is that large reductions in receptor number may merely reduce the rate at which the steady-state velocity is attained without affecting its final value: this has been shown for β-adrenergic receptors in turkey erythrocytes (Tolkovsky & Levitzki, 1978) as well as glucagon receptors in rat hepatocytes (Houslay *et al.*, 1980). Hence a reduction in the number of glucagon receptors should only reduce the sensitivity with which glucagon is capable of stimulating adenylate cyclase activity without affecting the final maximal specific activity attained. Hence it is most likely that the major defect accounting for the abolition of glucagon-stimulated adenylate cyclase activity in membranes from

obese animals is a gross perturbation of the interaction between the glucagon receptor and G_s. The nature of this lesion is unknown but considering the receptor-specific nature of the alterations in hormone-stimulated adenylate cyclase activity that were seen, the defect probably resulted from a non-functional glucagon receptor in membranes from obese animals, possibly as a result of a regulatory covalent modification. An increase in β-adrenergic receptor number could explain the enhanced maximal response to isoproterenol, but comprehensive binding studies would be necessary to address this question. However, the possibility that the β-adrenergic receptor was preferentially affected by a G-protein defect, such as a reduced 'tonic'GTP-dependent inhibitory effect on stimulation, is also likely.

To determine whether adipocyte membranes from the obese CBA/Ca mouse exhibited alterations in inhibitory regulation, both guanine nucleotide-mediated and receptor-dependent inhibitory phenomena were examined. The abolition of the 'tonic' inhibitory effect of G_i has been observed in adipose tissue from the Type I diabetic rat (Strassheim *et al.*, 1990), the obese (*ob/ob*) mouse (Bégin-Heick, 1985) and the obese (*fa/fa*) Zucker rat (Strassheim *et al.*, 1991b). This effect was monitored either by measuring Gpp(NH)p-dependent inhibition of forskolin-stimulated adenylate cyclase activity, or by measuring the ability of GTP to inhibit isoproterenol-stimulated activity. Gpp(NH)p was capable of eliciting a similar maximal inhibition of forskolin-stimulated adenylate cyclase activity in membranes from either lean or obese CBA/Ca animals. In contrast, whilst GTP was also capable of causing inhibition of isoproterenol-stimulated activity in membranes from lean and obese animals, the inhibition elicited in membranes from lean animals was much greater. Hence, in this respect adipocyte membranes from genetically obese CBA/Ca mice are similar to those from genetically obese (*ob/ob*) mice and obese Zucker rats, which do not exhibit a functional G_i activity with respect to GTP-dependent inhibitory effects (Bégin-Heick & Coleman, 1988; Strassheim *et al.*, 1991b). However, adipocyte membranes from obese (*ob/ob*) mice and

Zucker rats also displayed similarly attenuated Gpp(NH)p-dependent inhibitory effects, unlike the CBA/Ca mouse. This may imply the existence of a defect in G_i functioning which is distinct from that previously described for adipocytes from obese animals. It is possible that the aspect of G_i function measured by the ability of Gpp(NH)p to inhibit forskolin-stimulated adenylate cyclase activity activity is distinct from that measured by GTP-dependent inhibition of hormone-stimulated activity. With regard to this point, it has been suggested that inhibition of basal and forskolin-stimulated activities reflects the ability of $\beta\gamma$-subunits to inhibit adenylate cylase activity, whereas inhibition of hormone-stimulated activity represents mainly the functioning of $G_i\alpha$-subunits (Hildebrandt & Kohnken, 1990). Hence, the reduced ability of GTP to inhibit hormone-stimulated adenylate cyclase activity may reflect an attenuated functioning of $G_i\alpha$-subunits, which does not affect the ability of $\beta\gamma$-subunits to inhibit adenylate cyclase activity. However, it would be necessary to determine the effects of pretreating adipocytes with pertussis toxin to determine unequivocally that the altered response to GTP in the presence of isoproterenol represents a reduced 'tonic' inhibitory functioning of G_i. Given that inhibition of adenylate cyclase appears to be mediated by $G_i\alpha$-2 in several systems, including platelets (Simmonds *et al.*, 1989a) and possibly hepatocytes (Bushfield *et al.*, 1990a), it is possible that this protein has been modified such that it cannot function in the absence of an agonist-occupied inhibitory receptor. Such a phenomenon has been noted from adipocytes (Strassheim *et al.*, 1990) and hepatocytes (Bushfield *et al.*, 1990c) from streptozotocin-induced Type I diabetic rats, as well as adipocytes from the genetically obese (*ob/ob*) mouse (Bégin-Heick, 1985; Greenberg *et al.*, 1987) and the obese Zucker rat (Strassheim *et al.*, 1991b). Work on the streptozotocin-diabetic rat hepatocyte system suggests that the attenuation in inhibitory regulation is due to the phosphorylation and inactivation of $G_i\alpha$-2 (Bushfield *et al.*, 1990c).

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activity was monitored using the anti-lipolytic agents PIA, nicotinic acid and prostaglandin E_1. Whereas the maximal inhibitory effects of PGE_1 and nicotinic acid were unaltered in membranes from obese animals, the maximal inhibition achieved by PIA was slightly enhanced in membranes from obese animals. Therefore the attenuation in the GTP-dependent 'tonic' inhibitory functioning of G_i does not produce any gross alteration in receptor-dependent inhibitory phenomena. The slightly enhanced A_1 adenosine receptor-mediated inhibition of adenylate cyclase may reflect either an increased number or functioning of the A_1 adenosine receptor, although it may be a consequence of the possible loss of a 'tonic' inhibitory functioning of G_i in membranes from obese animals. The enhanced PIA-mediated inhibition could not be explained by any increased expression of any of the $G_i\alpha$-subunits or the total complement of β-subunits; indeed, levels of $G_i\alpha$-1, $G_i\alpha$-2 and $G_i\alpha$-3 were reduced by between 30 and 50% in membranes from obese animals. This situation bears some similarity to adipocyte membranes prepared from obese (*fa/fa*) Zucker rats, in which large reductions in the expression of $G_i\alpha$-1 and $G_i\alpha$-3 are not accompanied by any change in receptor-dependent inhibitory regulation, although 'tonic' inhibitory effects are abolished (Strassheim *et al.*, 1991b). A similar increase in A_1 adenosine receptor-mediated inhibition of adenylate cyclase has also been found in adipocyte membranes from the diabetic (*db/db*) mouse (Chapter 4) and has also been observed in adipocyte membranes from obese aged rats (Green & Johnson, 1989; Guidicelli & Pecquery, 1978).

Considering the heterogeneous nature of the changes in the regulation of adenylate cyclase activity in adipocyte membranes from genetically obese CBA/Ca mice, it is difficult to assess these changes with respect to their potential relevance in the development of obesity in this system. The effect of the enhanced β-adrenergic receptor-mediated activation of adenylate cyclase on increasing lipolytic rates would be offset by the abolition of a glucagon-stimulated activity. Thus the consequences of these changes on lipolytic rates *in vivo* would depend on the particular combination of stimulatory hormones that the adipocytes would be

exposed to at a given time. Therefore adipocytes from the CBA/Ca mouse are distinct from the Zucker rat and the genetically diabetic (*db/db*) mouse in which catecholamine-, glucagon- and secretin-stimulated adenylate cyclase activities, and thus the rates with which they stimulate lipolysis, would be reduced *in vivo*. Therefore any combination of stimulatory hormones would produce a reduced rate of lipolysis compared with lean controls in these systems. That this would not be the case for the obese CBA/Ca mouse implies that some other factors must be mainly responsible for the observed accummlation of triglyceride. For example, persistently elevated activities of cyclic AMP phosphodiesterases could produce sufficient reductions in intracellular cyclic AMP levels to cause a net increase in triglyceride synthesis: in this respect, an enhanced cyclic AMP phosphodiesterase activity has been noted in adipocytes from genetically obese (*ob/ob*) mice, although inhibition of this PDE activity by methylxanthines still results in reduced intracellular cyclic AMP levels compared with adipocytes from lean animals (Shepherd *et al.*, 1977). Insulin-stimulated lipogenesis is decreased in adipocytes from obese animals compared with lean animals (Mercer *et al.*, 1991), unlike adipocytes from the genetically diabetic (*db/db*) mouse which exhibit an enhanced insulin stimulation of lipogenesis (Kupiecki & Adams, 1974). However, other potential loci for defects which could explain the observed obesity include impaired activation or activity of cyclic AMP-dependent protein kinase, a reduced activity of hormone-sensitive lipase, the enzyme which catalyses triglyceride breakdown, or increased activities of lipogenic enzymes. Further work will need to determine which of these steps is responsible for the obese phenotype in the CBA/Ca mouse.

CHAPTER 6

Conclusions And Perspectives

The conclusions for each individual chapter of results have already been fully discussed. Therefore this section will be used to discuss potentially wider implications of the various conclusions that were reached in these studies of adenylate cyclase regulation.

The results from Chapter 3 suggest that a greater proportion of $G_s\alpha$- and $G_i\alpha$-subunits present in liver membranes from genetically Type II diabetic (*db/db*) mice, compared with those from lean animals, are in their free GTP-bound state. This was deduced from the discrepancies between relative assessments of G-protein α-subunit levels carried out both by immunoblotting and bacterial toxin-catalysed ADP-ribosylation, as well as being borne out by an apparently enhanced sensitivity of membranes from diabetic animals to stimulation by increasing concentrations of Gpp(NH)p. Whilst these studies have been concerned with the regulation of hepatic adenylate cyclase activity, the fact that βγ-subunits may be able to interact with several G-protein α-subunits suggests that such drastic reductions in levels of βγ-subunits in diabetic animals may have profound effects on other G-protein-linked signalling systems. Examples of such systems include regulation of phosphatidylinositol-specific phospholipase C, phospholipase A_2 and various G-protein-regulated ion channels.

The mechanisms by which βγ-subunits can control signalling events have been extensively studied for both the adenylate cyclase and retinal cyclic GMP-specific PDE systems. These studies have suggested that βγ-subunits may reduce basal (receptor-independent) GDP release from α-subunits since the rate of GDP release from holomeric G-proteins is one thousand-fold less than release from free α-subunits (Cerione *et al.*, 1985; Bourne *et al.* 1991). As a result, basal activity would be suppressed such that a hormone-stimulated "signal to noise" ratio would be maximized. Such a scenario has been proven for the adenylate cyclase system using both isolated S49 lymphoma cell membranes (Hildebrandt & Kohnken, 1990) and purified detergent-solubilised components (Cerione *et al.*, 1985).

However, both studies have pointed out that these effects are critically dependent on the ratio of α-subunits to βγ-subunits. Hence, depending on the relative abundance of various G-protein α-subunits controlling the effector enzymes mentioned above, some systems may be more susceptible to altered regulation by reduced levels of βγ-subunits than others. Indeed, the recent purification of multiple pertussis toxin-insensitive G-protein α-subunits from bovine brain by βγ-affinity chromatography, one of which (α_{42}) was shown to be present in liver by immunoblotting, suggests that signalling events other than the ones mentioned above will be subject to regulation by the concentration of βγ-subunits in the plasma membrane (Pang & Sternweis, 1990; Strathmann *et al.*, 1989) Also, with the heterogeneity of β- and γ-subunits becoming more apparent from molecular cloning studies, it may be that different βγ-complexes can interact preferentially with specific α-subunits, thereby adding another level of complexity to the system (Levine *et al.*, 1990).

In Chapter 4, studies on the regulation of adenylate cyclase regulation in adipocyte membranes from genetically Type II diabetic (*db/db*) mice demonstrated that lipolytic hormone-stimulated adenylate cyclase activity was severely impaired for three out of the four G_s-linked hormones tested, with a particularly large reduction being noted for β-adrenergic receptor-stimulated activity. Considering the fact that a similar reduction was noted in liver membranes from the same animals it would be of great interest to determine whether a reduction in β-adrenergic receptor-mediated stimulation of adenylate cyclase activity is observed in other insulin-sensitive tissues, especially in heart where cyclic AMP is thought to play an important rôle in mediating contraction (Bristow & Ginsberg, 1986). Also, as glucose transport is known to be profoundly affected by ligands which elevate intracellular cyclic AMP levels, altered responses to hormones which regulate adenylate cyclase activity in skeletal muscle, as well as fat and heart, could have profound effects on whole body glucose utilisation. This is because the major insulin-responsive glucose transporter, GLUT 4, is a substrate for cyclic AMP-

dependent protein kinase (Gould & Bell, 1990). However, considering the tissue-specific nature of the alterations in G-protein expression observed for the diabetic (*db/db*) mouse, it is possible that each tissue may display unique signalling defects.

Since inhibition of adenylate cyclase is relatively unaffected by the observed increase in expression of $G_i\alpha$-1 in adipocyte membranes from diabetic (*db/db*) animals, there may be consequences for other pertussis toxin-sensitive signalling events. One potential candidate is receptor-mediated inositol phosphate generation, which is completely abolished by pertussis toxin treatment in rat adipocytes (Moreno *et al.*, 1983).

In Chapter 5, it was argued that the heterogeneous nature of the defects in adenylate cyclase regulation in adipocyte membranes from obese CBA/Ca animals, compared with those from lean animals, suggested the existence of multiple defects in both stimulatory and inhibitory mechanisms. Of particular interest was the lack of any response to increasing concentrations of glucagon in membranes from obese animals. If such a phenomenon was also present in liver membranes from obese animals, this could severely perturb hormonal regulation of liver metabolism which would, in turn, have profound effects on whole body carbohydrate, lipid and protein metabolism. The marked potentiation in β-adrenergic receptor-mediated adenylate cyclase activity, if found in other tissues, could, for example, have opposite effects on the catecholamine-stimulated inotropic response in heart, and thus on the control of glucose transport in heart, fat and muscle compared with the scenario described above for the genetically diabetic (*db/db*) mouse. Similarly, from studies on adipocytes from obese CBA/Ca mice, the observation of a reduced 'tonic' inhibitory functioning of G_i in cells other than adipocytes could potentially produce constitutively elevated intracellular cyclic AMP levels and, in association with an insulin-resistant state, result in a net increase in the catabolism of carbohydrate and triglyceride reserves.

In conclusion, it can be seen that the development of an insulin-resistant

state is associated not only with an insensitivity to some of the actions of insulin, but also leads to changes in the responses to other hormones which regulate metabolism in insulin-sensitive tissues, such as liver and adipose tissues. In particular, the work described here on the regulation of adenylate cyclase activity shows that these alterations in responsiveness can involve changes in both stimulatory and inhibitory mechanisms, although distinct differences are observed between different animal models of insulin-resistance. As stated above, detailed studies are now necessary to assess any defects in the receptor/G-protein/phospholipase C pathway, which plays an important rôle in regulating hepatocyte and adipocyte metabolism in association with insulin and those hormones which control cyclic AMP levels. Indeed, considering the evidence which suggests that altered functioning of protein kinase C may be an important factor in the development of both adenylate cyclase defects and insulin resistance (Karasik *et al.*, 1990; Bushfield *et al.* 1990d), detailed investigations on the mechanisms by which intracellular sources of *sn*-1,2-diacylglycerol are regulated in diabetic states may provide important clues as to how the functioning of protein kinase C isoforms isoforms is altered. With respect to the alterations in expression of $G_i\alpha$-subunits found in many of these insulin-resistant syndromes, it has been suggested that the insulin receptor is capable of interacting non-covalently with the 41 kDa pertussis toxin substrate present in liver membranes, which presumably represents a mixture of $G_i\alpha$-2 and $G_i\alpha$-3 (Rothenberg & Kahn, 1988). If this is a reflection of a physiologically relevant interaction between the insulin receptor and either $G_i\alpha$-2 or $G_i\alpha$-3 which is involved in insulin receptor signal transduction, then either any reduction in the expression or any covalent modifications of these proteins could potentially account for some of the post-binding defects seen to characterise insulin's action in diabetic states.

However, any relationship between the observations made here, and by other workers, on animal model systems and the analogous signal transduction

systems in human diabetics remains to be rigorously defined.

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