ENZYMES OF CYCLIC AMP METABOLISM IN HEPATOCYTES

LI ZENG, B.Sc.

This thesis is presented for the degree of Doctor of Philosophy.

Division of Biochemistry and Molecular Biology,
Institute of Biomedical and Life Science,
University of Glasgow.

June, 1996.

Dedicated to my Mum

and

all the people I love

Acknowledgements

Here, my sincere thanks go to my supervisor, Professor Miles Houslay for his help, understanding, patience, enthusiasm and guidance throughout my PhD project and thesis. I would also like to thank the Division of Biochemistry and Molecular Biology, IBLS, University of Glasgow for the use of their facilities throughout my period of study.

I would like to thank John Brown Engineering Ltd and the University of Glasgow for their financial support during my studies.

My enormous thanks go to everyone in 'Lab A3' who have helped me in any way during my PhD. I enjoyed working in this lab and it was always fun!!! Also, I would like to thank everyone I know in the Department for their consideration, laughter and chatter.

However, I wish to tenderly acknowledge the following individuals for their love, support, and encouragement over the years of my PhD studies: My first special thanks go to Sandra, for her friendship and support over the years, for helping me out whether in academic or social life and for her patient help with my thesis. A special thanks also for Moira, who besides all of the above, also lent me the computer to make this thesis possible.

I will remember all these good and memorable times I had in Scotland.

My heartfelt thanks to my best Chinese friends An-xin (Cindy), Tu-yong (Esther) and MeiNa, for the excitement, enjoyment and happiness I shared with them as well as the hardships.

Tremendous thanks to everyone in my family, whose love, support, encouragement, understanding and belief in me has made all the difference. I know that I own you all a lot, but I'd still like to take this opportunity to say, don't worry about me, I will be fine!!! Last, thanks to God, for his blessing.

Abbreviations

AP-1	A-kinase anchoring protein
ATF	Activation transcription factor
ATP	Adenosine trisphosphate
BSA	Bovine serum albumin
CaM	Calmodulin
cAMP	Adenosine-3', 5'-cyclic monophosphate
cDNA	Complementary deoxyribonucleic acid
cGMP	Guanosine-3', 5'-cyclic monophosphate
Cilostimide	[4,5-dihydro-6[4-(1H-imadazol-1-yl)phenyl}-5-methyl-3(2H)-pyrazone]
CRE	Cyclic AMP responsive element
CREB	Cyclic AMP responsive element-binding
DAG	Diacylglycerol
DMSO	Dimethyl sulphoxide
DPD	Dunce-like phosphodiesterase (PDE4B)
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis (β-aminoethyl ether) N, N, N', N'-tetraacetic acid
EHNA	Erythro-9-(2-hydroxy-3-nonyl)-adenine
GDP	Guanosine diphosphate
G_i	Inhibitory G protein to adenylyl cyclase activity
G_s	Stimulatory G protein to adenylyl cyclase activity
G protein	GTP binding protein
GTP	Guanine trisphosphate
Hepes	N-2-Hydroxyethylpiperazine-N'-2-ethane-sulphonic acid
IBMX	1-isobutylmethyl-3-xanthine

Ki	The dissociation constant for the enzyme-inhibitor complex
Km	Michaelis constant, equal to the substrate concentration at which the reaction rate is half the maximum value
PBS	Phosphate buffered saline
PDE	Phosphodiesterase
PI-3 kinase	Phosphatidylinositol-3-kinase
PIP2	Phosphatidylinositol-4,5-bisphosphate
PKA	Cyclic AMP-dependent protein kinase
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol-12-myristate, 13-acetate
PMSF	Phenylmethylsulphonylfluoride
RD1	Rat dunce-like1 (PDE4A)
Rolipram	[4-{3-(cyclopentoxyl)-4-methoxyphenyl}-2-pyrrolidone]
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TEA	Triethanolamine
TEMED	N, N, N', N'-Tetramethylethylene diamine
TPA	12-O-tetradecanoylphorbol 13-acetate (PMA)

Adendda

β-ARK	β adreneric receptor kinase
CAT	Chloramphenicol acetyltransferase
CREM	Cyclic AMP responsive element modulator
DEPC	Diethyl pyrocarbonate
ICER	Inducible cAMP early repressor
IP_3	Inositol 1,4,5-trisphosphate
KLH	Keyhole limpet haemocyanin
LDH	Lactate dehydrogenase
PCA	Perchloric acid
PIP_2	Phosphatidyl inositol 4,5-bisphosphate
PtdSer	Phosphatidylserine
UCR	Up stream conserved region

Contents.

Chapter 1: Introduction.

List of Figures

List of tables

Summary

Incubation of hepatocytes or the SV40-DNA immortalised hepatocyte P9 cell line with cholera toxin led to a time-dependent activation of adenylyl cyclase activity which occurred after a defined lag period. When added together with cholera toxin, each of the hormones insulin and vasopressin was capable of attenuating the maximum stimulatory effect achieved by cholera toxin over a period of 60min through a process which could be blocked by the compounds staurosporine and chelerythrine. Attenuating effects upon cholera toxin-stimulated adenylyl cyclase activity could also be elicited using either the protein kinase C stimulating phorbol ester PMA (12-O-tetradecanoyl phorbol-13-acetate) or the protein phosphatase inhibitor okadaic acid. Alkaline phosphatase treatment of membranes reversed the inhibitory effect of PMA . Cholera toxin also stimulated the adenylyl cyclase activity of intact CHO and NIH-3T3 cells but this activity was insensitive to the addition of PMA. Overexpression of various protein kinase C isoforms in CHO cell lines did not confer sensitivity to inhibition by PMA upon cholera toxin stimulated adenylyl cyclase activity. It is suggested that the protein kinase C mediated phosphorylation of a membrane protein attenuates cholera toxin-stimulated adenylyl cyclase activity in hepatocytes and P9 cells. The cellular selectivity of such an action may be due to the target for this inhibitory action of protein kinase C being a particular isoform of adenylyl cyclase. This is believed to be type V adenylyl cyclase which provides the major activity in hepatocytes and P9 cells but is absent from both CHO and NIH-3T3 cells.

Multiple families of phosphodiesterase are differentially expressed and regulated in a cell-specific fashion. The work here focuses on identifying which members of the multigene PDE4 isoenzyme family are expressed in hepatocytes and P9 cells. This was done by RT-PCR transcript analysis and immunoblotting, showing that only the PDE4A and PDE4D families are represented. The effect of elevated cAMP on low K_m cAMP-specific PDE

expression in P9 cells was determined. Chronic treatment of P9 cells with the adenylyl cyclase stimulators forskolin and cholera toxin led to a profound increase in PDE4 activity. This was prevented by actinomycin D, an inhibitor of RNA synthesis. PDE4 up-regulation was due to an increased level of PDE4D3, a subtype product of the PDE4D gene, as determined by both transcript analysis and immunoblotting which detected an ~93kDa species, reflecting PDE4D3 protein. Although novel transcripts for PDE4B (PDE4B2) were detected associated with this PDE4 up-regulation, the protein product of this gene could not be detected in the P9 cells.

Hepatocytes from streptozotocin-induced diabetic rats showed decreased high affinity PDE activities, namely PDE2, PDE3 and PDE4 activities compared to normal animals. This was associated with changes in the PDE4 transcript profile noted for PDE4B2 and PDE4D3 forms.

Chapter 1
Introduction

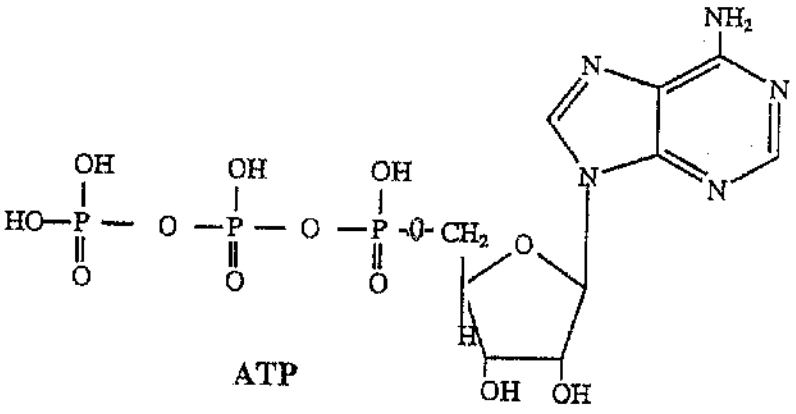
1.1 cAMP signalling

The intracellular signal triggers a cascade of molecular reactions the result of which culminates in the biochemical response. One such intracellular signal, or second messenger, is cyclic adenosine 3', 5'-monophosphate (cyclic AMP). Cyclic AMP has been shown to bind with high affinity to the regulatory subunits of a protein kinase, thereby activating the enzyme (Taylor, 1989). This enzyme has since been named cyclic AMP-dependent protein kinase (PKA). Cellular cyclic AMP homeostasis is maintained not only by regulating its synthesis by adenylyl cyclase (Cooper *et al.*, 1994; Taussig and Gilman, 1995), but also by control of its degradation, through the action of the cyclic nucleotide phosphodiesterases (PDEs) (Beavo *et al.*, 1994; Conti *et al.*, 1995b) (Figure 1.1). Since the discovery of cyclic nucleotides, their synthesis and its modulation by hormones, neurotransmitters and pharmacological agents are mechanisms which are now among the most well established in cellular signalling.

1.1.1 Cyclic AMP as a second messenger

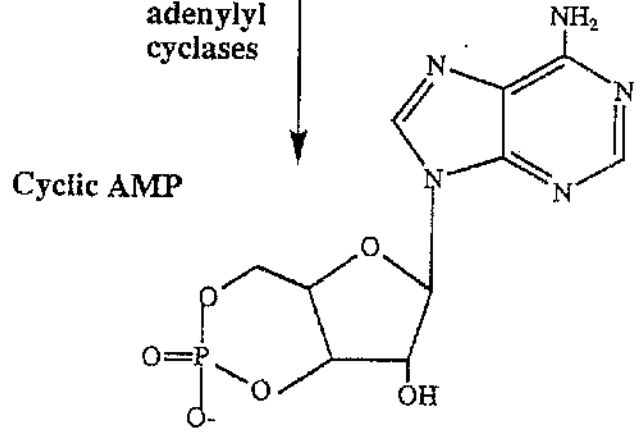
The second messenger cyclic AMP has shown to be central in the functioning of a diverse range of biological systems. Of great importance is the role of cyclic AMP in the hormonal control of blood glucose and fatty acid levels. Cyclic AMP has a dual effect in the hormonal control of lipolysis in adipocytes. On the one hand, cyclic AMP mediates the lipolytic action of hormones such as catecholamines, corticotrophin and glucagon (Steinberg *et al* 1975). The cAMP promotes lipolysis through the activation of cyclic AMP-dependent protein kinase, which phosphorylates and activates hormone sensitive lipase resulting in hydrolysis of stored triglyceride to liberate glycerol and free fatty acids (Stråfors *et al.*, 1984). Conversely, the degradation of cyclic AMP and the subsequent reduction in cyclic AMP-dependent protein kinase activity has been shown to be of functional importance in the antilipolytic

Figure 1.1 Diagram showing the action of adenylyl cyclase and cyclic AMP phosphodiesterase on ATP and cyclic AMP respectively.



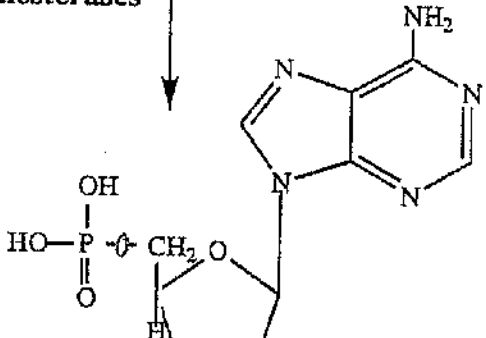
ATP

adenylyl cyclases



Cyclic AMP

cyclic AMP phosphodiesterases



5'AMP

action of insulin (Smith and Manganiello, 1988, Vasta *et al.*, 1992 and Anderson *et al.*, 1989).

In liver, cAMP exerts its influence at two levels. Firstly, it acutely regulates the activity of the key enzymes involved in glycolysis, gluconeogenesis and fatty acid metabolism (Strafors *et al.*, 1984). Secondly, it regulates gene transcription. Homeostatic control of blood glucose levels has been shown to be a cyclic AMP mediated event. Glucagon, the hormone produced by the alpha cells of the pancreas, is involved in the promotion of glycogenolysis, gluconeogenesis and the release of free fatty acids. The receptor for glucagon is coupled to the cyclic AMP synthesising enzyme adenylyl cyclase through the stimulatory G-protein G_S (Houslay, 1991b). In the liver, elevation of the intracellular levels of cyclic AMP, via activation of the glucagon receptor, triggers an enzymatic cascade initiated by PKA mediated phosphorylation (Cohen, 1985). Primarily this is the phosphorylation of glycogen phosphorylase, leading to the breakdown of glycogen and glucose release, and of glycogen synthase which results in the inactivation of the glycolytic enzyme pyruvate kinase (Johnson and Venezialek, 1980). This cyclic AMP signal in response to glucagon in hepatocytes has been shown to be transient (Heyworth *et al* ., 1983). Following the initial increase in cyclic AMP, a rapid desensitisation, which is a cAMP independent process, occurs (Heyworth and Houslay, 1983), in which the glucagon receptor is uncoupled from G_S stimulation. Several reports have also shown that this desensitisation of glucagon stimulated adenylyl cyclase is due to a protein kinase C mediated phosphorylation of the receptor (Murphy *et al.*, 1987, Murphy and Houslay, 1988; Savage *et al.*, 1995).

In liver, insulin opposes the action of glucagon and catecholamines on these processes. By restraining the level of cAMP it opposes PKA-mediated phosphorylation and gene transcription and has cAMP-independent effects on gene expression which favour the anabolic pathways. It enhances pyruvate

kinase levels and restores glucokinase levels which have been lowered by diabetes mellitus (Spence, 1983). In addition to its effects on carbohydrate metabolism, insulin has long been known to oppose the lipolytic action of cAMP both in liver and adipose tissue (Iliano and Cuatrecasas, 1972).

It is remarkable that the elevation of intracellular cAMP levels can also result in either stimulation or repression of specific gene expression (Borrelli *et al.*, 1992; Montminy *et al.*, 1986; Comb *et al.*, 1986) where cAMP abolishes the induction of pyruvate kinase (Munnich *et al.*, 1984) and impairs glucokinase expression (Pilkis, 1970), suggesting that complex, cell-specific molecular mechanisms must operate in the nucleus. cAMP is the major stimulus for the increased synthesis and impaired degradation of mRNA for the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK) (Hod *et al.*, 1986). cAMP-induced gene expression is important in many cellular responses, including the establishment of long-term memory (Borrelli *et al.*, 1992; Montminy *et al.*, 1986). cAMP inducible transcription factors (ICERs) have now also been identified, which are involved in the repression of transcription (Molina *et al.*, 1993). The kinetic expression of these transcription factors is characteristic of an early response gene, such as c-*fos*, c-*jun* and c-*rel* (Verma and Sassone-Corsi, 1987), with induction being rapid and transient. These are the first transcriptional repressor in the cyclic AMP signalling pathway whose functions are regulated by the modulation of its own intracellular levels and not by phosphorylation. It is thought to be important in the phenomenon of down-regulation of gene activity after a first burst of activation by cyclic AMP.

1.1.2 Termination of the cyclic AMP signal by phosphodiesterases (PDEs)

Another vital aspect of the cyclic AMP signalling pathway is its means of degradation. The only way in which the cells of eukaryotic organisms can terminate the cyclic AMP signal is through its hydrolysis catalysed by a

heterologous family of enzymes known as the cyclic nucleotide phosphodiesterases (PDEs) (Beavo *et al.*, 1994; Conti *et al.*, 1991). The degradation of cAMP through the action of PDEs presents a physiological function, the modulation of which may usefully be manipulated in the control of disease states such as depression and inflammation where changes in intracellular cAMP levels/PDE activity are known to occur.

However, some organisms such as Dictyostelium (Pitt *et al.*, 1992) can also extrude cyclic AMP from the cell, and this must occur in certain cells of the body as cAMP occurs in plasma and urine.

1.2 G-protein dependent signal transduction systems

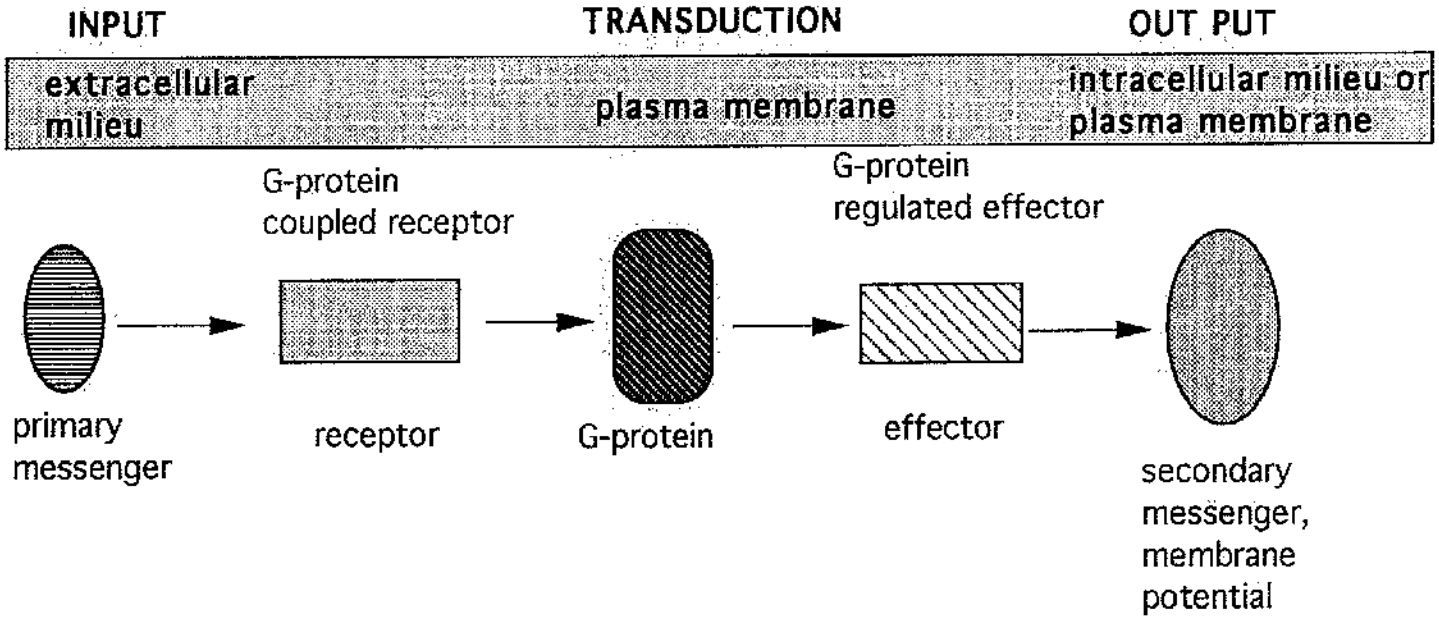
G proteins regulate the activity of effector enzymes in response to the interaction of specific ligands with appropriate receptors. Each G protein contains a guanine nucleotide-binding α subunit and a complex of tightly associated β and γ subunits. Upon activation of a G protein by an agonist-bound receptor, GDP is released from the α subunit in exchange for GTP. This binding of GTP causes conformational changes that result in dissociation of GTP-α from $\beta\gamma$, liberating two species which are capable of the regulation of downstream effectors (**Figure 1.2**).

1.2.1 G-protein subtypes:

Although G proteins consist of three distinct subunits, α, β and γ, they are generally defined by the nature of their α subunits (Gilman, 1987; Kaziro *et al.*, 1991). α is the guanine nucleotide binding component whilst $\beta\gamma$ subunits form a tightly, although non-covalently, associated complex. In the inactive state, heterotrimeric G proteins are bound to the inner leaflet of the plasma membrane via fatty acid modifications such as palmitoylation and myristylation (Spiegel *et al.*, 1991; Thissen and Casey, 1994).

Figure 1.2 Flow of information through G protein-dependent signal transduction systems

Adapted from Birnbaumer *et al.*, 1990


INPUT
TRANSDUCTION
OUT PUT
extracellular milieu
plasma membrane
intracellular milieu or plasma membrane
G-protein coupled receptor
G-protein regulated effector
primary messenger
receptor
G-protein
effector
secondary messenger, membrane potential

The heterotrimeric G-protein α subunits, of Mr between 39,000 and 50,000, bind and hydrolyze GTP and define the receptor and effector specificity of a G protein. To date, about twenty subtypes of α subunits, five β subunits and seven γ subunits have been isolated (Milligan, 1995). They are recognised to play a central role in signal transduction, as they couple a diverse array of receptors to intracellular effectors.

All α subunits contain sets of sequences highly homologous to sequences which in bacterial elongation factor TU are known to be involved in GTP binding and hydrolysis. The α subunits of all G proteins are substrates of one or both of two ADP-ribosyltransferases, the toxin of *Vibrio cholera* (cholera toxin) (Johnson *et al.*, 1978, Northup *et al.*, 1980) which depends on GTP for its activity (Kahn and Gilman, 1986) and the toxin of *Bordetella pertussis* (pertussis toxin) (Hildebrandt *et al.*, 1983; Bokoch *et al.*, 1983; Codina *et al.*, 1983) which depends on ATP for its action (Mattera *et al.*, 1986).

α subunits do not interact with receptors unless they first combine with βγ dimers (Florio and Sternweis, 1989; Navon and Fung, 1987). The overall import of free βγ dimers appears to be to promote interaction of the α subunits with receptors so that receptors may cause their activation by GTP. In addition, by dissociating from the activated α subunit, βγ dimers have the all-important role of facilitating the stabilization of the activated form of the G protein, allowing receptors to act catalytically and suppressing non-specific noise originating from the activity of unoccupied receptors (Milligan, 1996). However, it is becoming clear that βγ components may activate effectors system in their own right. They have been suggested to regulate potassium channels (Logothetis *et al.*, 1987), phospholipase A2 (Bourne, 1989), phospholipase C (Katz *et al.*, 1992), calmodulin (Katada *et al.*, 1987) and adenylyl cyclase (Codina *et al.*, 1988; Goldsmith *et al.*, 1988; Fdedrman *et al.*, 1992). Recently, Thomason *et al.*, (1994) also showed that platelet cytosol contains PI3-kinase activity that is sensitive to βγ subunit complexes. The

demonstration (Goldsmith *et al.*, 1988; Federman *et al.*, 1992) that βγ signalling can be hormone-specific, implies an important role in this field.

1.2.2 G-protein coupled receptors

Characterization of individual G protein-coupled receptor linked signalling pathways is one of the central issues in receptor research. All G protein-coupled receptors consist of a single polypeptide chain with seven putative transmembrane domains. Upon agonist binding to the receptor (**Figure 1.3**), it is thought that the conformation of the receptor changes, allowing the association of the G protein with cytoplasmic regions of the receptor. Receptor activation and subsequent binding of the G protein, promotes the exchange of GTP for GDP. Upon binding GTP, βγ subunits are released as a heterodimer, leaving the α subunit to interact with and modulate the activity of effectors such as adenylyl cyclase (Gilman, 1984). It has become increasingly evident that βγ subunits also have the ability to modulate the activity of certain classes of adenylyl cyclase (see below) (Tang and Gilman, 1991; Tang *et al.*, 1991), PLC (Camps *et al.*, 1992) and ion channels. The α subunit:effector interaction is terminated by an intrinsic GTPase activity which hydrolyses the terminal phosphate of GTP, returning the α subunit in its inactive GDP-bound state. In this state, it re-associates with the βγ complex, forming the heterotrimeric conformation once more, which is required for receptor interaction (**Figure 1.3**).

Given the numerous possible combinations of currently identified α, β and γ subunits, a wide diversity of receptor-G protein signalling pathways could be anticipated. Many receptors apparently interact with only one type of G protein, e.g. either G_s, G_i/G_o, or G_q/G_{11} (Ross and Gilman, 1977; Johnson *et al.*, 1978; Kuhn, 1980), although receptors apparently interacting with more than one G protein have been found. For example, glucagon receptors can mediate both stimulation of adenylyl cyclase, via G_s, and activation of

Figure 1.3 The G-protein Cycle

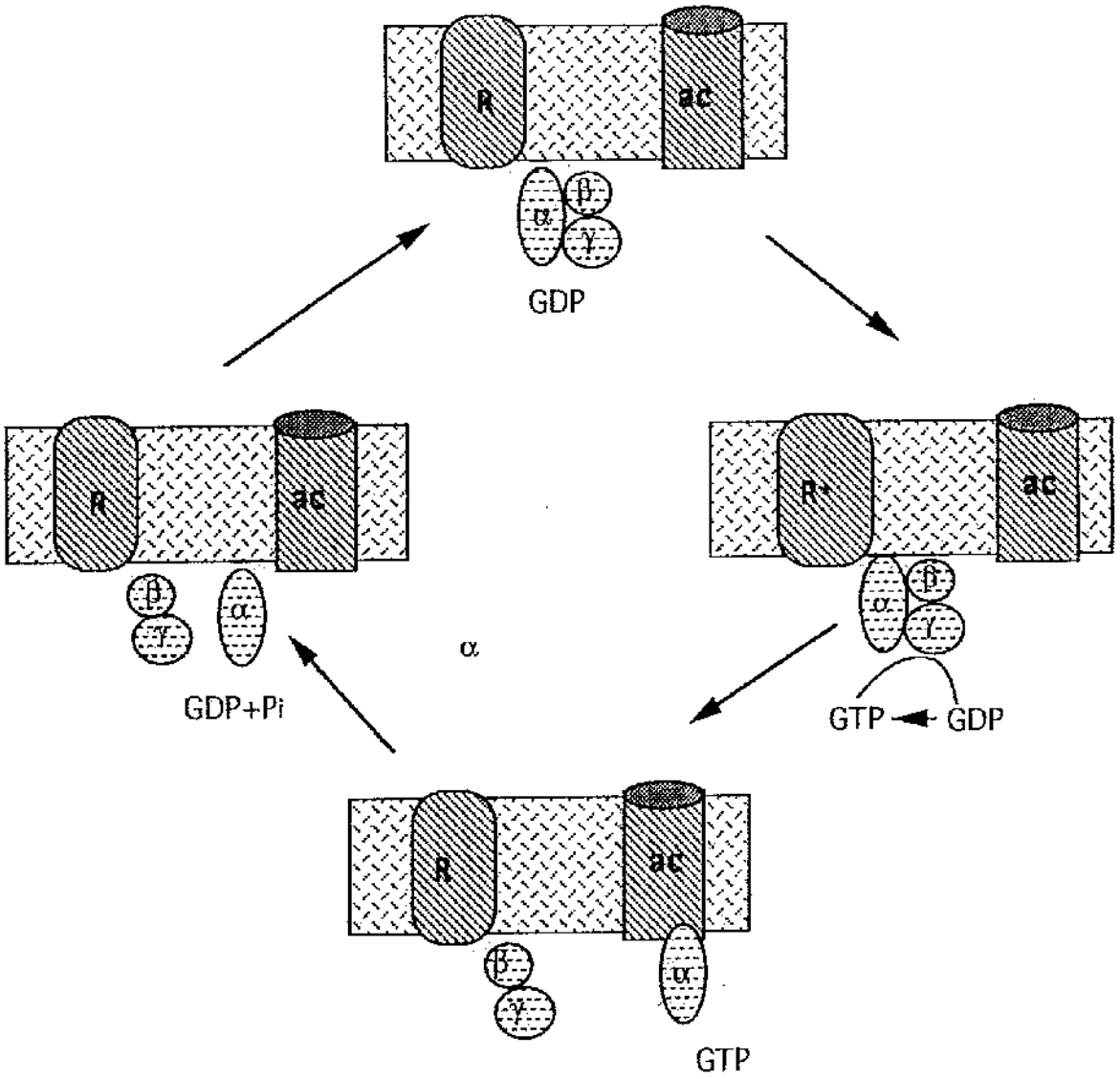
(a) G-protein in the basal state is holomeric and has GDP bound to the α subunit.

(b) Activated receptor (R) catalyses exchange of GDP for GTP; α subunit dissociates from the βγ component.

(c) In this example, GTP-bound α subunit interacts with the effector, although free βγ subunits can also modulate the activity of effector molecules.

(d) Intrinisic GTPase activity of the α subunit hydrolyses GTP and the trimer re-associates.

(Adapted from Spiegel, 1992)


R
ac
α
β
γ
GDP
R*
GTP
GDP
GDP+Pi
α
GTP

phospholipase C, presumably via G_q (Bitensky *et al.*, 1981; Northup *et al.*, 1980); TSH-induced activation of adenylyl cyclase and phospholipase C is mediated by G_s and G_q/G_{11} (Allgeier *et al.*, 1994). Receptors coupled to pertussis toxin-sensitive G_i/G_o proteins can interact with more than one member of this G protein class (Birnbaumer *et al.*, 1990).

1.2.3 Characterisation of G-proteins

G_s is expressed ubiquitously in mammalian cells. It is essential for stimulatory regulation of adenylyl cyclase by hormones. GTP at micromolar levels is required for efficient coupling. $G_{s\alpha}$ is encoded by a single gene, but differential splicing can produce up to four different polypeptides (Bray *et al.*, 1986) which have a tissue specific distribution (Mumbey *et al.*, 1986). One finding that two forms of $G_{s\alpha}$ (43kDa and 45kDa) could reconstitute fluoride and guanine nucleotide regulatory activity in the G_s-lacking mutant S49 cyc- cells with equal efficacy, led Graziano *et al.* (1987) to propose that there is no functional difference between the different forms. However, evidence has also been presented that the shorter form may have a greater ability to activate adenylyl cyclase (Walseth *et al.*, 1989).

Complementary DNA cloning of $G_{i\alpha}$ has revealed three different $G_{i\alpha}$ cDNAs: $G_{i\alpha}1$, $G_{i\alpha}2$ and $G_{i\alpha}3$ (Kaziro *et al.*, 1991). All of which are encoded by distinct genes. All three polypeptides have been purified from tissue sources and their distribution characterised. They show 85% amino acid sequence identity. $G_{i\alpha}2$ (40KDa) and $G_{i\alpha}3$ (41KDa) are ubiquitous but their relative amounts vary between tissues (Milligan, 1990). $G_{i\alpha}2$ is generally thought to be the form responsible for inhibition of adenylyl cyclase (Bushfield *et al.*, 1990b; McKenzie and Milligan, 1990), although there is evidence both for (Pobiner *et al.*, 1991) and against (McClue *et al.*, 1992) $G_{i\alpha}3$ also having such a role. $G_{i\alpha}3$ has been proposed to regulate sodium channels (Cantiello *et al.*, 1990), potassium channels (Mattera *et al.*, 1989) and Golgi trafficking (Stow *et*

al., 1991). $G_{i\alpha}1$ (41kDa) has a more limited tissue distribution, notably present in cerebral cortex, adipocytes and neural tissue-derived cell lines. It may serve to couple opiate receptors to inhibition of adenylyl cyclase (Attali and Vogel, 1989).

G_o, another pertussis toxin substrate, is expressed predominantly in neural tissues where it is thought to regulate calcium channels (McFadzean *et al.*, 1989). The pertussis toxin-insensitive G_q is a member of a group of α subunits thought to couple receptor activation of phospholipid hydrolysis by activating the β-isoform of phospholipase C (Simon *et al.*, 1991).

There is very strong conservation of α subunit structure between species, e.g. only one residue out of 394 differs between rat and human $G_{s\alpha}$. The amino acid sequences of $G_{i\alpha}2$, $G_{i\alpha}3$ and $G_{o\alpha}$ are 98% identical between mammalian species. This demonstrates that there is strong evolutionary pressure to maintain G-protein function.

1.2.4 Regulation of G-proteins

1.2.4.1 G_s, the stimulatory regulatory component of adenylyl cyclase

G_s constitutes a potential mixture of up to 16 proteins which have been unequivocally established as providing the stimulatory regulatory component of adenylyl cyclase. This was demonstrated by showing that it could reconstitute hormonal, NaF and guanine nucleotide regulation of adenylyl cyclase and confer cholera toxin sensitivity to the α_s-deficient adenylyl cyclase system of cyc-S49 cells (Codina *et al.*, 1984; Northup *et al.*, 1980). G_s has also been established as the component responsible for high-affinity binding of hormones to adenylyl cyclase stimulatory receptors as demonstrated by reconstitution of high-affinity binding to β-adrenergic receptors in cyc-membranes and its ability to confer high-affinity binding characteristics to fully purified β-adrenergic receptors after co-incorporation into phospholipid vesicles (Cerione *et al.*,

1984). G_s intriguingly, also seems to stimulate a class of Ca^{2+} channels (Yatani *et al.*, 1987).

1.2.4.2 Pertussis toxin substrates: G_i and G_o

Pertussis toxin has played a key role in defining our understanding of the involvement of G proteins in signal transduction through its ability to block inhibition of adenylyl cyclase systems (Ui, 1984). The first new G protein it helped to define was G_i which, by definition, mediates inhibition of adenylyl cyclase (Hildebrandt *et al.*, 1983). Inhibition of adenylyl cyclase by low concentrations of GTP had been shown as early as 1973 (Birnbaumer, 1973) and has since been studied extensively in the context of the mediation of the action if various inhibitory hormones. In GH4C1 pituitary cells, an excellent model to study the specificity of pertussis toxin-sensitive G proteins in coupling of receptors to effectors, all known three α_i subunits are expressed as well as the two types of α_o. These cells also express a range of effectors. However, Lui *et al.*, (1994) showed the specificity of the α subunits of G_{i2} and G_o in the coupling of specific receptors to inhibition of cAMP synthesis and calcium entry, respectively, in these cells.

1.2.4.3 Hormonal regulation of G-proteins

Down-regulation of plasma membrane receptors on prolonged exposure to agonist is responsible for reducing responses to specific hormones ie. homologous desensitisation. Altered expression of G-proteins may play a role, however, in heterologous desensitisation, i.e. desensitisation to multiple hormonal stimuli (Milligan and Green, 1991). (For more detail about desensitisation see the appendix). In this regard, $G_{s\alpha}$ has been found to be down-regulated by stimulatory signals (McKenzie and Miligan, 1990). Similarly, exposure to inhibitory agonists can cause down regulation of G_i (Green and Johnson, 1989). However both the mechanisms by which receptor sequestration and altered G-protein expression are determined remain unknown.

1.2.4.4 G-protein phosphorylation

Phosphorylation, a major control mechanism in metabolic regulation has been implicated in the regulation of G-protein function (Houslay, 1991a). Phosphorylation of GDP-bound G_i and $G_{o\alpha}$ subunits by the purified insulin receptor tyrosine kinase (O'Brien *et al.*, 1987) and phosphorylation of $G_{i\alpha}2$ (Krupinski *et al.*, 1988) have been recorded, but not the phosphorylation of $G_{s\alpha}$. Insulin, however, does not appear to elicit G_i phosphorylation in intact cells (Pyne *et al.*, 1989a), raising the possibility that the action of the insulin receptor is only an *in vitro* phenomenon. Phosphorylation of $G_{i\alpha}2$ has been detected in response to the phorbol ester TPA and calcium-mobilising hormones, which cause the production of diacylglycerol, suggesting that PKC might elicit such phosphorylation (Bushfield *et al.*, 1991). Indeed, it appears that PKC can phosphorylate the α-subunit of G_i2 on a specific serine residue, leading to changes in GTP-dependent inhibition of adenylyl cyclase (Morris *et al.*, 1994; Murphy *et al.*, 1989; Bushfield *et al.*, 1991).

1.2.4.5 Bacterial toxins

(a) Cholera toxin

Cholera toxin from *Vibrio cholerae* is the causative agent of the *diarrheal* disease cholera, and mediates its effects by increasing cAMP concentrations (Finkelstin, 1973). Although the human small intestinal mucosal cell is the normal target of the toxin, cholera toxin is an ubiquitious activator of adenylyl cyclase in most vertebrate cells (van Heyningen, 1983; Fishman, 1990). The structure of the homologous Escherichia coli heat-labile enterotoxin has been published (Sixma *et al.*, 1991). Cholera toxin is composed of an A subunit and a homopentameric B subunit (Fishman, 1990). Each subunit has a different function, the B subunit recognizes and binds to specific receptors on the cell surface which have been identified as the ganglioside G_{M1} (Fishman, 1990). The A subunit, which consists of two peptides, A_1 and A_2 linked by a

disulphide bond, activates adenylyl cyclase. The A_1 peptide transfers ADP-ribose from NAD^+ to the α subunit of the stimulatory G protein, G_s, obliterating the GTPase activity normally associated with this subunit. In the presence of GTP which causes the dissociation of G_s to release a free $G_{s\alpha}$-subunit, this ADP-ribosylation traps $G_{s\alpha}$ in its active GTP-bound form. This species causes the constitutive activation of adenylyl cyclase (Van Heyningen, 1977; Gilman, 1984; Birnbaumer *et al.*, 1985; Northup, 1985). The site of ADP-ribosylation is an arginine residue present in all variants of $G_{s\alpha}$; residue 201/202 in the long forms and 187/188 in the short forms (Northup, 1985).

The intervening steps between binding and activation are less well understood. There is a characteristic lag period after cholera toxin bind to the cell surface and before an increase in adenylyl cyclase activity is observed in hepatocytes (Houslay and Elliott, 1979 and 1981). At the end of the lag period, small amounts of A_1 peptide begin to be formed and their concentration increases in parallel with the increase in adenylyl cyclase activity (Kassis *et al.*, 1982). The site where A_1 is generated and the mechanism(s) involved are not known. How A_1 reaches $G_{s\alpha}$ which is located on the cytoplasmic face of the plasma membrane is also unclear. The molecular basis of this lag period remains to be defined, although it can be altered by changing temperature, cholera-toxin concentration and membrane fluidity (Houslay and Elliott, 1979). Two major models have been proposed. The first, based on photoabelling studies (Wisnieski and Bramhall, 1981; Tomasi and Montecucco, 1981), suggested that the cholera toxin A subunit may penetrate the membrane bilayer, undergoing reduction and releasing A_1 at the cytoplasmic face of the membrane where it can activate G_s (Fishman, 1990). In the other model, the lag time is in part, connected with a requirement for cholera toxin to undergo endocytosis before it can be processed and recycled back to the plasma membrane in order to act on G_s at the cytosolic surface of the plasma membrane (Houslay and Elliott, 1981; Janicot and Desbuquois, 1987; Janicot *et al.*, 1988 and 1991).

(b) Pertussis toxin

Pertussis toxin from *Bordetella pertussis*, the causative organism of whooping cough, ADP-ribosylates and inactivates the GDP-bound (trimeric) form of $G_{i\alpha}$ preventing its dissociation and abolishing both receptor-mediated and tonic inhibition of adenylyl cyclase. The site of action of the toxin is a cysteine residue located four residues from the C terminus within the receptor binding domain (Morgan, 1989). The α subunits of at least six G-proteins can be modified by pertussis toxin-catalysed ADP-ribosylation (Freissmuth *et al.*, 1989). Pertussis toxin has proven a particularly useful tool in identifying G_i-mediated events and substrates involved in signal transduction. Pertussis toxin treatment of cells, by removing inhibitory G_i input to the catalytic subunit, can enhance responses to stimulatory hormones (Heyworth *et al.*, 1984). Hormonal activity not mediated by G-proteins is not influenced by pertussis toxin treatment.

1.3 Adenylyl cyclase signalling

Adenylyl cyclase, the enzyme which catalyzes the conversion of intracellular ATP to cyclic AMP, is controlled dynamically by a variety of hormones, neurotransmitters and other regulatory molecules. The hormone-sensitive adenylyl cyclase system is comprised of three types of plasma membrane-associated components: heptahelical, G protein-coupled receptors for a variety of hormones, neurotransmitters and autocoids; stimulatory and inhibitory heterotrimeric G proteins and the catalytic entity itself (Taussig and Gilman 1995). So far, ten isoforms of adenylyl cyclase have been identified (Tang and Gilman, 1992; Taussig and Gilman 1995). These can be classified into three groups, with distinct structural and diverse regulatory properties (Krupinski et al., 1992; Kawabe et al., 1994; Yoshimura and Cooper, 1993; Jacobowitz *et al.*, 1993) (**Table 1.1**).

Table 1.1 Properties and regulation of adenylyl cyclase subtypes

Abbreviations used;

PKC, protein kinase C; CaM, calmodulin; AC, adenylyl cyclase.

References;

1. Jacobowitz *et al.*, 1993
2. Yoshimura and Copper, 1993
3. Tang and Gilman, 1991
4. Gao and Gilman, 1991
5. Pieroni *et al.*, 1993
6. Chen and Iyengar, 1993
7. Kawabe *et al.*, 1994
8. Ishikawa *et al.*, 1992
9. Krupinski *et al.*, 1992

Type of AC	Expression	Effect of G-protein		Ca^{2+}/CaM effect	PKC effect
		Gsα	βγ		
I	Brain[1]	+	-[3]	+	-[2]
II	Brain, lung	+	+[3]	0	+[6]
III	Olfactory	+	0	+	+ by α and ξ[1]
IV	Brain, others	+	+[4,5]	0	+[2]
V	Heart, others	+	0	-	+ by α and δ[7]
VI	Heart	+	0	-[8]	n.d.
VII	Kidney	+	+[9]	-	n.d.
VIII	Nervous system	+	n.d.	+	0

N.B. + stands for stimulated; - stands for inhibited; 0 stands for no effect; n.d. stands for not determined.

1.3.1 Divergent structure

1.3.1.1 Structure

Adenylyl cyclases have molecular weights of around 120kDa and are associated with the plasma membrane (Tang and Gilman, 1992) (**Figure 1.4**). A short cytoplasmic amino terminus is followed by six transmembrane spans (M1), a large (40kDa) cytoplasmic domain (C1), a second set of six transmembrane spans (M2) and a second cytoplasmic domain (C2) (Tang and Gilman, 1992). It is members of this complex and widely distributed group of adenylyl cyclases that are subject to stimulatory and inhibitory regulation by hormones and neurotransmitters, acting via the intermediacy of both receptors and heterotrimeric guanine nuceotide-binding regulator proteins (G proteins) (Gilman, 1987).

Among the different isoforms of adenylyl cyclase, the amino acid sequence is 50% homologous (Tang and Gilman, 1992). However, the two C1 and C2 domains (C1a and C1b) are more highly conserved (up to 93% sequence identity) and this relationship extends to corresponding domains of topographically similar adenylyl cyclases from both *drosophila* (Levin *et al.*, 1992) and *Dictyostelium* (Pitt *et al.*, 1992). The C1a and C2a domains are highly homologous to each other as well as to the catalytic domains of membrane-bound guanylyl cyclases and domains that are found in each of the subunits of cytosolic heterodimeric guanylyl cyclases. Based on these relationships, it is predicted that one or both of these domains of mammalian adenylyl cyclases is the site for catalysis of cyclic AMP synthesis (Taussig and Gilman, 1995).

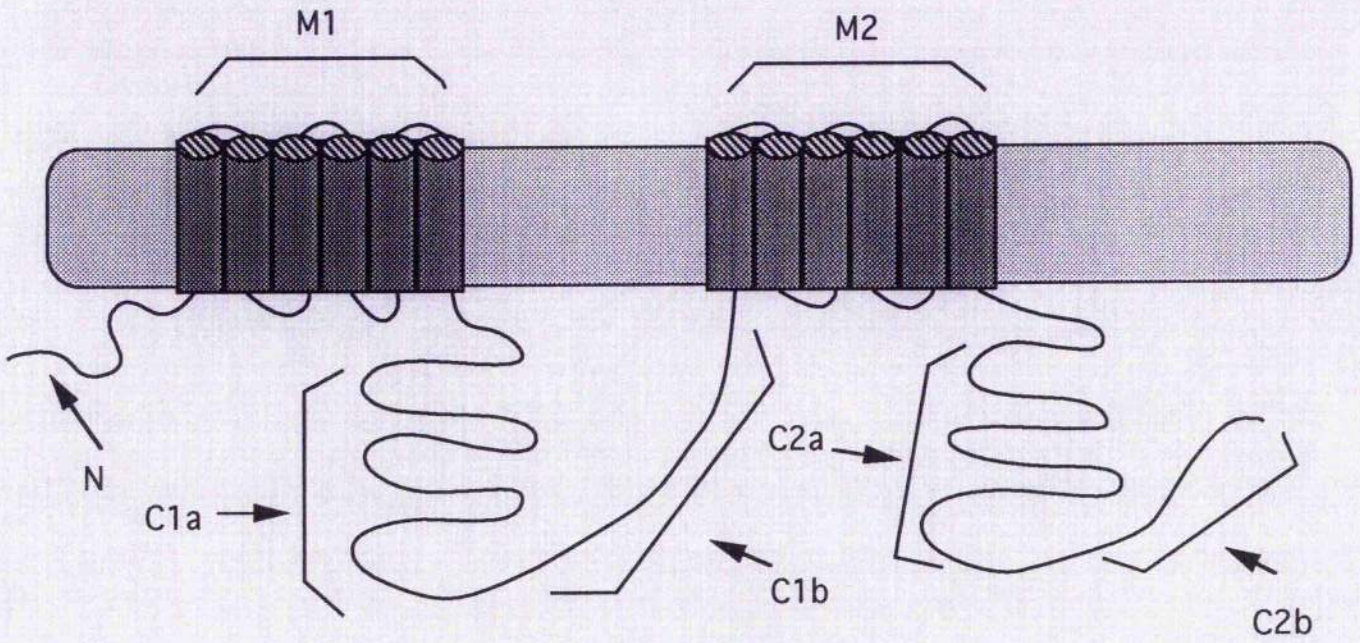
1.3.1.2 Structural correlation with activity

Expression of constructs encoding M1C1 and M2C2 in Sf9 cells, showed the enzymes to be regulated in characteristic, type-specific fashions by G protein subunits or calmodulin (Tang *et al.*, 1991). It is thus assumed that

Figure 1.4 Structure of adenylyl cyclase

The predicted topology of membrane-bound adenylyl cyclase is shown. Cylinders represent membrane-spanning regions; N, amino-terminal domain; M1, first set of membrane spanning regions; C1a and C1b, the first large intracellular cytoplasmic domain; M2, second set of transmembrane spanning regions; and C2a and C2b, second large intracellular domain.

Adapted from Taussig and Gilman, 1995.


M1
M2
N
C1a
C2a
C1b
C2b

the interaction between the C1 and C2 domains is essential for catalysis. It is also notable that point mutations, in either the C1a and C2a domains of the adenylyl cyclases, can impair enzymatic activity severely and that mutations in either domain can elevate the K_m for substrate. It has thus been speculated that both domains can bind ATP (Tang *et al.*, 1991).

The duplicated motif prompted the motion that adenylyl cyclase is composed of two "half-sites" that interact to provide catalytic activity (Taussig and Gilman, 1995). Expression of a "front half" construct of type I adenylyl cyclase, truncated to remove domain C1b, with the back half of type II adenylyl cyclase, which largely lacks C2b permits assembly of a functional adenylyl cyclase that responds very well to both forskolin and activated $G_{s\alpha}$. The variable C1b and C2b domains are thus not necessary for responses to these regulators. $G_{s\alpha}$ interacts with C1a/C2a and perhaps regulates interactions between these domains. C1b domain is likely to be a site of interaction of calmodulin with type I adenylyl cyclase (Vorherr *et al.*, 1993).

1.3.2 Molecular diversity in the adenylyl cyclase family

Molecular cloning studies have indicated that the diversity within the adenylyl cyclase family is greater than initially anticipated. Analysis of sequence relationships among the adenylyl cyclases permits some grouping (Krupinski *et al.*, 1992). Type II, IV, VII and IX are clearly more related to each other than to the others ; a similar relationship exists between type V and type VI (**Table 1.1**). The regulatory properties of these isoforms appear to reflect their evolutionary relationships (Taussig and Gilman, 1995). Types I, III, VIII are equally dissimilar from the others (Cali *et al.*, 1994; Tang and Gilman, 1992; Iyengar, 1993). Type V and VI possess long N-terminal domains (149-163 amino acids) compared to other types (about 70 amino acids). This disparity in N-terminal lengths between different cyclase types has prompted exploration of N-terminal function. Truncation of the first 52 amino acid residues of type I

adenylyl cyclase was shown to decrease activity to 10% of that seen in wild-type adenylyl cyclase (Tang *et al.*, 1991), suggesting an important role in secondary structure or membrane orientation.

Application of low stringency hybridization and polymerase chain reaction techniques has now permitted isolation of six additional full-length clones (types II-VI and VIII) (Krupinski *et al.*, 1989; Bakalyar and Reed, 1990; Ishikawa *et al.*, 1992). The dendrogram of the alignment gives a qualitative indication of the amino acid similarity among the various products (**Figure 1. 5**). Relationships among these 57 amino acid fragments are qualitatively similar to what is observed if the complete protein sequences of types I-VI are aligned. The dendrogram indicates the existence of at least five different subfamilies amongst the adenylyl cyclases (**Figure 1.5**). Types I, III and VIII define their own branches of the family, while types V and VI (only one amino acid difference in this region, but only 83% nucleotide identity) are the most closely related of any two sequences (Krupinski *et al.*, 1992). The type VII peptide is 84% identical with that of type II, and together with type IV enzymes, these three proteins probably form a distinct subfamily. Once the type VII cDNA has been expressed, it will be of interest to note if it also can be activated synergistically by G protein βγ subunits in the presence of an active $G_{s\alpha}$, a distinguishing property of the type II and IV isoforms (Feinstein *et al.*, 1991; Gao and Gilman, 1991; Tang and Gilman, 1991).

However, definition of the extent of molecular diversity in this family is probably not yet complete with alternatively spliced transcripts of adenylyl cyclases having also been suggested (Wallach *et al.*, 1994).

1.3.3 Tissue distribution of adenylyl cyclases

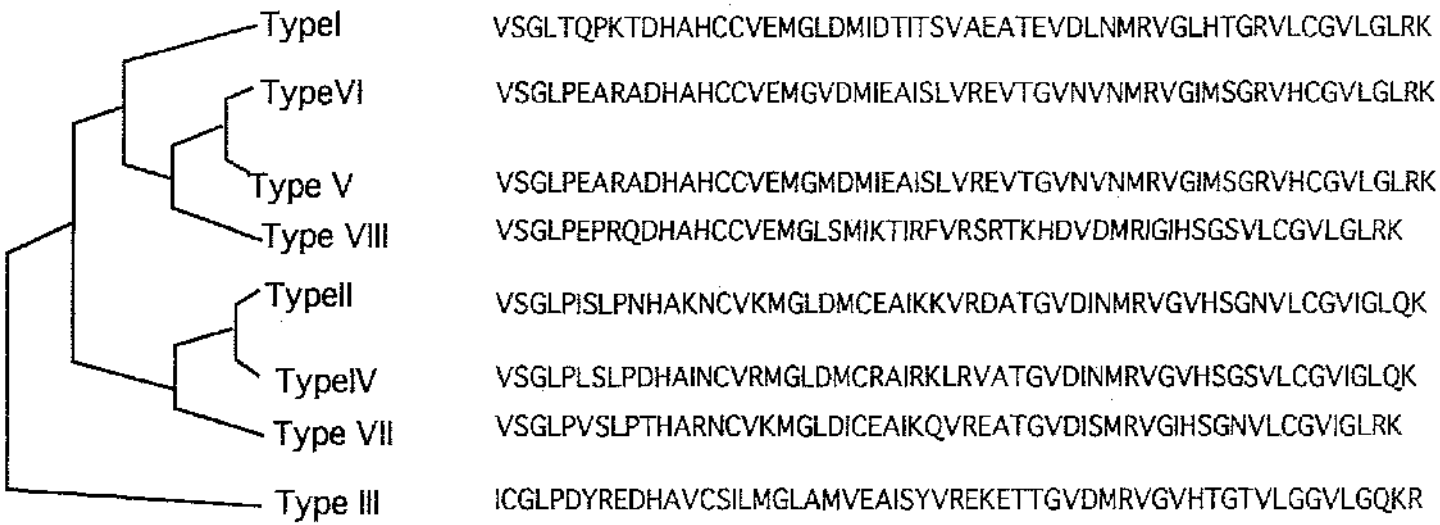
Documentation of the cellular and subcellular localization of the different isoforms of adenylyl cyclase in mammalian tissues has been hindered by the low levels of expression (generally 0.01-0.001% of membrane protein) and

Figure 1.5 Comparison of the amino acid sequences from a region conserved among members of the adenylyl cyclase family.

All of the amino acid sequences presented were obtained by translating nucleotide sequences derived from rat cDNA templates. The alignment of the rat sequences and the corresponding dendrogram of the alignment is from the program Clustal in the Intelligenetics PC/Gene sequence analysis package.

The dendrogram indicates the existence of at least five different subfamilies among the adenylyl cyclases. Types I,III and VIII define their own branches of the family, while types V and VI (only one amino acid difference in this region, but only 83% nucleotide identity) are most closely related of any two sequences. The type VII peptide is 84% identical with that of type II and together with type IV these three proteins probably form a distinct subfamily.

Adapted from Krupinski *et al.*, 1992.


TypeI
TypeVI
Type V
Type VIII
TypeII
TypeIV
Type VII
Type III
VSGLTQPKTDHAHCCVEMGLDMIDTITSVAEATEVDLNMRVGLHTGRVLCGVLGLRK
VSGLPEARADHAHCCVEMGVDMIEAISLVREVTGVNVNMRVGIMSGRVHCGVLGLRK
VSGLPEARADHAHCCVEMGMDMIEAISLVREVTGVNVNMRVGIMSGRVHCGVLGLRK
VSGLPEPRQDHAHCCVEMGLSMIKTIRFVRSRTKHDVDMRIGIHSGSVLCGVLGLRK
VSGLPISLPNHAKNCVKMGLDMCEAIKKVRDATGVDINMRVGVHSGNVLCGVIGLQK
VSGLPLSLPDHAINCVRMGLDMCRAIRKLRVATGVDINMRVGVHSGSVLCGVIGLQK
VSGLPVSLPTHARNCVKMGLDICEAIKQVREATGVDISMRVGIHSGNVLCGVIGLRK
ICGLPDYREDHAVCSILMGLAMVEAISYVREKETTGVDMRVGVHTGTVLGGVLGQKR

limited availability of high affinity, isoform-specific antibodies (Cooper *et al.*, 1994). Most information thus comes from analysis of patterns of mRNA expression (Krupinski *et al.*, 1992). On such a basis, all isoforms of adenylyl cyclase appear to be expressed in the brain, apparently in region-specific patterns (**Table 1.1**).

The type I enzyme is largely restricted to the nervous system and regulated by Ca^{2+}/camodulin (Xia *et al.*, 1993); the type II enzyme appears to be restricted to the hippocampus and cerebellum (Furuyama *et al*,. 1993); the type III is found predominantly in olfactory neuroepithelium (Bakalyar and Reed, 1990), whereas the types IV, V, VI and VII are differentially expressed primarily in peripheral tissues, such as heart, kidney, liver, lung and skeletal muscle (Gao and Gilman, 1991; Krupinski *et al.*, 1992). Evidence indicates that type VIII adenylyl cyclase is specifically expressed in the brain but not in other tissues such as heart or liver (Cali *et al.*, 1994). Northern analysis of brain regions indicates that human type IX is also restricted to the CNS, being most abundant in the caudate, cerebellum and hippocampus (Helluvo *et al.*, 1993).

1.3.4 Diverse regulatory properties

Studies on the regulation of the isoforms of mammalian adenylyl cyclases reveal a wealth of common and disparate features (Kawabe *et al.*, 1994; Jacobowitz *et al.*, 1993; Yoshimura and Cooper, 1993). All isoforms are activated by both forskolin and the GTP-bound α subunit of the stimulatory G protein G_s. All are inhibited by certain adenosine analogues termed P-site inhibitors. However, all of the isoforms of adenylyl cyclase are further regulated in type-specific patterns by other inputs, partularly including those that are dependent on Ca^{2+} or that arise from other G protein subunits (**Table 1.1**).

1.3.4.1 Regulation by G-protein subunits

G-proteins consist of α, β and γ subunits. Activation of heterotrimeric G proteins results in the dissociation of two regulatory moieties, the GTP-bound α subunit and a dimer of the β and γ subunits. The adenylyl cyclases from murine S49 cells and platelets, that were originally characterized with regard to the effects of G proteins, are activated by $G_{s\alpha}$ and are largely insensitive to the G protein βγ subunit complex. Regulation of adenylyl cyclases by $G_{s\alpha}$ has been appreciated for some time and was the basis for discovery of this G protein (Cooper *et al.*, 1994). However, knowledge of direct interactions of other G protein subunits with specific isoforms of adenylyl cyclase is more recent.

(a) $G_{s\alpha}$:

All of the cloned adenylyl cyclase species appear to be equally well-regulated by $G_{s\alpha}$, either when reconstituted with activated $G_{s\alpha}$ or when expressed in Sf9 cells and stimulated with GTP-γ-S (Tang, 1992; Iyengar, 1993). However, in intact HEK 293 cells, the Ca^{2+}-actived types I and VIII were not readily stimulated by PGE1 and the endogenous G_s (Yoshimura and Cooper, 1992; Cali *et al.*, 1994). Under similar circumstances, however, other adenylyl cyclases such as the II, V or VI forms were well stimulated. This may have resulted from high endogenous levels of βγ subunits of G-proteins, exerting inhibitory effects on type I and VIII.

(β) βγ:

Smigel (1986) and Katada *et al.*, (1987) first noted inhibition of type I adenylyl cyclase activity by the G protein βγ subunit complex, but in neither case was it clear that the effect was exerted directly. Only recently was it noted that prominent inhibition by βγ of type I adenylyl cyclase expressed in Sf9 cells occurred with the purification of the expressed protein permitting the

demonstration of the direct interaction of adenylyl cyclase with this subunit complex (Taussig *et al.,* 1993). Notably, however, when the effects of βγ on other isoforms were tested, surprising stimulatory effects were observed with type II (Tang and Gilman, 1991) and type IV adenylyl cyclase (Gao and Gilman, 1991). More interestingly, stimulation of these enzymes by βγ is highly conditional in that whilst it was detectable, the magnitude of stimulation by βγ was enhanced in the presence of $G_{s\alpha}$. Stimulation of type II adenylyl cyclase by βγ required significantly higher concentration of βγ than of $G_{s\alpha}$, and the source of the βγ was presumed to be contributed by G proteins such as G_i or G_o oligomers, which are far more abundant than G_s, at least in brain (Taussig and Gilman, 1995). This provides a clear mechanism for cross-talk between signalling pathways.

(c) G_i:

G_i proteins ($G_{i\alpha}1$, $G_{i\alpha}2$ and $G_{i\alpha}3$) were discovered as substrates for pertussis toxin and as the G protein oligomers responsible for inhibitory regulation of adenylyl cyclase activity. The three $G_{i\alpha}$ proteins can inhibit type I adenylyl cyclase (Taussig *et al.,* 1993) but the effect is not as prominent as that observed with βγ. Furthermore, inhibition of type I adenylyl cyclase activity by G_i is largely absent when the $G_{s\alpha}$-stimulated activity was examined (Taussig and Gilman, 1995). Indeed inhibition was largely confined to an action observed in the presence of calmodulin or forskolin. Type I adenylyl cyclase can also seemingly be inhibited by $G_{o\alpha}$ (Cooper *et al.,* 1994). Types II, III and VI were all well inhibited by $G_{i\alpha}2$ (Chen and Iyengar, 1993) when expressed in HEK 293 cells. However, the inhibition of type II could be offset by pretreating transfected cells with phorbol ester (Jacobowitz *et al.,* 1993). Thus, there appears to be a type-specific potential for modulation of $G_{i\alpha}$-mediated inhibition. This finding may also have some bearing on the ability of protein kinase C to stimulate selectively type II adenylyl cyclase (Yoshimura and

Cooper, 1993); i.e. whether PKC directly effects type II adenylyl cyclase or indirectly modifies $G_{i\alpha}$ interactions with adenylyl cyclase, remains to be determined (Kawabe *et al.*, 1994). Type V and VI adenylyl cyclases expressed in Sf9 were inhibited by $G_{i\alpha}$ subunits, with no apparent difference in potency between $G_{i\alpha}1$, $G_{i\alpha}2$ and $G_{i\alpha}3$.

1.3.4.2 Regulation by Ca^{2+}:

Changes in intracellular Ca^{2+} can have profound effects on cellular concentrations of cyclic AMP if appropriate isoforms of adenylyl cyclase are present (Cooper, 1991).

Types I and VIII adenylyl cyclase, and type III to a lesser extent, were markedly stimulated by nanomolar concentrations of Ca^{2+} (Cooper *et al.*, 1994). In the presence of calmodulin, intracellular cyclic AMP concentrations rose dramatically when transfected cells expressing these isoforms were exposed to agonists that elevate intracellular Ca^{2+} (Caldwell *et al.*, 1992). Progress has been made in determining the sequences responsible for Ca^{2+} regulation, via calmodulin, of type I adenylyl cyclase. Vorherr *et al.*, (1993) identified two potential calmodulin binding domains in the sequence of bovine type I adenylyl cyclase. A peptide corresponding to one of these domains (amino acids 495-522), which is equivalent to the cytosolic region just prior to the seventh transmembrane-spanning domain, could totally preclude the activation by Ca^{2+}/calmodulin of bovine brain adenylyl cyclase. Further support that the amino acid sequence 495-522 in type I adenylyl cyclase is a calmodulin binding site came from site-directed mutagenesis (Bakalyar and Reed, 1990). Unlike type I adenylyl cyclase, stimulation of type III adenylyl cyclase by Ca^{2+}/calmodulin required concomitant stimulation by other factors, such as forskolin or $G_{pp}[NH]_p$ (Choi *et al.*, 1992). This might suggest that the calmodulin binding site is inaccessible in the basal state and only becomes accessible upon activation. In addition, the type III adenylyl cyclase is also 100

times less sensitive to Ca^{2+} than the type I enzyme (Choi *et al.*, 1992) indicating structural differences. Of the remaining adenylyl cyclase subtypes, both forms V and VI are inhibited by Ca^{2+}, independent of the addition of exogenous calmodulin, and three forms, II, IV and IX are insensitive to Ca^{2+} as determined in various types of expression assays (Cooper *et al.*, 1994).

1.3.4.3 Regulation by phosphorylation

The intracellular cyclic AMP concentrations achieved in response to exogenous regulators are highly dependent on the state of phosphorylation of components of hormone-sensitive adenylyl cyclase systems. This is particularly obvious in the case of the receptors for stimulatory and inhibitory ligands, which are desensitized and down-regulated following phosphorylation by various kinases such as cyclic AMP dependent protein kinase, protein kinase C and a variety of receptor-specific kinases that view the agonist-bound receptors as preferential substrates (Dohlman *et al.*, 1991). Only a few reports of phosphorylation of G protein subunits exist like $G_{i\alpha}2$ (Bushfield *et al.*, 1990, Murphy *et al.*, 1989; Morris *et al.*, 1994), however more studies are available to investigate phosphorylation of adenylyl cyclase itself.

Feedback inhibition of adenylyl cyclase in response to phosphorylation by cyclic AMP-dependent protein kinase was detected in chick hepatocytes and the S49 lymphoma cell line (Premont *et al.*, 1992). These cells share the type VI enzyme that contains two consensus sites for phosphorylation by cyclic AMP-dependent protein kinase, one on which is also conserved in the closely related type V isoform (Premont *et al.*, 1992).

Recent exploration of the regulation of adenylyl cyclase by protein kinase C was extensively prompted by the use of the PKC activator-phorbol esters. Reports indicated that the activity of type II adenylyl cyclase, expressed by transfection, could be augmented substantially by stimulation of protein kinase C (Jacobowitz *et al.*, 1993; Yoshimura and Cooper, 1993; Lustig *et al.*,

1993). Kawabe and co-workers (1994) have demonstrated the phosphorylation of type V adenylyl cyclase in vitro by PKC and this effect was specific for the α and δ isoforms of PKC, suggesting cross-talk between this adenylyl cyclase and both G_q-mediated phosphalipid pathways (for PKC-α) and growth factor tyrosine kinase-mediated pathways (for PKC-δ).

The actual site of phosphorylation has proved difficult to determine because of the numerous potential PKC phosphorylation sites in many of the adenylyl cyclases. Nevertheless, type II adenylyl cyclase contains a unique, putative PKC phosphorylation site at serine 871, which is located in the second cytoplasmic loop (Yoshimura and Cooper, 1993).

There is less consensus with regard to effects of protein kinase C on type I adenylyl cyclase, this Ca^{2+}/calmodulin stimulated enzyme would seem a logical candidate for feedback regulation by a Ca^{2+}-activated kinase (Cooper *et al.*, 1994).

1.3.5 Forskolin: A potent activator of adenylyl cyclase

The diterpene forskolin (Lindner, et al., 1978) from the roots of Coleus forskolhlii (Bhat *et al.*, 1977) is a unique and potent activator of adenylyl cyclase. Its ability to reversibly activate cAMP-generating systems in intact cells clearly distinguishes it from other stimulating agents such as (a) cholera toxin, which activates cAMP-generating systems in intact cells in an irreversible fashion by covalently modifying the guanyl nucleotide-binding subunit and (b) fluoride, which is ineffective in activating the enzyme in intact cells as like guanyl nucleotides and divalent cations it requires access to intracellular sites. Thus, forskolin represents an invaluable agent for the general activation of adenylyl cyclase in intact cells and, hence, for the investigation of the relationship of cAMP levels to physiological functions in a variety of systems. The stimulation of adenylyl cyclase is rapid and potent, with 10μM forskolin generally giving half maximal activation. Maximal stimulation by 100μM

forskolin is generally greater than that achieved with fluoride or non-hydroysable GTP analogues and there is little variation in the kinetics of its action between cell types. Forskolin stimulated intracellular cAMP in the intact cells usually reaches a maximum level after 5-10 minutes. Thereafter, any fall in cAMP level can usually be attributed to cAMP phosphodiesterase activity or cAMP extrusion, as homologous desensitisation to forskolin has not been observed.

Forskolin can act synergistically with both weak and strong agonists in elevating cAMP (Seamon and Daly, 1983) and can reveal responses to hormones which are too low to observe in the absence of forskolin (Darfler *et al.*, 1982). This synergistic action is more easily observed in intact cells than in plasma membranes. These observations led to the suggestion that forskolin has two sites of action (Barovsky *et al.*, 1984) *viz.* A low affinity site for its action on the catalytic subunit of adenylyl cyclase and a high affinity site responsible for its potentiation of hormone action. However the high affinity response requires functional G_s (Darfler *et al.*, 1982), suggesting that forskolin potentiates other hormones by an action on G_s. Brooker *et al.*, (1983) suggested that forskolin, in exerting its low affinity effect on the catalytic subunit, may act on a distinct protein susceptible to inhibition of protein synthesis.

The majority of forskolin's pharmacological actions can be attributed to cAMP elevation and protein kinase A activation (Seamon and Daly, 1986) and indeed, all, adenylyl cyclase isoforms discovered to date can be activated by this diterpene (Seamon and Daly, 1983). However, a number of cAMP-independent actions of forskolin have also been reported, for example an inhibitory effect on glucose transport (Klip *et al.*, 1988).

1.3.6 Adenylyl cyclase signalling in P9 cells

1.3.6.1 SV40-immortalised hepatocytes

Immortalisation means acquisition of an unlimited proliferative potential. The SV40 virus can be used to immortalise and transform various cell types by transfection, using genetic material prepared from virions. This leads to the establishment of a stable cell line (Chou, 1989). Woodworth *et al.*, (1986) generated such a cell line by transfection of rat hepatocytes with SV40. The large T antigen induces changes in the host cell, bringing about immortalisation and eventually transforming them to tumorigenicity. A high concentration of T antigen is thought to be required for immortalisation and expression above a certain level is necessary for tumorigenicity (Efrat and Hanahan, 1989). These transfected rat hepatocyte cells did not initially show a fully transformed morphology, but developed this after a time in culture. Such cell lines, at least at low passage, have been suggested as beings more useful than hepatoma cells as a model system for the study of drug metabolism (Nairn *et al.*, 1990). Indeed, for up to 22 passages after transfection, gene products were expressed in SV40-immortalised cells at similar levels to those in native hepatocytes (Nairn *et al.*, 1990). Chromosomal damage and cytogenetic abnormalities observed in SV40-immortalized cells, as compared to native rat hepatocytes, are lower than in other established cell lines (MacDonald *et al*,. 1991). This closer genotypic relation to native cells has caused workers to consider the value of SV40-immortalised cells as model systems. Interestingly, the immortalised cell line was found to retain the pathway for glutathione synthesis and possessed glutathione S-transferase activity comparable to rat hepatocytes (Nairn *et al.*, 1990). As cell lines differ phenotypically from their native cell types, some have been subjected to agents and conditions which promote differentiation in order to produce cells with more native characteristics.

1.3.6.2 Analysis of the signalling system in an immortalized hepatocyte cell line

P9 cells transformed by SV40 retain a variety of differentiated enzyme activities characteristic of hepatocytes, namely glucose-6-phosphatase, glycogen phosphorylase & bilirubin in glucuronyltransferase. Both glucagon and prostaglandin E1 (PGE1)-stimulated adenylyl cyclase activities are also retained, albeit at decreased levels compared with native hepatocytes (Livingston *et al.*, 1995). The loss of responsiveness appears to be due to the low level of glucagon receptors in the plasma membrane. Meanwhile, the levels of the G-protein subunits $G_{i\alpha 2}$, $G_{i\alpha 3}$, G_β and the "long" form of $G_{s\alpha 2}$ (45kDa) were shown to be 4-fold higher relative to native hepatocytes, whereas those of the "short" form of $G_{s\alpha 2}$ (42kDa) were lower by 40%. Associated with this were marked alterations in the guanine nucleotide regulation of adenylyl cyclase. Receptor-mediated stimulation, achieved by either PGE1 or glucagon, was apparent in P9 cells, although the latter was only evident upon amplification with forskolin. Glucagon-stimulated cyclic AMP accumulation in P9 cells did not exhibit desensitisation in contrast to hepatocytes, nor was the phosphorylation of $G_{i\alpha 2}$ evident. This cell line was used to show that signalling through high-affinity insulin receptors can attenuate the G_s-coupled-receptor stimulation of adenylyl cyclase activity and increase the expression of the inhibitory G-protein $G_{i\alpha 2}$ and $G_{i\alpha 3}$

P9 cells thus appear to provide the first example of an immortalized hepatocyte cell line which exhibits a G-protein-regulated adenylyl cyclase activity and a functional, high-affinity insulin-signalling system (Livingston *et al.*, 1995). Culture of P9 cells with low, physiological, concentrations of insulin increases the expression of both G_i and G_i, suggesting that the expression of these two inhibitory G-proteins may be directly regulated by insulin action in both hepatocytes and P9 cells (Livingston *et al.*, 1995). Adenylyl cyclase signalling in the P9 cell line, unlike marker protein expression, is considerably divergent from that of native rat hepatocytes.

However, the cell line may still be useful at low passage for studying aspects of cyclase signalling, insulin action and the activities of other second messenger systems.

Indirect evidence has been provided for differences in cAMP phosphodiesterase activity between P9 cells and their native rat hepatocyte counterparts. The nature of the cAMP degradation pathway depends on the cell phenotype and evidence has previously been presented that the complexity of this process may be greater in differentiated cells (Conti *et al.*, 1991). As P9 cells are rapidly dividing and cAMP is inextricably involved in growth control (Roesler *et al.*, 1988), there is good reason to expect differences in its regulation between the two cell types. The phosphodiesterases expressed in P9 cells would merit formal investigation.

1.4 Cyclic AMP-specific Nucleotide Phosphodiesterases

The enzyme activity that catalyzes the hydrolysis and inactivation of cAMP and cGMP second messengers in cells is contributed by a family of cyclic nucleotide phosphodiesterases (PDEs). These elicit the hydrolysis of the 3' phosphate ester bond to give the corresponding 5' nucleoside monophosphate, thus rendering the signal inactive (Butcher and Sutherland, 1962). These isozymes are differentially expressed in individual cell types and are differentially regulated. Currently more than 30 different isozymes have been identified due to the presence of both multiple genes together with alternative splicing (Beavo *et al.*, 1994; Bolger, 1994; Conti *et al.*, 1995b; Houslay and Kilgour, 1990; Reeves and England, 1990).

1.4.1 Mutiple families of PDEs

Cyclic nucleotide hydrolysing PDEs exhibit distinct biochemical characteristics and can be divided into at least seven functional classes (Beavo *et al.*, 1994; Conti *et al.*, 1991) **(Table 1.2).**

Table 1.2 Summary of cyclic nucleotide phosphodiesterase isoforms.

Isoenzyme Type	Effectors	Substrate Specificity	Selective Inhibitor
PDE1 formerly Type-I, Ca^{2+}/CaM- stimulated PDE	Stimulated by calcium/calmodulin	Differing K_m for cAMP and cGMP depends on the tissue	vinpocetine
PDE2 formerly Type-II, cGMP-stimulated PDE	cAMP activity is stimulated by low (μM) [cGMP]	High K_m for both cAMP and cGMP	EHNA
PDE3 formerly Type-III, cGMP-inhibitied PDE	cAMP activity is inhibited by low (μM) [cGMP]	Low K_m for both cAMP and cGMP	milrinone cilostamide amrinone
PDE4 formerly Type-IV, cAMP- specific PDE	Specific for cAMP	Low K_m for cAMP only	rolipram Ro20-1724
PDE5 formerly Type-V, cGMP-binding cGMP-specific PDE	Specific for cGMP located in the periphery eg. heart or lung	Isoforms with high and low K_m for cGMP only	zaprinast dipyridamole
PDE6 formerly Type-V, Photoreceptor PDE	Specific for cGMP Located in rods and cones of visual system	Micromolar K_m for cGMP	dipyridamole
PDE7 formerly Type-VII, cAMP-specific rolipram-insensitive PDE	Specific for cAMP	Very low K_m for cAMP only	None reported Insensitive to rolipram and IBMX

(i) PDE1, which can hydrolyse both cyclic GMP and cyclic AMP and whose activity is stimulated by calcium/calmodulin (Ca^{2+}/CaM); (ii) PDE2 hydrolyses cyclic AMP and cyclic GMP, with the activity being stimulated by micromolar concentrations of cyclic GMP; (iii) PDE3 specifically hydrolyses cyclic AMP in a manner which is inhibited by micromolar concentrations of cyclic GMP; (iv) PDE4 enzymes are cyclic AMP specific PDEs which are insensitive to cyclic GMP, and whose activity is attenuated by the selective inhibitor rolipram; (v and vi) PDE5 and PDE6 enzymes specifically hydrolyse cyclic GMP, but differ in their structure and tissue distribution; (vii) PDE7 specifically hydrolyses cyclic AMP and is insensitive not only to cyclic GMP but also to the non-selective PDE inhibitor isobutylmethylxanthine (IBMX) and the PDE4 selective inhibitor rolipram.

The PDE enzyme family shows a diverse range of properties that are exemplified by their differential sensitivity such as responsiveness to phosphorylation i.e. can regulate PDEs by various kinases (Beltman *et al.*, 1993; Degerman *et al.*, 1990; Houslay and Kilgour, 1990; Kilgour *et al.*, 1989; Manganiello *et al.*, 1990a; Pyne *et al.*, 1989; Swinnen *et al.*, 1989b); their inhibitor specificities and cofactor requirements (Degerman *et al.*, 1990; Hall and Hill, 1992; Houslay and Kilgour, 1990; Moore *et al.*, 1991; Reeves and England, 1990; Swinnen *et al.*, 1989b); their hormonal regulation (Conti *et al.*, 1995b; Sette *et al.*, 1994b; Swinnen *et al.*, 1991); their differential expression patterns (Engels *et al.*, 1995; Engels *et al.*, 1994; Lobban *et al.*, 1994) and their intracellular localisation (Lobban *et al.*, 1994; Shakur *et al.*, 1993). Members of this diverse family thus have the potential to modulate cyclic AMP metabolism to the specific requirements of the cell, including being able to integrate regulatory signals from phospholipid (DiSanto and Heaslip, 1995), tyrosyl (O'Connell *et al.*, 1996) and nitric oxide (Genaro *et al.*, 1995) signalling pathways.

1.4.2 Structure of cyclic nucleotide PDEs

It has become apparent that PDEs are multidomain proteins, with distinct catalytic and regulatory regions (**Figure 1.6**).

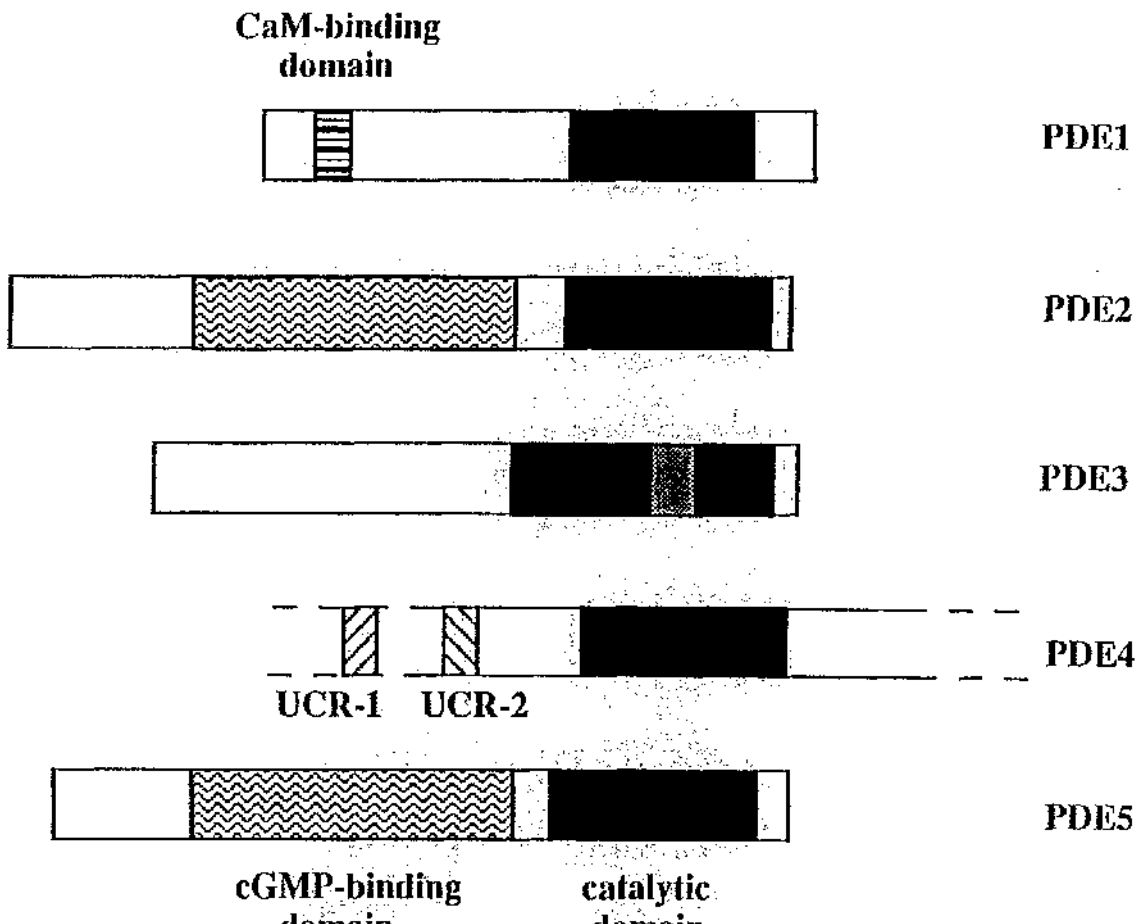
1.4.2.1 Catalytic domain of cyclic nucleotide PDEs

All the vertebrate and insect PDEs that have been isolated to date contain a putative catalytic domain, located in the carboyxl half of the protein, with approximately 30% amino acid identity (Beavo and Reifsynder, 1990). The catalytic domains of members of any one PDE class are more strongly related than those of any two different classes. As primary amino acid sequences have been determined, it has become clear that there is a conserved region of about 270 amino acids (35kDa) present in nearly all PDE isoenzymes, displaying with typically 60 to 90% or greater homology between PDE families (Charbonneau *et al.*, 1986). Outside the catalytic region, there is no region of sequence common to all PDE classes.

The conservation of this central 270 amino acid region led to the proposal that this was the catalytic domain, and this has been supported by a number of studies. The region was shown to contain a motif (Glu-Leu-Ala-Leu-Met-Tyr-Asn) which is also found in the regulatory subunits of cyclic AMP-dependent protein kinases and which has been suggested forms a cyclic AMP binding sequence motif (Scott, 1987). Limited proteolysis of different PDE isoenzymes, such as PDE1 and PDE2, has revealed that the catalytic domains of these enzymes are distinct from their regulatory domains (Kincaid *et al.*, 1985; Stroop *et al.*, 1989). A 35-40kDa fragment was produced in this way, which had a cyclic AMP hydrolysing activity, but could no longer be regulated by Ca^{2+}/CaM or cyclic GMP respectively. Furthermore, site-directed mutagenesis of residues within the conserved domain has revealed that selected amino acids in this central region are essential for catalysis (Jin *et al.*, 1992). Among these are a number of invariant histidine residues found in PDEs obtained from divergent sources (Charbonneau, 1990; Charbonneau *et al.*,

Figure 1.6 Schematic representation of the catalytic and regulatory domains of cyclic nucleotide phosphodiesterases.

The diagram compares (from top to bottom), Ca^{2+}/CaM stimulated, PDE1; cyclic GMP-stimulated, PDE2; cyclic GMP-inhibited, PDE3; cyclic AMP-specific, PDE4 and cyclic GMP-specific, PDE5. The black area indicates the conserved catalytic domain of about 270 amino acid residues between almost all PDEs. PDE1 contains a CaM binding domain in the N-terminal region of the PDE (horizontal lines). PDE2 and PDE5 contain a homologous non-catalytic cyclic GMP binding domain (wavy-lined area). PDE3 has a 44 amino acid insert within its catalytic domain, unique to this class of PDEs. PDE4 is shown to contain upstream conserved regions (UCR1 and UCR2) upstream of the conserved catalytic domain unique to this class of PDEs.


CaM-binding
domain
PDE1
PDE2
PDE3
PDE4
UCR-1
UCR-2
PDE5
cGMP-binding
domain
catalytic
domain

1986; Francis *et al.*, 1994). These amino acids appear to be important in sustaining enzymatic activity, as mutation of these residues abolished PDE activity (Jin *et al.*, 1992).

1.4.2.2 Regulatory domains of cyclic nucleotide PDEs

Members of each class of PDEs often share additional sequence motifs. These motifs are located in the amino termini of the protein and are generally postulated to have regulatory functions e.g. PDE1 are regulated, at least in part, by a presumptive calmodulin-interaction domain near their N-terminal region (Charbonneau *et al.*, 1991) and PDE2 are regulated by a cGMP-binding domain in the amino-terminal region of the protein. The interaction of PDE6 with their regulatory γ-subunit is also believed to occur in their amino-terminal regions (Stryer, 1986; Chabre and Deterre, 1989). The cAMP-specific PDEs also contain two conserved amino-terminal regions, called upstream conserved regions 1 and 2 (UCR1 and UCR2), which may have regulatory functions (Bolger, 1994; Bolger *et al.*, 1996).

PDE2, PDE5 and PDE6 all possess a conserved domain which is separate from the catalytic domain and believed involved in cGMP-binding Charbonneau *et al.*, 1989) (**Figure 1.6**). The degree of homology in this region is high (30% between PDE2 and PDE6) (Gillespie and Beavo, 1989; Stroop and Beavo, 1991). Studies using limited proteolysis on PDE2 have shown that cyclic GMP binding can be separated from the catalytic activity and is localised to a 60kDa chymotryptic fragment (Stroop and Beavo, 1991). PDE2 binds cyclic GMP with high affinity (Martins *et al.*, 1982) via both an allosteric domain and a catalytic domain (Charbonneau *et al.*, 1986; Erneux *et al.*, 1985; Yamamoto *et al.*, 1983). This results in the display of complex kinetics due to actions mediated through the cyclic GMP-binding regulatory site (Erneux *et al.*, 1982; Manganiello *et al.*, 1990; Pyne *et al.*, 1986; Stroop *et al.*, 1989). However, removal of the regulatory site by limited proteolysis has been

shown to abolish the cooperativity of catalysis of cyclic AMP (Stroop and Beavo, 1992). This implies that the regulatory and catalytic domains are indeed separate.

1.4.3 Cyclic AMP specific PDE4

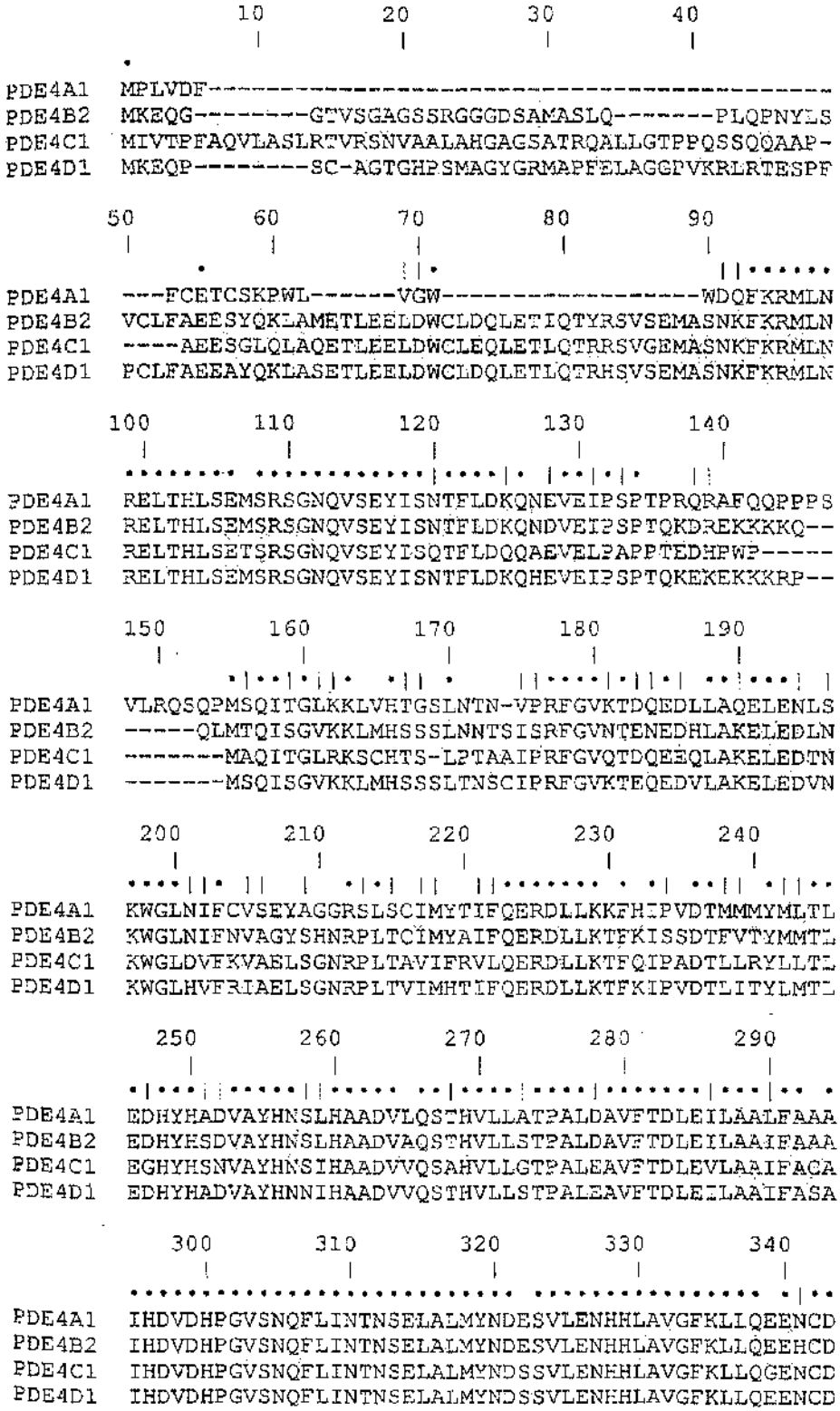
The cAMP-specific (PDE4) cyclic nucleotide phosphodiesterases (PDEs) are a diverse family of proteins that are important regulators of intracellular signalling (Beavo *et al.*, 1994; Conti *et al.*, 1991). Study of the structural and biochemical properties of the PDE4 isoenzymes may lead us to understand the PDE4 family and the functional basis of this diversity in the regulation of intracellular signalling.

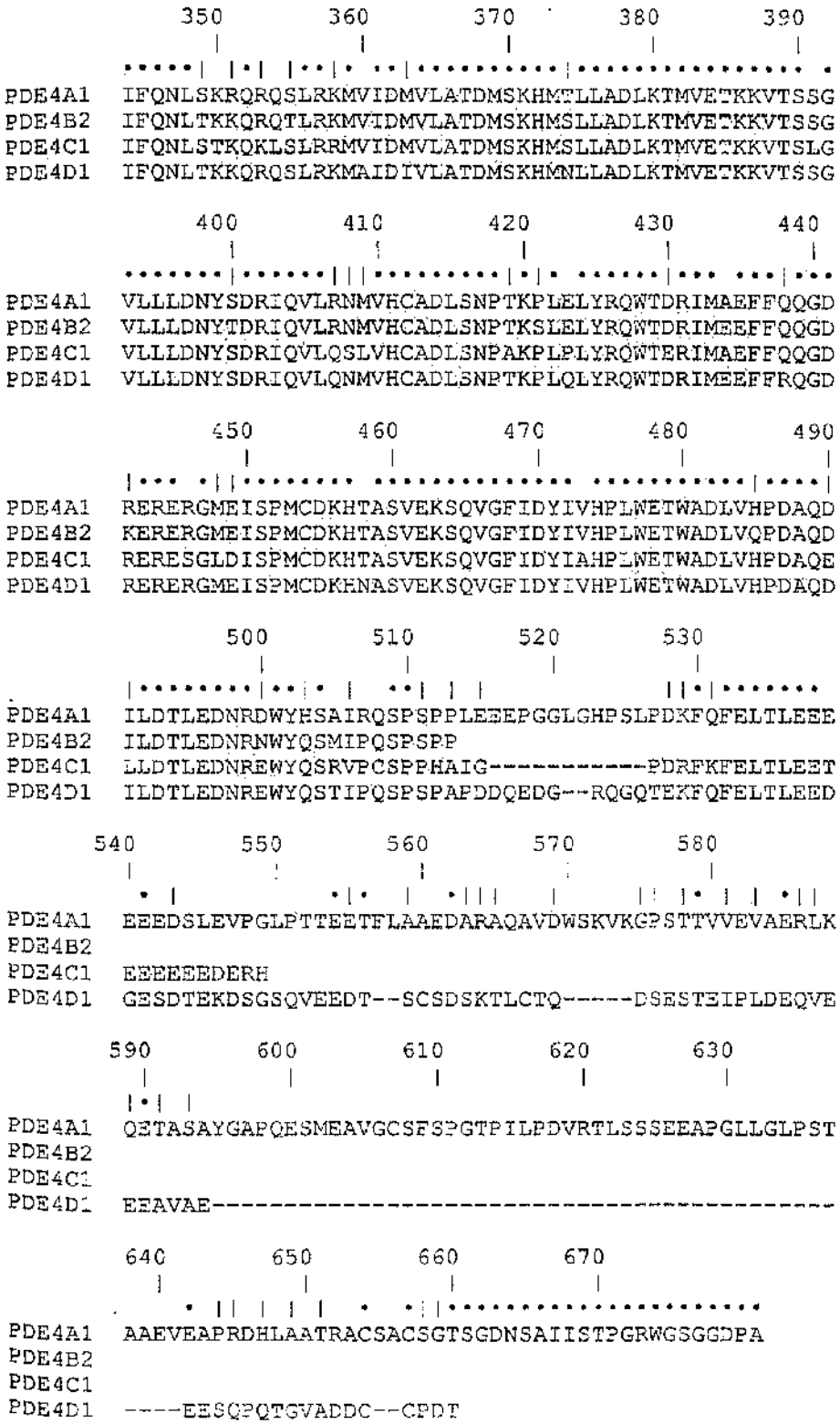
1.4.3.1 Structural features and tissue expression of PDE4

Inspection of PDE4 cDNAs suggested that mammalian PDE4 are coded for by four different genes, A, B, C and D (Davis *et al.*, 1989; Swinnen *et al.*, 1989a; Bolger *et al.*, 1993; Engels *et al.*, 1994). Analysis of the amino acid sequences of the four rat (**Figure 1.7**) and human PDE4 reveals three distinct highly conserved regions. One of these conserved regions is the putative catalytic region (Jin *et al.*, 1992), the two other regions, which are located in the amino-terminal regions of the proteins, are called UCR1 and UCR2 (Upstream Conserved Region). Although these regions are strongly conserved between organisms as evolutionarily divergent as *Drosophila melanogaster* and humans, they have no close homologues in any other sequences in the GenBank or EMBL data bases (Bolger *et al.*, 1993). Additionally, UCR1, but not UCR2, undergoes alternative splicing (Bolger, 1994) suggesting that these conserved regions are likely to be important in the functioning or regulation of these proteins. For example, the different proteins encoded by various rat PDE4D mRNAs differ in their regulation by phosphorylation (Sette *et al*, 1994) and

Figure 1.7 Comparison of primary amino acid sequences of rat PDE4A, B, C and D.

The peptide sequences of rat PDE4 isoforms have been aligned. The PDE sequences are as follows from top to bottom: PDE4A (RD1) (Accession Nos: M26715, Jo4554); PDE4B2 (rat PDE4) (Accession Nos: M25350, M28413); PDE4C1 (RPDE36) (Accession Nos: L27061) and PDE4D1 (rat PDE3.1) (Accession Nos: M25349, M28412). •denotes an identical amino acid residue shared in all sequences. The area of conservation in the middle of the proteins, the catalytic domain (residues 220-500) and the two conserved regions N-terminal to this UCR2 (residues 176-200) and UCR1 (residues 94-130), are also of note.




350 360 370 380 390
PDE4A1 IFQNLSKRQRQSLRKMVIDMVLATDMSKHMTLLADLKTMVETKKVTSSG
PDE4B2 IFQNLTKKQRQTLRKMVIDMVLATDMSKHMSLLADLKTMVETKKVTSSG
PDE4C1 IFQNLSTKQKLSLRRMVIDMVLATDMSKHMSLLADLKTMVETKKVTSLG
PDE4D1 IFQNLTKKQRQSLRKMAIDIVLATDMSKHMNLLADLKTMVETKKVTSSG
400 410 420 430 440
PDE4A1 VLLLDNYSDRIQVLRNMVHCADLSNPTKPLELYRQWTDRIMAEFFQQGD
PDE4B2 VLLLDNYTDRIQVLRNMVHCADLSNPTKSLELYRQWTDRIMEEFFQQGD
PDE4C1 VLLLDNYSDRIQVLQSLVHCADLSNPAKPLPLYRQWTERIMAEFFQQGD
PDE4D1 VLLLDNYSDRIQVLQNMVHCADLSNPTKPLQLYRQWTDRIMEEFFRQGD
450 460 470 480 490
PDE4A1 RERERGMEISPMCDKHTASVEKSQVGFIDYIVHPLWETWADLVHPDAQD
PDE4B2 KERERGMEISPMCDKHTASVEKSQVGFIDYIVHPLWETWADLVQPDAQD
PDE4C1 RERESGLDISPMCDKHTASVEKSQVGFIDYIAHPLWETWADLVHPDAQE
PDE4D1 RERERGMEISPMCDKHNASVEKSQVGFIDYIVHPLWETWADLVHPDAQD
500 510 520 530
PDE4A1 ILDTLEDNRDWYHSAIRQSPSPPLEEEPGGLGHPSLPDKFQFELTLEEE
PDE4B2 ILDTLEDNRNWYQSMIPQSPSPP
PDE4C1 LLDTLEDNREWYQSRVPCSPPHAIG----------PDRFKFELTLEET
PDE4D1 ILDTLEDNREWYQSTIPQSPSPAPDDQEDG--RQGQTEKFQFELTLEED
540 550 560 570 580
PDE4A1 EEEDSLEVPGLPTTEETFLAAEDARAQAVDWSKVKGPSTTVVEVAERLK
PDE4B2
PDE4C1 EEEEEEDERH
PDE4D1 GESDTEKDSGSQVEEDT--SCSDSKTLCTQ-----DSESTEIPLDEQVE
590 600 610 620 630
PDE4A1 QETASAYGAPQESMEAVGCSFSPGTPILPDVRTLSSSEEAPGLLGLPST
PDE4B2
PDE4C1
PDE4D1 EEAVAE----------
640 650 660 670
PDE4A1 AAEVEAPRDHLAATRACSACSGTSGDNSAIISTPGRWGSGGDPA
PDE4B2
PDE4C1
PDE4D1 ----EESQPQTGVADDC--CPDT

those encoded by different rat PDE4A transcripts differ in cellular localization and activity (Bolger *et al.*, 1996).

(a) PDE4A:

The full length cDNA encoding RD1 (PDE4A1) was isolated from a rat brain library by homology cloning using the *dunc* cyclic AMP PDE gene and expressed in yeast (Henkel-Tigges and Davis, 1989). This enzyme is specifically expressed in brain and may be involved in mood regulation (Henkel-Tigges and Davis, 1989).

Comparison of the sequences of RD1 with the dunce-like cyclic AMP PDE4B1 (DPD) and the *Drosophila* dunce PDE (Davis *et al.*, 1989) has identified a central region which is well conserved among these enzymes (approx.75%). However in RD1, over the C-terminal region of approx. 170 amino acids and an N-terminal region of approx. 25 amino acids there is very considerable heterogeneity (> 90%). The possibility that the unique N-terminal region of RD1 provides a domain which confers functional attributes upon this enzyme has been explored (Shakur *et al.*, 1993). When transiently expressed in COS-1 cells then both the full-length RD1 and an N-terminal truncated form (Met^{26}-RD1) was produced. Of these, the full length RD1 was found to be exclusively membrane-associated with Met^{26}-RD1 being located exclusively in the cytosol fraction. These species also showed a marked difference in their thermostability. Thus, the N-terminal alternatively spliced region of RD1 appears to serve as a signal for membrane association and also confers enhanced thermostability upon this PDE4A enzyme. That the unique N-terminal 25 amino acid domain of RD1 is responsible for determining membrane association was confirmed by showing that it could direct the normally soluble, cytosolic enzyme CAT (chloramphenicol acetyltransferase) to the identical membrane locality as RD1 (Scotland & Houslay, 1995). Thus, the N-terminal domain of RD1 contains structural information that targets proteins

to the membrane. Furthermore, immunoblot analyses done with a PDE4A specific anti-serum also identified RD1 as a membrane bound protein species upon immunoblotting of cerebellum homogenates in rat brain (Shakur *et al.*, 1995). COS cells transfected with RD1 cDNA showed RD1 to be predominantly localized to plasma membranes but also associated with the Golgi apparatus and intracellular vesicles. Pooley *et al* ., (1996 submitted) also showed that stable transfection of RD1 into thyroid FTC-133 cells led to localisation exclusively in the Golgi apparatus.

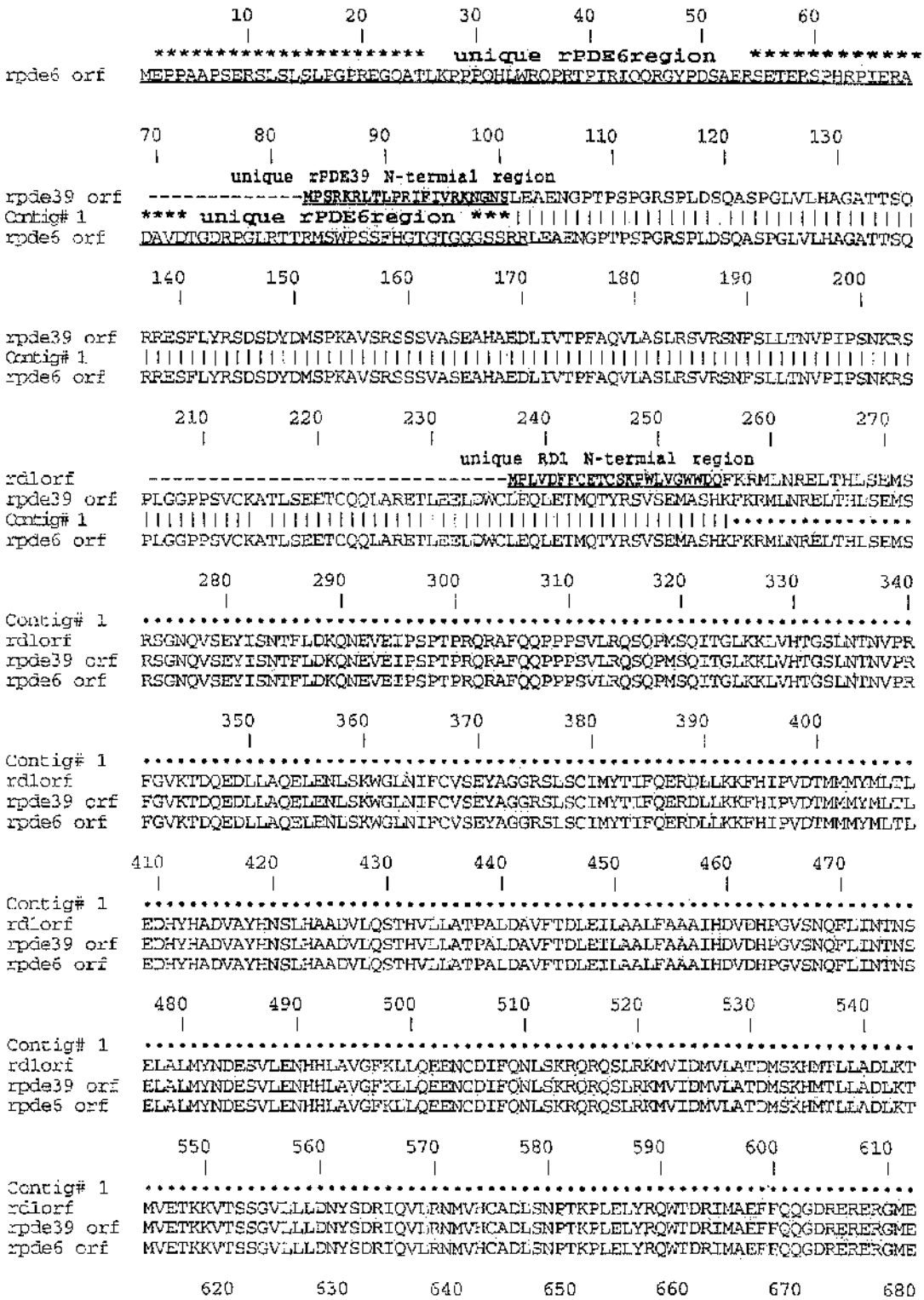
A second rat PDE4A transcript, rPDE6 cDNA, is also expressed in brain. This is a protein of 844 amino acids that is present in both particulate and cytosolic forms (Bolger *et al.*, 1994; McPhee *et al.*, 1995). Furthermore, analysis of brain regions shows that the expression patterns for RD1 and rPDE6 are very different (Bolger *et al.*, Shakur *et al.*, 1995), perhaps suggestive of distinct functional roles for these two species. Recently, a further alternatively spliced mRNA transcript from the rat PDE4A locus, called rPDE39 has been identified (Bolger *et al.*, 1996). This is a rolipram-inhibited, cAMP-specific PDE of 763 amino acids that is found in both particulate and cytosolic forms. rPDE39 (**Figure 1.8**) has a structure consistent with alternative splicing at its extreme 5' end. When expressed in COS cells it migrates as a ~98kDa protein and a very similar size, of ~97kDa, is seen on immunoblotting of testis extracts. This tissue-specific distribution of the rPDE39 protein suggests that this PDE4A isoform has a specific functional role. Indeed, the highly distinct expression patterns of all PDE4A isoforms suggests that alternative splicing leads to the expression of proteins with specific functional roles in the cells that they are expressed in.

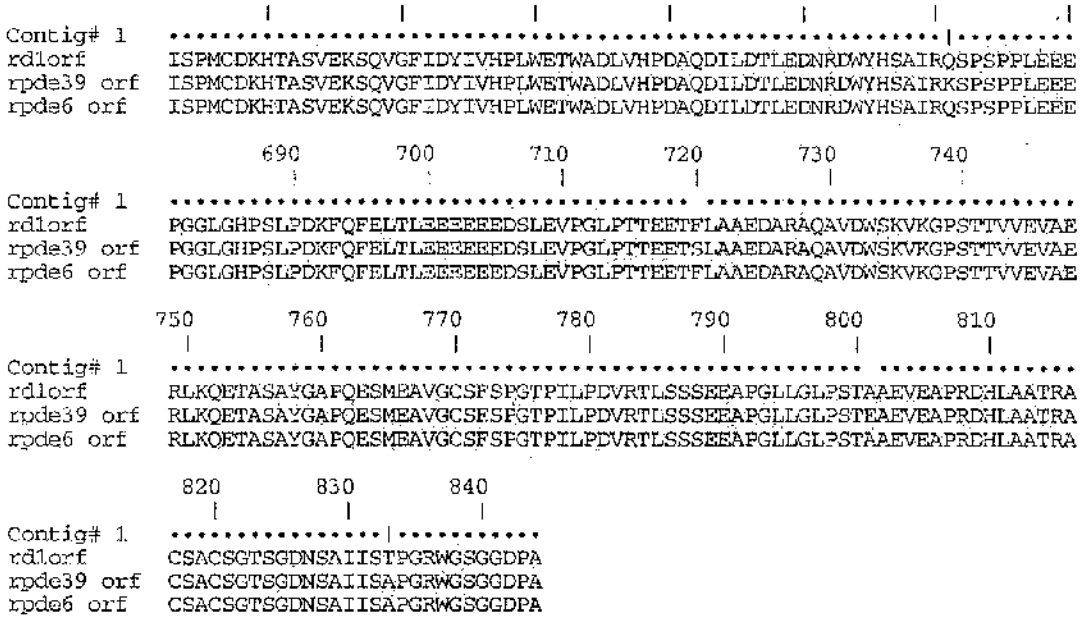
(b) PDE4B:

Splice variants of the PDE4B locus are targeted to different regions of rat brain (Lobban *et al.*, 1994), indicating that differential splicing may

Figure 1.8 Comparison of primary amino acid sequences of rat PDE4A splice variants.

The peptide sequences of rat PDE4 isoforms have been aligned. The PDE sequences are as follows from top to bottom: RD1 (Accession No: M26715); rPDE39 (Accessopm No: L36467); rPDE6 (RPDE36) (Accession No: L27057). •denotes an identical amino acid residue shared in all sequences. * denotes amino acid residues unique to the N-terminal of PDE6. Residues underlined are expressed only in the PDE isoform indicated.


unique rPDE6region
unique rPDE39 N-termial region
unique RD1 N-termial region
rpde6 orf
rpde39 orf
Contig# 1
rdlorf


Contig# 1
rd1orf
rpde39 orf
rpde6 orf
ISPMCDKHTASVEKSQVGFIDYIVHPLWETWADLVHPDAQDILDTLEDNRDWYHSAIRQSPSPPLEEE
ISPMCDKHTASVEKSQVGFIDYIVHPLWETWADLVHPDAQDILDTLEDNRDWYHSAIRKSPSPPLEEE
690
700
710
720
730
740
PGGLGHPSLPDKFQFELTLEEEEEEDSLEVPGLPTTEETFLAAEDARAQAVDWSKVKGPSTTVVEVAE
750
760
770
780
790
800
810
RLKQETASAYGAPQESMEAVGCSFSPGTPILPDVRTLSSSEEAPGLLGLPSTAAEVEAPRDHLAATRA
820
830
840
CSACSGTSGDNSAIISTPGRWGSGGDPA

contribute to tissue specific-as well as subcellular-localisation of these enzymes (Houslay *et al.*, 1995). Analysis of the PDE4B gene (Monaco *et al* 1994) has shown that PDE4B1 (DPD) and rat PDE4B2 ("pde4") are splice variants of the same gene. From comparison of the protein sequences it can be seen that rat PDE4B2 has an extended N-terminus of 48 amino acids (**Figure 1.7**). It is possible, however, that products larger than PDE4B1 can be transcribed according to which methionine initiation codon is utilised. However, Lobban *et al.*, (1994) have shown that PDE4B1 and rat PDE4B2 are likely to be the only products *in vivo* of the PDE4B gene, given that only one immunoreactive species was detected with the antisera specific for rat PDE4B2 and one which recognised rat PDE4B1/PDE4B2. Functional and tissue specific expression of PDE4B isoforms have been demonstrated in the rat brain (Lobban *et al.*, 1994). PDE4B1 was found to be exclusively cytosolic and was selectively expressed in hippocampal, cortex, hypothalamus and striatum regions. Rat PDE4B2 on the other hand was located at the membrane of all brain regions with the exception of the midbrain (Lobban *et al* ., 1994).

(c) PDE4C:

Compared with the products of other PDE4 genes, PDE4C shows a very restricted distribution, for only two sequences corresponding to this gene have been found to date, one each in rat and human tissue (Swinnen *et al.*, 1989; Bolger *et al.*, 1993). This mRNA is found in testis (Swinne *et al.*, 1989) but there are few or no transcripts in brain (Bolger *et al.*, 1994). Recently, Obernolte *et al.*, (1996) have identified four PDE4C splice variants by analysis of cloned human cDNAs from foetal lung and testis. The possibility of tissue-specific PDE4C isoforms and non-catalytic proteins should be considered in evaluating how PDE4C may modulate cellular responses to cAMP.

(d) PDE4D:

The pattern of expression of multiple variants from rat PDE4D gene (Monaco *et al.*, 1994; Sette *et al.*, 1994) has shown that the heterogeneity of the different mRNAs lies in the 5'-region. The corresponding protein variants contain a common region encoded by exons 2 to 11 (Monaco *et al.*, 1994) and divergent amino-terminal regions derived from the assembly of different upstream exons (Sette *et al.*, 1994). The rat PDE4D1 and PDE4D2 mRNAs are generated by a start site controlled by an intronic promoter, which has been shown to be activated in Sertoli and FRTL-5 cells (Vicini *et al.*, 1994). Another variant rat PDE4D3, detected in the brain and FRTL-5 cells, derives from the activity of a different promoter and transcription of upstream exons (Sette *et al.*, 1994a). A fourth variant (PDE4D4) was also detected in human (Bolger *et al.*, 1993) and rat (Vicini and Conti, unpublished results), suggesting the presence of a third, distinct promoter. Human PDE4D1, PDE4D2 and PDE4D3 have been transiently expressed in human kidney 293 cells (Nemoz *et al.*, 1996). A 72kDa protein was expressed in cells transfected with PDE4D1 cDNA, a novel 67kDa protein in cells transfected with PDE4D2 cDNA and a 93kDa protein for cells transfected with PDE4D3 cDNA (Nemoz *et al.*, 1996). Therefore, recombinant human PDE4D2 and PDE4D3 proteins have molecular weights identical to those previously determined for the corresponding rat isoenzymes (Sette *et al.*, 1994b).

Interestingly human PDE4D1, PDE4D2 and PDE4B (PDE4 splice variant) lack the UCR1 (upsteam conserved region), a region of high homology with the drosophila dunce PDE sequence, which is present at the N-terminus in human and rat PDE4D3 and other PDE4 variants (Conti *et al.*, 1995b, Bolger *et al.*, 1993; Sette *et al.*, 1994b), this might suggest an important function for the region encoded by this sequence (Bolger *et al.*, 1993). However, the existence of PDEs lacking this region in both rat and human, indicates that it is not essential for catalysis. The UCR1 region may play a role in the regulation of

enzyme activity and, indeed, the consensus site for PKA-mediated phosphorylation in rat PDE4D3 which confers activation of this enzyme resides within this region (Sette and Conti, 1995).

The complexity of the PDE4 family is apparent from the existence of over twenty splice variants discovered to date (Bolger, 1994). The importance of such a large family could be to enable cells to maintain a high degree of regulation on cyclic AMP degradation. Each PDE4 locus appears to have distinct tissue distribution, suggesting that these alternatively spliced transcripts have specific and subtle differences in regulation and sub-cellular targetting of samples.

1.4.3.2 Cellular Regulation of cyclic AMP specific PDE4

It has been well established that cyclic AMP is at the centre of the control of many cellular functions, therefore enzymes involved in the metabolism of cyclic AMP, such as the PDE4 family, are poised to play a crucial role in these functions.

(a) Regulation of cyclic AMP PDE by hormones:

Many hormones exert their actions on target cells by elevating the intracellular concentration of cAMP. Glucagon through its second messenger cyclic AMP, stimulates both glycogenolysis and gluconeogenesis in hepatocytes, whereas insulin can serve to antagonize this action by presumably depressing intracellular cyclic AMP concentrations (Loten and Sneyd, 1970).

The only known mechanism for the degradation of cyclic AMP is by the action of phosphodiesterases; these therefore provide potentially important regulatory sites. Evidence consistent with this has come, for example, from studies on hepatocytes. In these cells, a gentle homogenization procedure followed by a rapid subcellular fractionation of hepatocytes on a Percoll gradient was developed (Heyworth *et al.*, 1983) to resolve subcellular membrane fractions and identify associated cyclic AMP PDEs. Using such a

procedure, at least three, high-affinity cyclic AMP PDEs species were found to be activated by insulin in hepatocytes (Heyworth *et al.*, 1983 and 1984). However, only one of these enzyme activities, the so-called 'dense-vesicle' PDE, which is located in a unique intracellular vesicle fraction, was also stimulated by glucagon (Heyworth *et al.*, 1983). Indeed, this enzyme is the only PDE identified in liver that is known to be stimulated by glucagon. Phosphorylation of the 'dense-vesicle' PDE by protein kinase A was shown by Kilgour and co-workers (1989). This 'dense-vesicle' enzyme is a PDE3, cAMP-specific enzyme. Its ability to be rapidly regulated by protein kinase A action and by insulin is typical of this enzyme family, as has been shown in detail by Manganiello *et al.*, (1990) for adipocytes.

The so-called peripheral plasma membrane PDE was also activated by insulin. From *in vitro* studies, it appeared that this enzyme became phosphorylated through a cAMP mediated pathway since it was demonstrated that cAMP, as well as ATP, were required for the insulin activation of the plasma membrane PDE. Furthermore, inhibitors of cAMP-dependent kinase blocked the insulin activation of the plasma membrane PDE activity (Pyne *et al.*, 1989). However, studies done in intact cells indicated that this enzyme may be tyrosyl phosphorylated (Pyne *et al.*, 1989), indicating differences in the regulation seen in intact cells. This peripheral plasma membrane enzyme is inhibited by Ro1724 and appears to be a PDE4 enzyme, although the subtype is unclear. Furthermore, it was also noted that cholera toxin served to mimic the insulin-stimulated phosphorylation event (Heyworth *et al.*, 1983) suggesting that the action of a guanine nucleotide regulatory protein could regulate this enzyme.

It is clear that at least two phosphodiesterase isozymes play key roles in the hormonal regulation of glycogenolysis in the liver. The stimulation of PDE3 enzymes by glucagon contributes to the transitory nature of the glucagon stimulated rise in cAMP and both a PDE3 and PDE4 enzyme mediate the ability

of insulin to lower intracellular cAMP levels. However, insulin's activation of these two enzymes cannot account in full for the ability of insulin to decrease intracellular cyclic AMP concentrations (Heyworth *et al.*, 1983) suggesting that additional enzyme(s) must be activated by a rapidly reversible process. In hepatocytes, the so-called cyclic GMP-activated cyclic AMP phosphodiesterase (Pyne *et al.*, 1988), but not the other insulin-regulated phosphodiesterases, is activated by a soluble 'mediator' preparation (Pyne *et al.*, 1988).

(b) Up-regulation of cyclic AMP specific PDE4 activity by intracellular cAMP:

The activity of certain subtypes of PDE4 (4A-4D) can be increased *in situ* by either a short term regulatory process involving protein phosphorylation or by a long term regulatory process involving increased gene expression (Conti *et al.*, 1991; Sette *et al.*, 1994a and 1994b). Both of these regulatory mechanisms are cAMP-dependent and can be triggered by a variety of activators of adenylyl cyclase (Bourne *et al.*, 1974; Conti *et al.*, 1991; Torphy *et al.*, 1992). This raises the possibility that PDE4 activity can be regulated *in vivo* by various hormones, drugs, and growth factors (Conti *et al.*, 1991).

In the FRTL-5 thyroid cell line PDE4 activity is elevated in response to thyroid stimulating hormone (TSH), which is known to elevate intracellular cyclic AMP (Sette *et al.*, 1994a). This hormone dependent cyclic AMP regulation in FRTL-5 cells involves an initial rise in cyclic AMP which returns to basal level within minutes. This return to basal occurs despite the constant presence of hormone, due to an activation of PKA and subsequent phosphorylation and activation of PDE4D. This investigation demonstrated that splice variants from the same locus could be differentially regulated on stimulation of the cell by a particular hormone (Sette *et al.*, 1994b). Differential regulation led to short-term activation of PDE4D3, via changes in phosphorylation status and long-term regulation by stimulating *de novo* protein synthesis of two other splice variants PDE4D1 and PDE4D2. In addition

agents that elevate cyclic AMP has also been demonstrated to cause to an increase in rat PDE4D3 expression in immature Sertoli cells on stimulation with follicle stimulating hormone (FSH) (Monaco *et al.*, 1994; Sette *et al.*, 1994a; Swinnen *et al.*, 1991) and in cardiac myoblasts (Kovala *et al.*, 1994).

Also in the U937 human monocyte cell line it has been shown that the up-regulation of PDE activity by agents that elevate cyclic AMP content was due to an increase in PDE4 alone (Torphy *et al.*, 1992). Recently, when they used prostaglandin E2 (PGE2), an agent that activates adenylyl cyclase by a non-β-adrenoceptor-mediated mechanism, then after 4h treatment, cAMP accumulation was substantially decreased in cells in which PDE4 activity had been up-regulated (Torphy *et al.*, 1995). The difference in PGE2-stimulated cAMP accumulation between control and PDE4 up-regulated cells was greatly reduced in the presence of rolipram, consistent with the notion that an increase in PDE4 activity was responsible for the heterologous desensitization. Furthermore, Manning and co-workers (1996) showed that prolonged β-adrenoceptor stimulation increased PDE activity in monocytes. This was due to the up-regulation of PDE4. At least two of the four subtypes which were are up-regulated in blood monocytes were PDE4A and PDE4B (Manning *et al.*, 1996).

Clearly PDE4 enzymes are extremely important for the regulation of cyclic AMP signalling pathway and this regulation process is mediated by cAMP-dependent protein kinase A.

(c) Phospholipid regulation of cyclic AMP specific PDE4:

There is also evidence for activation of PDE4 via interaction with the phospholipid signalling system. Marcoz *et al.*, (1993) demonstrated that in rat thymocytes, physiological concentrations (10μg/ml) of mixed phosphatidic acid (PAs) species increased the activity of two subtypes of PDE4. DiSanto and Heaslip (1993) reported the seperation of two PDE4 species from U937 cells (referred to as Peak 1 and Peak 2 PDE4), and found that the Peak 2 PDE4 was

activated by phosphatidic acid (PA) (DiSanto and Heaslip 1994). Further studies (1995) were conducted to determine that rolipram's effectiveness versus Peak 2 PDE4 depended on the state of activation of the PDE by phosphatidic acid. Other phospholipids, such as lysophosphatidic acids (LPAs) and phosphatidylserines (PSs) were also found to activate "Peak 2 PDE4" (DiSanto and Heaslip 1995). However, phosphatidylcholines (PCs), phosphatidylethanolamines (PEs) and diacylglycerol (DAG) did not activate PDE4 (DiSanto & Heaslip 1995). It has also been demonstrated that exogenous PA could not stimulate either PDE2 or PDE3 preparations which had been partially purified from rat thymocytes, and that neither PDE1 from porcine brain nor PDE3 from rat adipocytes were sensitive to modulation by PA or PS (Marcoz *et al.,* 1993; Macaulay *et al.,* 1983; Wolff and Brostrom, 1976). Little information, however, is available as to whether human PDE4 is regulated by phospholipids in cells or whether such regulation would be produced specifically by individual phospholipid species. It has been suggested that phospholipid-induced PDE4 activation may represent a mechanism for rapid stimulus-specific modulation of PDE4 activity and hence cyclic AMP concentrations in cells expressing phospholipid-sensitive PDE4 isoforms (DiSanto *et al.,* 1995).

(d) SH3 domain interaction with PDE4A:

The N-terminal domain of the PDE4A family appears to have a variety of functional roles such as membrane targeting (Shakur *et al.,* 1993; Scotland and Houslay, 1995; Shakur *et al.,* 1995; McPhee *et al.,* 1995), regulation of enzyme activity (Vmax) (Shakur *et al.,* 1995; McPhee *et al.,* 1995) and conferring susceptibility to phosphorylation by protein kinase A (Monaco *et al.,* 1994). The N-terminal splice region of rPDE6 is rich in both proline and arginine residues. This is characteristic of proteins able to interact with Src homology 3 (SH3) domains (Pawson and Gish, 1992; Mayer and Baltimore,

1993; Terasawa *et al.*, 1994; Musacchio *et al.*, 1992). SH3 domains have been identified in a variety of cytoplasmic tyrosyl protein kinases as well as in cytoskeletal and adaptor proteins (Pawson and Gish, 1992; Mayer and Baltimore, 1993; Terasawa *et al.*, 1994; Musacchio *et al.*, 1992). These SH3 containing proteins perform a crucial role in recruiting specific proteins so that they can take part in signalling cascades and also in conferring distinct subcellular localisations upon proteins which bind to them (Pawson and Gish, 1992; Mayer and Baltimore, 1993; Terasawa *et al.*, 1994; Musacchio *et al.*, 1992). O'Connell *et al.*, (1996) have shown that rPDE-6 can bind to the SH3 domain of v-Src by virtue of its N-terminal splice region. This provides the first direct connection between an enzyme involved in the cyclic AMP signalling pathway and SH3 domain expressing proteins and identifies selectivity in this at the level of both the particular SH3 domain species and the particular PDE4A splice variant (O'Connell *et al.*, 1996).

1.4.3.3 Inhibition of cAMP specific PDE4

Rolipram is a potent and selective inhibitor of PDE4 and has been reported to inhibit PDE4 from different cellular sources (Henkel-Tigges and Davis, 1990; McLaughlin *et al.*, 1993; Nemoz *et al.*, 1989; Torphy *et al.*, 1992). Rolipram is known to interact with PDE4 in at least two distinct fashions: either by binding to high affinity binding sites at very low concentrations (Kd=0.4 to 6nM) or by a lower affinity interaction (Ki=0.06 to 1μM) which is presumed to occur at the enzyme catalytic site (Schneider *et al.*, 1986; Torphy *et al.*, 1992). In general, rolipram's inhibition of cAMP catabolism by PDE4 has been correlated only with rolipram's low affinity interaction (Torphy *et al.*, 1992).

The association of PDE4 hydrolytic activity and rolipram binding raises the question of the molecular relationship between the antagonism of rolipram binding and inhibition of PDE4 activity. Several studies have shown (Pinto *et*

al., 1993; Barnett *et al.*, 1995; Jacobitz *et al.*, 1994) that there is a lack of correlation between rolipram binding and PDE4 inhibition. Enzyme kinetic studies have, however, implied that the binding site for rolipram and the active site of cAMP hydrolysis may be identical. Although it cannot be excluded that the high-affinity binding site is located on the same protein as the PDE4 hydrolytic activity.

1.5 Cyclic AMP dependent protein kinase (PKA)

Cyclic AMP-dependent protein kinase (PKA) has been found to be the only protein, apart from cyclic AMP phosphodiesterases, that binds cyclic AMP with high affinity, in eukaryotic cells. This has led to the suggestion that all the downstream effects of cyclic AMP are mediated through protein phosphorylation via PKA (Taylor *et al.*, 1990).

1.5.1 Structural and tissue distribution of PKA isoforms

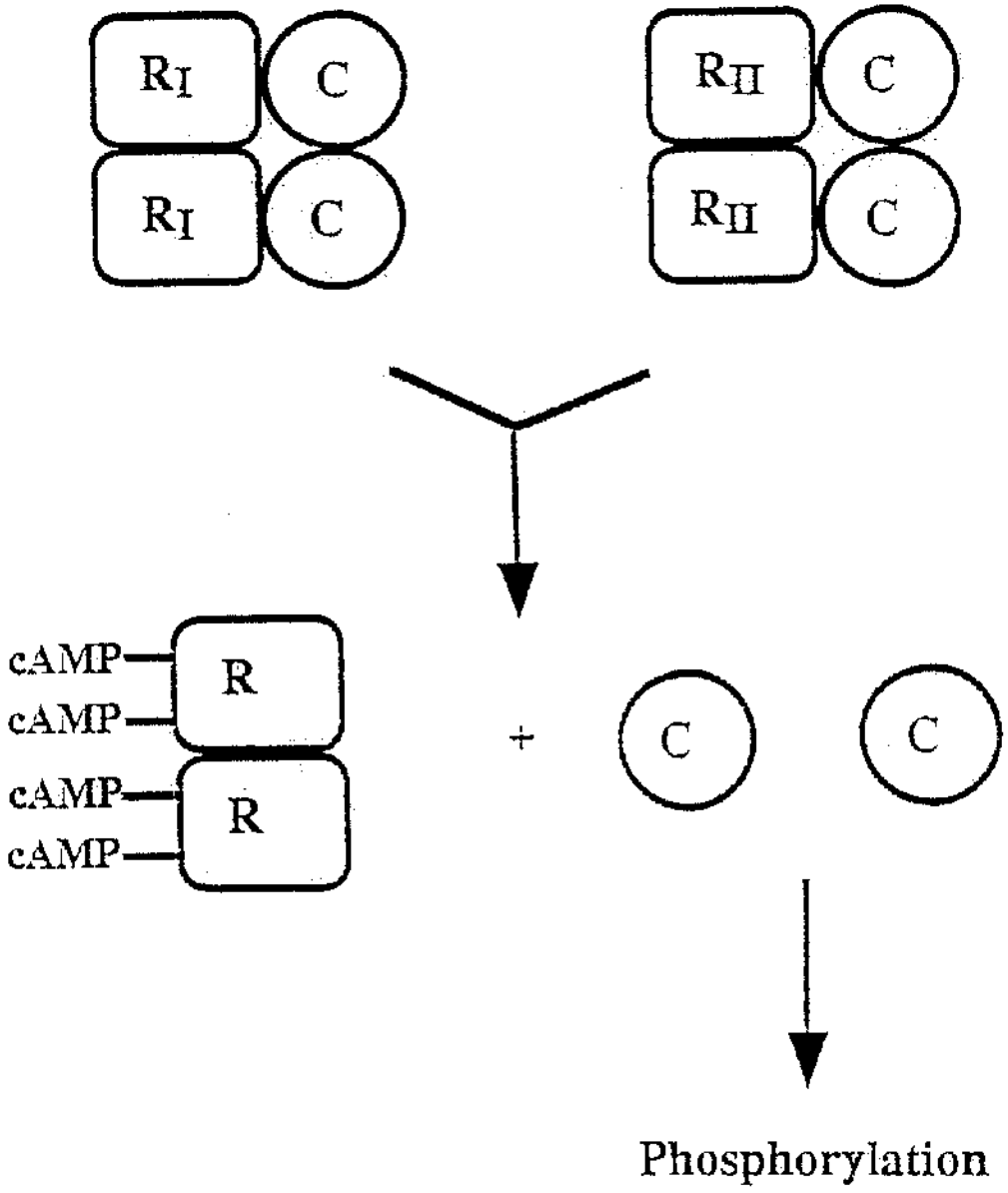
PKA exists as two major isoforms, type-I and type-II, that differ with respect to their regulatory (R) subunits, as identified by their differing molecular weights, antigenicity, amino acid sequence and affinity for cyclic AMP analogues. Isotypes of each of these regulatory domains have also been identified; RI (α and β) and RII (α and β) subunits, as well as the catalytic (C) (α, β and γ) subunit (**Figure 1.9**). Expression of these isoforms varies between tissues, with type-II PKA being present in all cells, whereas the tissue distribution of type-I PKA is more restricted (Corbin *et al.*, 1977; Rubin *et al.*, 1972). Intracellular localisation also varies, with RI isoforms found primarily in the cytosolic compartment of the cell, although a fraction has been found associated with the membranes of erythrocytes (Rubin *et al.*, 1972) and associated with the T cell receptor of activated lymphocytes (Skalhegg *et al.*, 1994). The RII isoforms are generally particulate, with up to 75% of the

Figure 1.9 Diagrammatic representation of protein kinase A.

RI and RII; Regulatory subunits

C; Catalytic subunits

cAMP; Adenosine 3', 5' cyclic monophosphate


R_I
C
R_I
C
R_{II}
C
R_{II}
C
cAMP
cAMP
cAMP
cAMP
R
R
+
C
C
Phosphorylation

cellular RII pool being associated with the plasma membrane and cytoskeletal compartments (Nigg *et al.*, 1985; Salavatori *et al.*, 1990).

In all cases, the inactive holoenzyme form of PKA is a tetrameric conformation, consisting of two R subunits and two C subunits (R_2C_2) (Taylor, 1989) (**Figure 1.9**). High affinity binding of 4 molecules of cyclic AMP to the R subunits, activates the complex by dissociation of the two catalytic subunits from the R subunit dimer (Beebe and Corbin 1986), thereby, liberating the catalytic subunits to phosphorylate substrate proteins or to migrate to the nucleus (Nigg, 1990). This can be summerised in the reaction scheme:

$$R_2C_2 + 4 \text{ cyclic AMP} \text{-----} R2 \bullet (\text{cyclic AMP})_4 + 2C$$

Studies have shown that the RII subunit has an additional function, namely the localisation of PKA (Scott, 1991).

The active catalytic subunits are able to phosphorylate their physiological substrates (serine in the context X-R-R-X-**S**-X) within a number of cytoplasmic and nuclear proteins. Both catalytic and regulatory subunits have been postulated to have a role in the transcriptional regulation of cyclic AMP-responsive genes (Lalli and Sassone-Corsi, 1994). However it appears that the catalytic subunits are both necessary and sufficient for this response.

1.5.2 Phosphorylation of target proteins by PKA

In order to perform specific phosphorylations that are necessary in the tight regulation of cellular metabolism one might expect that the activation of a kinase would be compartmentalised, in some way, to ensure targeting and specificity of phosphorylation of the desired substrate. The proposal of selective activation of PKA-pools, which are co-localised with key substrates, may provide the opportunity for this to occur. The type-I PKA isoenzyme has shown to be associated with the TCR/CD3 complex upon TCR ligation (Skalhegg *et al.*, 1994). Interestingly, the binding of cAMP to the inactive PKA R2C2 holoenzyme, much of which is anchored in the perinuclear region

of the cytoplasm through membrane-associated anchoring proteins (known as AKAPs) (Scott and Carr, 1992), releases the active C subunit, which is then competent to phosphorylate its substrates on a single serine residue (ser 133). This phosphorylation results in increased transactivation activity, without a significant effect on CREB DNA-binding activity (discussed in the later section). The extent of ser-133 phosphorylation correlates directly with the rate of transcription of cAMP-responsive genes (Hagiwara *et al.*, 1993)

A number of different AKAPs specific for type-II PKA have been identified in the literature by virtue of their molecular weights, subcellular localisation and differential hormone induction (Carr *et al.*, 1993; Carr *et al.*, 1992; Keryer *et al.*, 1993; McCartney *et al.*, 1995). Interestingly, these AKAPs do not appear to anchor PKAII alone, but have recently been reported to bind the protein phosphatase calcineurin (Coghlan *et al.*, 1995). It has been noted that the first 5 amino acids of each RII subunit are critical for AKAP interaction (Hausken *et al.*, 1994). However, it appears that AKAPs contain additional binding sites, possibly responsible for targeting them to cellular structures, such as DNA binding domains (Coghlan *et al.*, 1994) and cytoskeletal binding domains (Glantz *et al.*, 1993). This added control coupled to a dynamic system, may allow the redistribution of PKAII to occur in response to external stimuli. The ability of signalling molecules to be grouped around AKAPs (Coghlan *et al.*, 1995) may allow tight regulation of the phosphorylation state and thus activity of key substrates.

1.5.3. Modulation of transcription factors by PKA

As discussed above, binding of cyclic AMP to the PKA-holoenzyme causes the dissociation of the two regulatory subunits from the two active catalytic subunits (Krebs and Beavo, 1979; Taylor, 1989). The catalytic subunits are now free to phosphorylate serine/threonine residues of target proteins. Indeed, a fraction of this subunit population translocates into the

nucleus, apparently through passive diffusion, where it phosphorylates its nuclear targets. Lalli and Sassone-Corsi, (1994) showed one of the major nuclear PKA substrates to be the transcription factor CREB (cAMP responsive element binding protein). CREB binds to a palindromic response element (TGACGTCA), known as the cAMP-regulated enhancer (CRE), in cAMP-inducible genes. Thus the modulation of transcription factor activities, such as CREB or activation transcription factor (ATF) families, is thought to occur via phosphorylation. CREB/ATF proteins are the targets for several protein kinases including PKC and PKA, with phosphorylation of these factors stimulating or repressing transcription (de Groot *et al.*, 1993; Yamamoto *et al.*, 1988). Different forms of CREB/ATF proteins bind to different CRE sites, or even the same sites, with differing affinities following phosphorylation by such kinases.

CREB/ATF regulatory proteins are characterised by a leucine-zipper domain and a basic DNA binding domain that recognise the CRE site. These proteins have an important role in regulating basal gene transcription or induced gene transcription in response to cyclic AMP elevation. This family of transcription factors also share a certain degree of homology with members of the activator protein-1 (AP-1) family, *Fos/Jun* proteins. Indeed, hetero-dimerisation can occur between the families as well as homo-dimerisation, which creates enormous complexity, leading to a higher order of regulation, involving cross-talk between lipid and cyclic nucleotide signalling at the level of gene transcription (Altschul *et al.*, 1990; Hai and Curran, 1991; Landschulz *et al.*, 1988).

Attenuation or inhibition of cyclic AMP-stimulated gene transcription requires dephosphorylation of transcription factors to occur, representing a key mechanism in the negative regulation of CREB activity. It appears that protein phosphatase 2A (PP2A) and protein phosphatase 1 (PP1) oppose the action of PKA (Hagawara *et al.*, 1992; Wadzinski *et al.*, 1993).

Another class of transcription factors involved in the transduction of cyclic AMP effects on transcriptional regulation are CRE-modulators (CREM) (Meyer and Habener, 1993). The complexity of the CREM protein repertoire is enhanced by alternative splicing mechanisms giving rise to various forms of CREM which can be activators or repressors of transcription (Foulkes and Sassone-Corsi, 1992).

1.6 Perspectives of the protein kinase C family in cell regulation

Activation of PKC is an early event in a wide range of signal transduction processes. However, PKC "activity" in represented by a large gene family of isoenzymes differing remarkably in their structure, expression, mode of activation and in substrate specificity (Parker, 1994; Nishizuka, 1992, Hug and Sarre, 1993). The role of specific PKC isoenzymes has yet to be fully elucidiated.

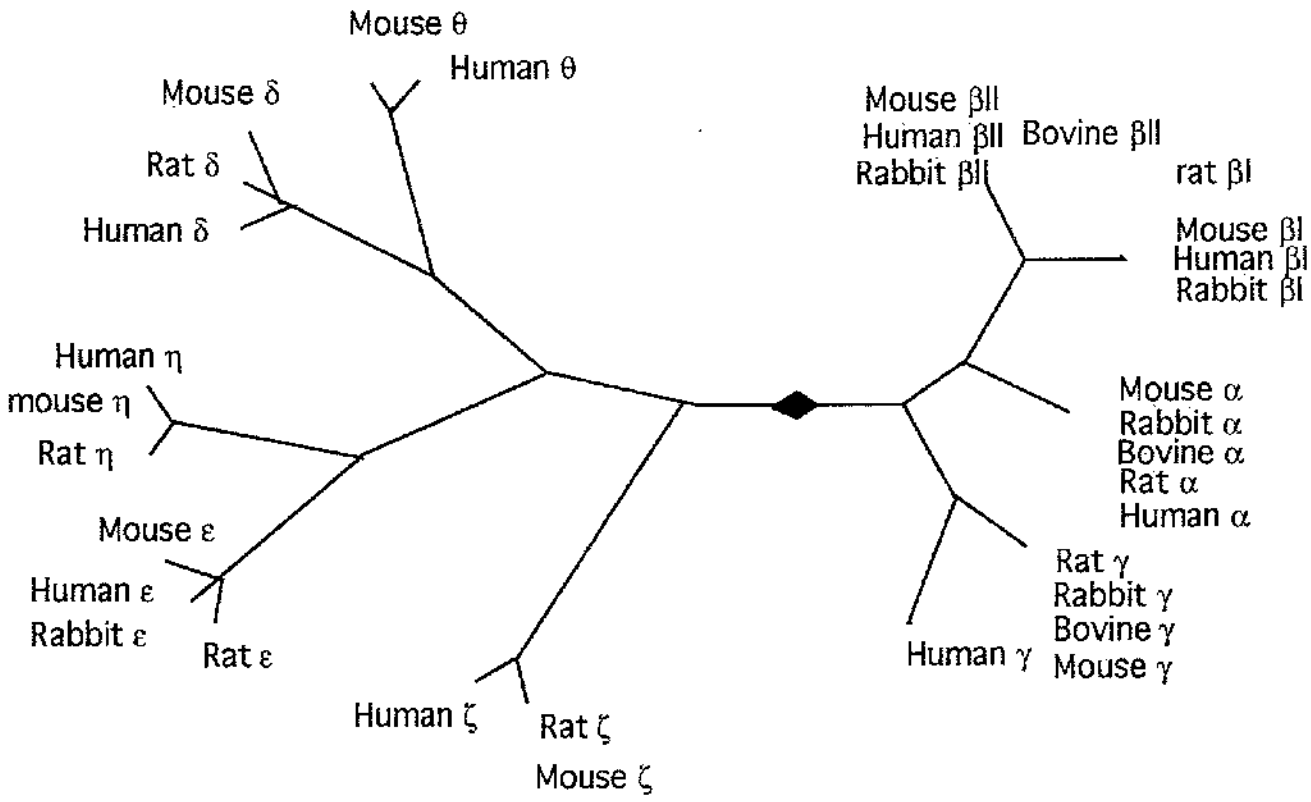
1.6.1 Classification of PKC isoenzymes

The division of the PKC family into subfamilies is based on the primary structure of the polypeptides and the implications of this on functional similarity within subfamilies have been discussed extensively (Nishizuka, 1992; Hug and Sarre, 1993). From early biochemical studies and purifications (Huang *et al.*, 1986a), PKC would seem to represent a gene of at least three isoforms α, β, γ. So far, however, the cDNAs coding twelve different PKC isoforms from different species and tissues or cell lines have been identified (Nishizuka, 1992; Asaoka *et al.*, 1992; Parker *et al.*, 1989; Hug and Sarre, 1993). These can be divided into Ca^{2+}-dependent or conventional cPKCs (α, βI, βII, γ) and the Ca^{2+}-independent or novel nPKCs (δ, ε, η, θ) (Ohno *et al.*, 1990). PKC-ζ constitutes what could be considered a separate branch (Parker *et al.*, 1994) as shown in **Figure 1.10**. PKC-ι (Bacher *et al.*, 1991 and 1992), λ and μ have not been included in the dendrogram as no sequence data are available.

Figure 1.10 Phylogenetic relationships between mammalian PKC isotypes

The diamond represents a common PKC ancestor. Evolutionary distance (bar, 10% difference) is based upon alignment of the amino acid sequence of the PDE isotypes using the GCG multiple sequence alignment program.
Adapted from Dekker and Parker, 1994.

10 %


Mouse θ
Human θ
Mouse δ
Rat δ
Human δ
Mouse βII
Human βII
Rabbit βII
Bovine βII
rat βI
Mouse βI
Human βI
Rabbit βI
Human η
mouse η
Rat η
Mouse α
Rabbit α
Bovine α
Rat α
Human α
Mouse ε
Human ε
Rabbit ε
Rat ε
Rat γ
Rabbit γ
Bovine γ
Mouse γ
Human γ
Human ζ
Rat ζ
Mouse ζ

However, it has been suggested that PKC-μ is a member of the PKC-δ, ε, η, θ subfamily whilst PKC-ι and λ are related to PKC-ζ (Dekker and Parker, 1994).

1.6.2 Activation of PKC

Activation of cPKC is achieved in stimulated lipid metabolism, i.e. in signalling by phospholipases and phosphatidylinositol kinases (Meldrum *et al.*, 1991). For the cPKCs, a model of activation was sufficient and convincing that included (1) the generation of DAG and Ins (1,4,5) P_3 from plasma membrane-associated PtdIns (4,5) P_2 by the action of phospholipase C. Hormones such as glucagon, vasopressin and angiotensin which all elicit a rapid, transient increase in hepatocyte PKC activity (Tang and Houslay, 1992) act by this route. (2) the release of Ca^{2+} from intracellular storage sites stimulated by Ins(1,4,5)P_3, (3) the binding of Ca^{2+} to (the C2 region of) PKC and subsequent translocation of the enzyme to the plasma membrane, (4) Activation at the plasma membrane (via the C1 region) by DAG and PtdSer, the latter being constitutively present in the membrane (Bell and Burns, 1991). PKC is also known to act as a direct "receptor" protein and can be activated by phorbol esters; substances known to interfere dramatically with proliferative and differentiation events by promoting oncogenic transformation of cells *in vivo in situ*. In this model, phorbol ester would mimic the action of DAG and, by its persistence in the cellular membrane, lead to a long-term activation of PKC (Gschwendt *et al.*, 1991).

On the other hand, nPKC show cofactor Ca^{2+}-independence, whilst PKC-ζ is not activated by phorbol ester/DAG. Such behaviour is of great interest since it indicates that the signal transduction pathways in which PKC isotypes may be involved *in vivo* are not restricted to those leading to the generation of DAG but might also include those that activate the phosphatidylinositol 3-kinase (PI3-kinase) pathway. In addition controversy

surrounds the question (Farese *et al.*, 1992; Glynn *et al.*, 1986; Vaartjes *et al.*, 1986) of whether insulin-induced increases in DAG result in PKC activation. However, there is now considerable evidence that DAG derived from phosphatidylcholine (PC) hydrolysis (Laviada *et al.*, 1991) and the *de novo* pathway (Lee *et al.*, 1989) can translocate and activate PKC.

Recently it has been shown that other components of glycerolipid metabolism can be activators of PKC, at least in vitro (Lee and Bell, 1991; Chahan *et al.*, 1991). Cytokines such as IFN-α, IL-1 and IL-3 which have been reported to induce phosphatidylcholine hydrolysis, but not inositol phospholipid turnover (Duronio *et al.*, 1989) can activate PKC β and ε. Thus, the signal-induced production of a distinct second messenger or activator may actually decide which PKC is activated and indeed the activation potency of different phorbol ester derivatives on PKC isoenzymes reveals quite distinct differences. (Ryves *et al.*, 1991)

1.6.3 Structure of PKC isoenzymes

Amino acid structure of PKC can be divided into conserved and so-called function domains (C1-C4) which are separated by variable regions (V1-V5) (**Figure 1.11**). The C-terminal regions C3-V5 have been defined in all PKC isoforms as the catalytic domain, which is separated by the V3 region from the N-terminal regulatory domain.

The N-terminal V1 region of the PKC isoforms is a short stretch of aproximately 20 amino acids for which no function has been attributed. At the beginning of the C1 region, a sequence motif is located that is similar to the consensus sequence found in the phosphorylation sites of prominent PKC substrates; the so called pseudosubstrate domain. A peptide reflecting this can act to inhibit kinase activity both in *vitro* and in *situ* (House and Kemp, 1987; Eicholtz *et al.*, 1990; Shen and Buck, 1990). The cysteine-rich region within the C1 domain consists of two zinc finger motifs each with six cysteine residue.

Figure 1.11 Domain structure of PKC isoenzymes

All PKC isoenzymes consist of constant (C) and variable (v) regions. The cysteine-rich repeats in the C1 region and the ATP binding site in the C3 region are indicated by arrowheads. The arrow points to the hinge region in the V3 domain which separates the regulatory from the catalytic domain.

The functions of these domains are:

C1: The first conserved domain cotains a cysteine rich motif which is tandemly repeated in most but not all PKC isotypes.

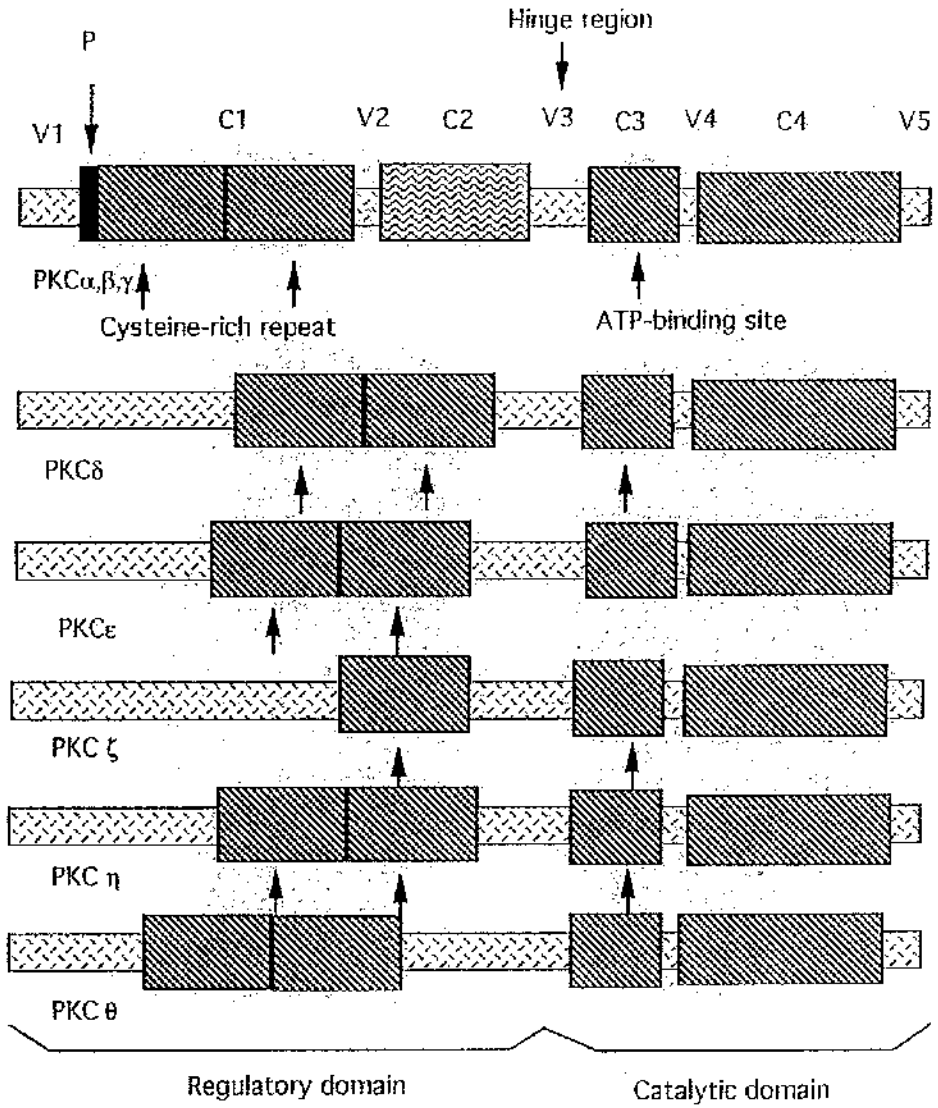
C2: The second conserved domain present in only some PKCs appears to be responsible for confering Ca^{2+} -dependence.

C3 and C4: The conserved regions 3 and 4 cover the kinase domain. All the characteristic protein kinase sequences are present in this region.

V3: The third variable region includes the sites of cleavage associated with proteolytic activation.

V5: This C-terminal variable region is divergent among all the PKCs and also covers the splice variants β1 and β2.

P: The pseudosubstrate site acts to inhibit kinase activity before effector binding.


P
Hinge region
V1
C1
V2
C2
V3
C3
V4
C4
V5
PKCα,β,γ
Cysteine-rich repeat
ATP-binding site
PKCδ
PKCε
PKC ζ
PKC η
PKC θ
Regulatory domain
Catalytic domain

This is a potential DNA-binding motif found in transcription factors like GAL4 (Pan and Coleman, 1990). However, no such DNA-binding activity has been demonstrated for PKC, but the regulatory subunit alone, generated by proteolytic cleavage in the V3 hinge region, may bind to this. The use of deletion mutants of different PKC isoforms has revealed that the cysteine-rich region is necessary for DAG and phorbol ester binding (Muramatsu *et al.*, 1989; Kaibuchi *et al.*, 1989; Burns and Bell, 1991). The Ca^{2+}-independent nPKCs lack the C2 region, which is thought to represent the Ca^{2+} binding site, as it contains many acidic amino acids which are thought to participate in Ca^{2+} binding (Ohno *et al.*, 1987). The V3 or hinge region separates the regulatory domain from the catalytic domain. This region is sensitive to proteolytic cleavage by trypsin or Ca^{2+}-dependent neutral peptidase. The C3 region contains the ATP-binding site. However, here PKC-ζ differs slightly from the consensus ATP-binding motif in that it contains an alanine instead of a glycine at position 264. The C4 region contains the substrate binding site and the phosphate transfer region. The aspartate residue here is thought to be responsible for the transfer of the phosphate group to substrates.

It is possible to give functional assignments to generalized PKC domains by comparing the varying degrees of identity between these enzymes. PKC-ζ contains only one zinc finger and does not bind DAG or phorbol ester (McGlynn *et al.*, 1992). This is in agreement with the finding that (Parker *et al.*, 1994), at least *in vitro*, PKC-ζ exhibits a constitutive protein kinase activity and is resistant to activation-induced down-regulation. PKC-α is more resistant to proteolytic digestion that β and γ (Hug and Sarre, 1993). Could this be evidence that the V3 region of different PKC isoforms exhibit certain features that modify the susceptibility to proteolytic cleavage?

1.6.4 Tissue expression

The tissue distribution of the PKC isoenzymes has been determined mostly by Northern blotting and, more recently, by Western blotting analyses using isoenzyme-specific antibodies. All PKC isoforms known so far are expressed in brain to varying amounts. PKC α, βI/II, δ and ζ seem to be ubiquitously distributed, e.g. in brain, lung, spleen, thymus and skin (Nishizuka, 1988; Wada *et al.*, 1989; Yoshida *et al.*, 1988; Schaap *et al.*, 1989), whilst PKC γ is exclusively found in the central nervous system, e.g. brain (Nishizuka, 1988), and PKC–η is strongly expressed in skin and lung and only slightly in brain and spleen (Osada *et al.*, 1990). PKC-θ is predominantly expressed in skeletal muscle and to a clearly lower extent, in lung, spleen, skin and brain (Osada *et al.*, 1992). PKC-α and ε seem not to be present in liver, where PKC-β is the major isoenzyme (Rogue *et al.*, 1990). The isoforms of PKC therefore differ in tissue distribution and amounts expressed.

Employing the same techniques, PKC isoenzyme expression patterns of selected cell lines from various origins have been determined. PKC-α, βI/βII, δ, ε and ζ seem to be the most ubiquitously expressed isoenzymes, whilst PKC γ is restricted to a neuronal cell line (PC12). Information on PKC η and θ is yet too limited. PKC βI/βII are absent from mouse and rat fibroblasts, and the absence of PKC ε appears to be a feature of cells from the myeloid but not erythroid lineage of the haematopoietic system, i.e. macrophages, platelets and the promyelocytic leukaemia cell line HL60.

A true picture of PKC isoenzymes in a distinct signalling pathway may become evident once it is possible to define a given cell type unequivocally by its PKC isoenzyme expression pattern and by its signal response potential. Little is known about how the expression of various PKC isoenzymes are regulated, due to the fact that information on the gene and promoter structure is

limited. So far, only the promoters of the rat PKC-γ (Chen *et al.*, 1990) and the human PKC β (Niino *et al.*, 1992) gene have been cloned.

With the detection of the various PKC isoenzymes, it became clear not only that there are tissue-specific expression patterns but that the amount and number of PKC isoenzymes varied within a given tissue depending on its developmental stage (Nishizuka, 1988). This again indicates that a certain set of PKC isoenzymes is necessary to guarantee the ordered sequence of proliferation and differentiation events which leads to and maintains the characteristics of a give tissue.

1.6.5 Regulation of PKC activity

Understanding the regulation of each PKC subspecies is a prerequisite essential for determining the potential roles of this enzyme family in signal transduction. The variable regions found in each PKC subspecies may play key roles in governing the individual enzymological characteristics and possibly their specific localization and function. Recently the biochemical properties of the different PKC isoenzymes have been investigated with respect to autophosphorylation, proteolytic activation/degradation and mechanisms of attenuation.

1.6.5.1 Inhibitors of PKC

Both regulatory (phospholipid-and phorbol ester-binding site) and catalytic domains (peptide-and ATP-binding sites) on PKC offer targets for inhibitor design.

Examples of inhibitors directed against the regulatory site include sphingosine (Hannun and Bell, 1987) and calphostin C (Kobayashi *et al.*,, 1989). The latter is a perylenequinone which is competitive with phorbol ester and is three orders of magnitude more potent against PKC (IC_{50}=50nM) than against any other protein kinase. However, the mechanism of action of this

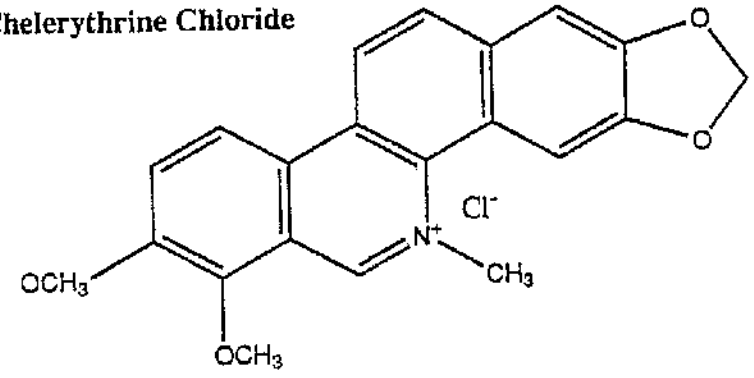
compound appears to involve photo-activation to yield a short-lived species which reacts with PKC resulting in permanent inactivation of phorbol ester binding (Bruns *et al.*, 1991).

Peptide sequences directed against the substrate binding site, for instance the pseudosubstrate sequence PKC19-36 (Smith *et al.*, 1990), whilst being potent and selective inhibitors do not penetrate cells, thus limiting their potential use.

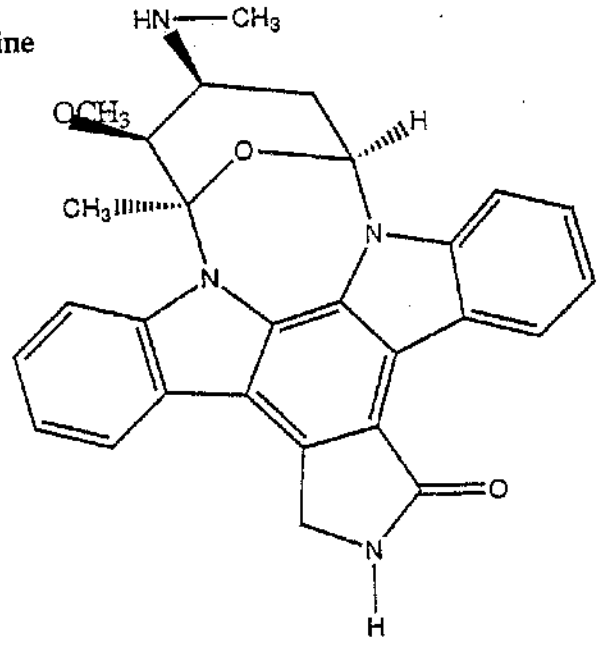
By far the greatest progress has been make with the design of ATP-competitive inhibitors of PKC. An alternative approach to the design of selective ATP-competitive inhibitors was provided by the identification of the microbial metabolite staurosporine as a potent, but non-selective, protein kinase inhibitor (Tamaoki *et al.*, 1986; Elliott *et al,* 1990). Replacement of the methylamino substituent on the pyranose ring in this molecule with -OH (**Figure 1.12**) resulted in a considerable loss in potency against PKC (Osada *et al.*, 1990), suggesting the presence of a cation-binding site on the enzyme. The benzophenanthridine alkaloid, chelerythrine which, unlike staurosporine, blocks not only PKC but also a variety of other protein kinases (Ruegg and Burgess, 1989), is one of the most potent, selective inhibitors of PKC interacting with the catalytic domain of the enzyme. Chelerythrine did not alter any activity of other protein kinases such as protein tyrosine kinase, Ca^{2+}/calmodulin-dependent protein kinase or cAMP-dependent kinase (PKA). These inhibitory agents are useful tools for clarifying the role of PKC in mediating functional responses in cells. For example, PKC plays a key role in cellular growth and differentiation (Nishizuka, 1986), the effects of the inhibitor chelerythrine showed potent cytotoxic effects on L-1210 cells (Herbert *et al.*, 1990) where it prevents tumour cell growth. Given that bisindolylmaleimide PKC inhibitors are potent inhibitors of T-cell proliferation *in vitro* (Nixon *et al.*, 1991) and can also effect T-cell-mediated inflammation *in vivo*, this implies that PKC inhibitors may have therapeutic potential in the treatment of diseases.

Figure 1.12 Structure of the serine/threonine protein kinase inhibitors chelerythrine and staurosporine.

Chelerythrine Chloride



Staurosporine



1.6.5.2 Proteolytic regulation of PKC

PKC was originally detected as an undefined protein kinase, which was present in many tissues and could be activated by limited proteolysis with calpain (protease) (Inoue *et al.*, 1977). This hydrolytic cleavage occurred at one or two specific sites in the V3 hinge region and thus produced two distinct fragments; a protein comprising the regulatory domain and a protein containing the kinase domain which is catalytically active in the absence of any activators (Kishimoto *et al.*, 1989). *In vitro*, this proteolytic activation can be achieved by a limited trypsin treatment (Huang *et al.*, 1989), though *in vivo*, activation of PKC and translocation to the cell membrane is thought to be a prerequisite for proteolytic cleavage.

The physiological significance of this proteolysis has not yet been unequivocally established. Two alternative possibilities may be considered. Firstly, this proteolysis may be a process to activate the PKC molecule, and the resulting protein kinase fragment may play some role in the control of cellular functions (Melloni *et al.*, 1986; Fournier, 1987). It is also possible that the regulatory fragment has some roles in the control of gene expression since it contains a putative DNA-binding motif. Secondly, in contrast, the limited proteolysis may be a process to initiate the degradation of PKC, eventually depleting the enzyme from the cell. PKC isoenzymes display different sensitivities to proteolysis. Compared to PKC β and γ, PKC α is relatively resistant to both calpain-and trypsin-mediated proteolysis (Kishimoto *et al.*, 1989; Huang *et al.*, 1989).

1.6.5.3 Down-regulation of PKC

In many cell types (Young *et al.*, 1987; Ase *et al.*, 1988), prolonged treatment with phorbol esters resulted in the complete depletion of cellular PKC level in a process termed down-regulation. This process prevented permanent kinase activation. PKC isoenzymes exhibit quite extreme differences in their

susceptibility to down-regulation *in vivo* (Kishimoto *et al.*, 1989; Huang *et al.*, 1989; Kochs *et al.*, 1993; Schaap *et al.*, 1990) which is in agreement with observations *in vitro*.

1.6.6 Phosphorylation of substrates

cPKC (Ca^{2+}-dependent PKC) isoenzymes appeared to be non-specific Ser/Thr kinases *in vitro*. Thus histones H1 or histones IIIS, myelin basic protein (MBP), protamine or any other basic protein or peptide could be used as an efficient substrate as long as it contained the phosphorylation site motif xRxxS/TxRx. Kinase activities of cPKC towards histone IIIS, protamine or MBP are very similar (Marais and Parker, 1989; Burns *et al.*, 1990). Members of the nPKC (Ca^{2+}-independent PKC) group differ significantly from cPKC isoenzymes in that they exhibit a rather poor kinase activity towards histone IIIS, MBP, protamine or protamine sulphate (Schaap and Parker, 1990; Olivier and Parker, 1991; Dekker *et al.*, 1992). Thus, it was sometimes necessary to use synthetic peptides derived from the respective pseudosubstrate sequence to detect kinase activity of nPKC isoforms, a fact that may explain the difficulties in detecting new PKC isoenzymes by biochemical analysis, i.e. the appropriate substrates are not yet available.

PKC is known to play an important role in phosphorylation of target proteins including various membrane proteins such as receptors (Houslay, 1991), the inhibitory guanine nucleotide regulatory protein $G_{i\alpha}2$ (Morris et al, 1994, Bushfield *et al.*, 1990; Murphy *et al.*, 1989) and adenylyl cyclase (Kawabe *et al.*, 1994).

In the absence of pure/specific isotype-specific PKC stimulators/inhibitors, it is difficult to assess the exact consequences of the activation of a certain isotype in terms of substrate phosphorylation and biological effect. However, a variety of stable cell lines have been generated (Chin *et al.*, 1993., Lehel *et al.*, 1994) that overexpress PKC isotypes,

allowing the examination of their effects on biological processes or with specific proteins of interest.

By over-expressing PKC isotypes in Chinese hamster ovary cells, it has been possible to determine that insulin receptor phosphorylation can be mediated *in vivo* by PKC-α, βI and γ, but not ε (Chin *et al.*, 1993). Stimulation of PKC-α, βI and γ, but not of PKC-ε by TPA inhibited the insulin-dependent activation of PI-3 kinase in these cells, indicating that phosphorylation by specific PKC isotypes inhibits insulin-stimulated responses (Chin *et al.*, 1993). The same results were found in both BC3H-1myocytes (Vila *et al.*, 1989) and rat adipocytes (Graves *et al*, 1985) where both insulin and TPA rapidly and comparably phosphorylated the same specific peptide fragments from several typical PKC substrates, e.g. 40,000 and/or 80,000 molecular weigh proteins.

In various cell types, the activation of PKC has been shown to result in alterations in the regulation of adenylyl cyclase activity (Houslay, 1991a). In some systems enhanced basal and agonist-stimulated actions have been observed, whereas, in others, a loss of receptor-mediated stimulation was recorded. The underlying molecular basis for these differences may lie in the cell-specific expression of particular control systems, where changes may be dependent on the expression of particular isoforms of PKC, adenylyl cyclase and cAMP phosphodiesterase, as well as the susceptibility of particular receptors to be phosphorylated. However, evidence from a number of studies (Houslay, 1991a, Bushfield *et al.*, 1991) has indicated that the inhibitory regulation of adenylyl cyclase, mediated through G_i, can be prevented by the action of PKC. Studies by Houslay, (1991a) and Bushfield *et al.*, (1990a and 1990b) indicated that PKC activation can lead to the phosphorylation of the α-subunit of the G_{i2} isoform on ser^{144} and to the loss of GTP-elicited G_i functioning in hepatocytes (Houslay, 1991a; Bushfield *et al.*, 1991a and 1991b; Morris *et al.*, 1994), U-973 cells (Daniel-Issakani *et al.*, 1989) and platelets

(Halenda *et al.*, 1986; Crouch *et al.*, 1988; Yatomi *et al.*, 1992). Such phosphorylation, however, only appears to occur in certain cell types and an additional mechanism for the PKC-mediated loss of G_i inhibition may exist, such as phosphorylation of the catalytic unit of type II adenylyl cyclase (Chen and Iyengar, 1993).

The phosphorylation of various transcription factors by PKC and other signal-transducing kinases has recently been revealed (Meek and Street, 1992). It should also be noted that amongst PKC substrates there might be several other protein kinases like S6 kinase or raf kinase.

Most protein kinases exhibit a rather pronounced autophosphorylation which is often, but not necessarily, linked to a modulation of kinase activity (Miller and Kennedy, 1986). Detailed studies on the role of autophosphorylation have mostly been carried out with cPKC isoenzymes and have revealed that it is an intramolecular reaction at serine and threonine residues on both the regulatory and catalytic domains (Huang *et al.*, 1986b) and strictly dependent on the presence of activators, as the enzymes Km value for ATP is about 10-fold lower than that for substrate phosphorylation (Huang *et al.*, 1986b). Autophosphorylation seems not to be a prerequisite for PKC activity. Protein phosphatases 1 and 2A are capable of dephosphorylating PKC without loss of PKC activity.

Based on the observation presented above, it is apparent that a large body of evidence has accumulated to indicate that PKC has a dual action, providing positive forward, as well as negative feedback, control over various steps in cell signalling processes.

1.7 Diabetes induced alterations in hepatocyte signalling pathways

Diabetics have long been known to have higher metabolic stress responses than non-diabetics ie. the catabolic pathways of glycogenolysis and

lipolysis are more readily activated in response to catecholamines (Shamoon and Daly, 1980). Most (80-90%) cases are type 2 (non-insulin dependent) diabetes in which insulin resistance is present in the major insulin target tissues *viz.* adipose tissue, muscle and liver (Shamoon and Daly, 1980). Such 'resistance' in liver being responsible for most of the fasting hyperglycemia observed in diabetes (Gerich, 1990). Insulin resistance encompasses a number of defects, including impaired receptor tyrosine kinase activity, reduced glucose transport, reduction in activities of enzymes involved in glucose metabolism and abnormal responses to other hormones (Defronzo *et al.*, 1982; Swislocke *et al.*, 1989).

1.7.1 Defects in hepatocyte adenylyl cyclase signalling in diabetes

Liver, being an insulin sensitive tissue, has been extensively used for studies of adenylyl cyclase signalling in diabetic animals. In diabetes, liver turns from a glucose-storing to a glucose-producing tissue. This follows a decrease in plasma insulin and increased plasma levels of the anti-insulin hormones, noradrenaline, adrenaline, glucagon and cortisol (Tamborlane *et al.*, 1979; Cryer, 1980). This increased liver responsiveness to anti-insulin hormones has also been observed in insulin-treated diabetic animals (Shamoon and Daly, 1980).

A common event of elevated cAMP was observed in liver from animal models of both type 1 diabetes e.g. streptozotocin-induced diabetic rats (Pilkis *et al.*, 1974) and type 2 diabetes e.g. the obese Zucker rat and db/db mice (Herberg and Coleman, 1977). Studies of diabetic rats have revealed either increased (Soman and Felig, 1978), decreased (Srikant *et al.*, 1977; Bhathena *et al.*, 1978) or unchanged (Chamras *et al.*, 1980) numbers of hepatic glucagon receptors, as well as elevated (Lynch *et al.*, 1989) or reduced (Pilkis *et al.*, 1974) basal and agonist-stimulated adenylyl cyclase activity in liver plasma membranes from chemically-induced diabetic rats. One possible explanation is

that animals differ in their toxic responses to diabetogenic agents. Changes in G-protein expression and function in liver from diabetic animals is suggested as being in part responsible for the altered adenylyl cyclase signalling. Gawler *et al.*, (1987) observed marked enhancement in the ability of glucagon to activate adenylyl cyclase in hepatocyte plasma membranes from diabetic rats which they suggested was due to a decreased level of expression of the inhibitory G-protein G_i. Extended analysis by Bushfield *et al.*, (1990a) investigated the effect of streptozotocin-induced diabetes on the expression of the stimulatory G-protein, G_s, on the different froms of G_i and on G-protein β-subunits in both hepatocytes and liver, as well as analysing the phosphorylation state of $G_{i\alpha}2$ (Bushfield *et al.*, 1990). The decreased level of $G_{i\alpha}2$, reduced by about 50%-80% in diabetic animals compared with that found in normal animals, was consistent with the effect of diabetes in diminishing the number of $G_{i\alpha}$ subunits available for pertussis-toxin-catalysed ADP-ribosylation in hepatocyte membranes (Houslay *et al.*, 1989). Also Bushfield *et al.*, (1990b) found a reduction in the levels of $G_{i\alpha}3$ and the 42 kDa form of $G_{s\alpha}$ in hepatocyte plasma membranes from diabetic rats, as well as increased expression of the adenylyl cyclase catalytic subunit. As $G_{i\alpha}1$ is not expressed in liver and $G_{i\alpha}3$ is not thought to mediate adenylyl cyclase inhibition this finding suggested a dysfunctional $G_{i\alpha}2$. It was suggested in hepatocytes from diabetic animals, that the residual $G_{i\alpha}2$ appears to be fully phosphorylated, at the putative 'C-kinase site' (Bushfield *et al.*, 1990a), under basal conditions. The decrease in the activity of this G-protein by phosphorylation through PKC can be shown by treatment of normal rat hepatocytes with agents which can activate PKC. This phosphorylates $G_{i\alpha}2$ leading to the loss of guanine nucleotide inhibition of adenylyl cyclase. It is tempting to speculate that such an increased phosphorylation of $G_{i\alpha}2$ may characterize many insulin-resistant states.

Changes in the ability of G-proteins to be labelled by pertussis toxin have been observed in diabetes. Lynch *et al.*, (1989) found reduced pertussis

toxin-catalysed ADP-ribosylation of total G_i in hepatocyte membranes from diabetic animals. The ability of pertussis toxin to label G_i in human diabetic liver has also been noted to be impaired (Caro *et al.*, 1991). They also noticed that the ability of insulin to attenuate pertussis toxin-catalysed ADP-ribosylation of G_i was impaired in liver tissue from diabetic humans. These findings tie in with the view of Rothenbery and Kahn (1988) who suggested insulin's ability to inhibit pertussis toxin-labelling of G-proteins implied a functional interaction between the two signalling systems, possibly by insulin influencing G_i conformation. Recently, Moxham and Malbon (1996) used cells from transgenic mice harbouring inducible expression of RNA antisense to the gene encoding $G_{i\alpha}2$ (Moxham and Malbon, 1993; Moxham *et al.*, 1993) to show that $G_{i\alpha}2$ is critical for insulin action. The $G_{i\alpha}2$ deficiency creates a model for insulin resistance characteristic of non-insulin-dependent diabetes mellitus (Reaven, 1995), implicating $G_{i\alpha}2$ as a positive regulator of insulin action (Moxham and Malbon, 1996). These findings again raise the possibility of an abnormality of G_i function in diatetes.

1.7.2 Diabetes induced alterations in PKC expression in hepatocytes

Activated PKC has indeed been reported in diabetes as a result of increased diacylglycerol levels formed from excess glucose (Greene *et al.*, 1987) and may impair insulin action by increasing insulin receptor phosphorylation. The observations of loss of tonic GTP-elicited G_i function (see above) also suggest a profound effect upon the level of expression and the distribution of PKC isoforms in hepatocytes of streptozotocin-induced diatetic rats.

Tang *et al.*, (1993) demonstrated that an overall increase in the expression of PKC-α, in both membrane and cytosol fractions of diabetic rat hepatocytes, coupled with translocation of a portion of the PKCα ~81KDa species to the membrane fraction. Streptozotocin-induced diabetes elicited a

marked increase in the amount of cytosolic PKC-βII, this was also accompanied by the ability to detect immunoreactive PKC-βII in membranes from the hepatocytes of diabetic rats, implying that translocation of PKC-βII from the cytosol had occurred (Tang *et al.,* 1993). The increase in the levels and altered distribution (between membrane and cytosol compartments) of certain protein kinase C isoforms may thus provide an explanation for the aberrant increase in the level of protein kinase C-mediated phosphorylation of $G_{i\alpha}2$ seen in the hepatocytes of streptozotocin-induced diatetic rats (Bushfield *et al.,* 1990b).

The changes in PKC expression are likely to have widespread effects, some of which may in themselves affect the action of insulin on target tissues. For the insulin receptor itself is known to provide a substrate for this enzyme (Takayama *et al.,* 1988; Houslay, 1989; Koshio *et al.,* 1989; Lewis *et al.,* 1989) with such modifications being implicated as attenuating at least certain of the functions fo the receptor and thus exerting a selective effect on its different signalling actions (Houslay, 1989). It may be then that aberrant expression of specific PKC isoforms can alter cell signalling pathways leading to selective hormone resistant states.

1.7.3 Diabetes induced changes in cAMP phosphodiesterase activity

As well as defects at the level of the membrane, decreased cAMP phosphodiesterase activity may account in part for the increased catecholamine and glucagon responsiveness of diabetic liver. In fact, Solomon *et al.,* (1986) found a reduction in the activity of the high affinity low K_m cAMP phosphodiesterase in liver from streptozotocin diabetic rats which would allow the exaggerated intracellular cAMP responcs. Meanwhile, streptozotocin-induced diabetic rats show elevated levels of glucagon and heightened glucagon-stimulated adenylyl cyclase activity in hepatocytes (Gawler *et al.,*

1987; Bushfield *et al.*, 1990b). As such, it is likely that cAMP levels in these cells will have been chronically elevated upon onset of diabetes.

Calmodulin is a ubiquitous, highly conserved protein throughout the phylogenetic scale, in addition to its capacity to bind Ca^{2+}, it serves as an important biochemical regulator as both CaM-dependent protein kinase(s) and cyclic nucleotide phosphodiesterase(s) have been identified (Cheung, 1970; Smoake and Solomon, 1989). Evidence presented in Smoake *et al.*, (1995) suggested that low K_m PDE is CaM dependent, as three different CaM antagonists reduced the activity of low K_m PDE. Also, insulin stimulated the activity of this PDE in isolated membranes in the absence of these inhibitors but not in their presence (Smoake *et al.*, 1995).

This concept of CaM involvement in insulin regulation of PDE was suggested by Solomon (1981, 1986, 1987) with the observations of a reduction in both CaM and PDE activity from liver and adipose tissues from streptozotocin diabetic and spontaneously diabetic rats. PDE and CaM activities were returned to normal with insulin teatment (Solomon *et al.*, 1979 and 1987). Hence, PDE alterations are known to occur in the diabetic state.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Tissue culture plastic ware:

Costar Co	Filters (0.22μm)
U.S.A	Falcon tissue culture flasks 75cm^2
	Multiple cell plates
	50ml centrifuge tubes
	Tissue culture pipettes

2.1.2 Tissue culture medium:

Gibco Life Technologies	Hams-F12 medium
Paisley, UK	Dulbecco's modification of Eagle's medium (10x)
	Foetal calf serum
	Glutamine (200mM)
	Sodium bicarbonate (7.5%)
	Penicillin/Streptomycin (10000 IU/ml)
	Trypsin/EDTA

2.1.3 Radio chemicals:

Amersham PLC, Amersham	[8-^{3}H]Adenosine-3'5'-cyclic
Buckinghamshire, U.K.	monophosphate
	[5, 8-^{3}H] Adenosine 3'5'cyclic monophosphate
	[^{125}I]-labelled anti-rabbit IgG
	[^{14}C]-labelled protein molecular weight markers

2.1.4 General reagents:

2.1.4.1 Chemical compounds:

BDH, MERCK Ltd	Glucose
Lutterworth, Leics, U.K.	Hepes
	EDTA
	Sodium dodecyl sulphate
	Citric acid
	Glycine
	Methanol
Sigma	Tris
Poole, Dorest, U.K.	Tween-20
	EGTA
	Triton X-100
Fisons Scientific Equipment	Sucrose
Loughborough, U.K.	Sodium Citrate
	Glycerol
	Dimethyl sulphoxide
	Sodium Acetate
Boehringer	Triethanolamine hydrochloride
West Germany	

2.1.4.2 Biochemical reagents:

Rhone Merieux	Sagatal
Harlow, Essex, U.K.	
Worthington Biochemical Co	Collagenase
Freehold, New Jersey, U.S.A.	
Sigma	Cytochalasin B
Poole, Dorest, U.K.	Theophylline
	Aprotinin

Antipain

Pepstatin A

Benzamidine hydrochloride

Phenylmethylsulphonyl fluoride

Sodium pyruvate

ATP

GTP

GppNHp

BSA

Calmodulin (bovine brain)

Cyclic-AMP

Cyclic-GMP

Dextran T-500

Dowex1X8-400 (chloride form 200-400mesh)

3-isobutyl-1-methylxanthine

Snake venom (*Ophiophagus hannah*)

Bromophenol blue

TEMED

Ponceau stain

β-mercaptoethanol

Collagen

Cholera toxin

12-O-tetradecanoylphorbol 13-acetate

Insulin

Glucagon

Vasopressin

Alkaline phosphatase

Streptozotocin

	Staurosporine
	Chelerythrine
	Calphostin C
	Actinomycin D
Peptide Research Foundation	Leupeptin
London, U.K.	
BDH, Merck Ltd	Universal indicator
Lutterworth, Leics, U.K.	Charcoal (Norit Gsx)
	Dithiothreitol
Calbiochem	Forskolin
Cambridge, U.K.	Pansorbin
Boehringer Co	Diabur-Test 5000
Lewes, East Sussex	Nicotinamide adenine dinucleotide
	(reduced U.K.form)
	Creatine phosphate
	Creatine kinase
Bio-rad Laboratories Ltd	30% Acrylamide/bis acryclimide mix
Hertfordshire, U.K.	(29:1)
	Bradford reagent
National Diagnostics	Ecoscint, scintillation fluid
Buckinghamshire, U.K.	
M & B	Ammonium persulphate
Dagenham, UK.	
BRL	Pre-stained molecular weight markers
Paisley, Scotland	
Amersham Life Science	ECL(Amersham enhanced
Buckinghamshire, U.K.	chemiluminescence)
SAPU	HRP Anti-rabbit IgG

Scotland, U.K.	
Gift from Aktiengesellschaft	Rolipram
Berlin, Germany	
Pfizer Central Research	Cilostimide
Sandwich, Kent, U.K.	
Gift from Dr. M. Wigler	cDNA encoding DPD (PDE4B splice
Cold Spring Harbor	variant)
Long Island, U.S.A.	

2.1.5 Molecular Biology reagents:

Pharmacia Biotechnolgies	First strand cDNA synthesis kit
Herts, U.K.	
Sigma	Tri-reagent
Poole, Dorest, U.K.	Ethidium bromide
	Diethyl pyrocarbonate (1%)in water
Promega	Taq polymerase
Southampton, U.K.	$MgCl_2$
	Taq buffer
	Deoxyribonucleoside trisphosphates
	Molecular weigh markers
	Wizard mini preps (DNA purification system)
Stratagene	"Taq Start Antibody"
Cambridge, U.K.	"Perfect match"

2.1.6 Equipment:

Fison	100µm mesh screens
Loughborough, U.K.	
Progene	PCR amplification machines

Cambridge, U.K.	
Belmont Instrument	Perfusion equipment (Watson-Marlow
Glasgow, Scotland, U.K.	502S)
Amicon	Pressure cell 8050 Max 75Psi,
Gloucestershire, U.K.	5.3kg/cm^2

2.1.7 Animal resources

Male adult Sprague-Dawley rats were purchased from commercial sources with weight range between 200-250g. Settled animals were used as follows: (1) Injection with streptozotocin in order to induce diabetes or (2) dose with sagatal by i.p.(intraperitoneal) injection in order to anaesthetise the animal. This was checked by testing eye and leg reflexes (for consciousness) after which the perfusion procedure was carried out as described in method section 2.3.1.

2.1.8 Cell lines

P9 cells: This is a SV40-immortalised hepatocyte-derived cell line described by first by Livingstone *et al.*, (1994). This cell line was generated by transfection of rat hepatocytes with SV-40 to produce "hepatocyte-like" cells with an unlimited proliferative potential.

CHO cells: Wild-type CHO cells and cells transfected so as to overexpress the α, β-II, γ an ε forms of protein kinase C were generously given by Dr. Richard A. Roth, Department of Pharmacology, Stanford University, U.S.A. The generation and characterization of these cell lines, which were also transfected so as to overexpress the human insulin receptor, are described in Chin, *et al.*, (1993).

2.2 Media, buffers and standard solutions

Chinese Hamster Ovary (CHO) cell were cultured in HAM-F12 medium supplemented with 1% (v/v) penicillin/streptomycin, 1% (v/v) glutamine and 10% (v/v) foetal calf serum

P9, NIH-3T3 and COS-7 cell were cultured in DMEM(x1) medium supplemented with 1% (v/v) penicillin/streptomycin 1% (v/v), glutamine 10% (v/v) and foetal calf serum

2% cell culture medium DMEM (x1) supplemented with 1% (v/v) penicillin/streptomycin 1% (v/v), glutamine 2% (v/v) and foetal calf serum.

Serum-free medium DMEM (x1) supplemented with 1% (v/v) penicillin/streptomycin, 1% (v/v) glutamine

Cell freezing buffer; supplement appropriate medium with
2 5% foetal calf serum and10% DMSO.

Rat hepatocyte perfusion buffer: Ca^{2+} free buffer containing final concentration of

25.3mM $NaHCO_3$

4.8mM KCl

1.2mM $MgSO_4$

1.2mM KH_2PO_4

120mM NaCl

5mM glucose
equilibration with 95%O_2:5%CO_2

For Ca^{2+} containing buffer add 1.28mM $CaCl_2$.

Incubation buffer : As Ca^{2+} containing buffer but containing final concentrations of 2% (w/v) BSA & 10mM Hepes.

Collagenase containing buffer: 40mg collagenase (added immediately before use) and 2% (w/v) BSA in Ca^{2+} containing buffer.

Protease inhibitor cocktail	2mM Aprotinin
	2mM Antipain
	2mM Pepstatin A
	2mM Leupeptin
	2mM Benzamidine
	0.1mM PMSF
	diluted 1:1000 in buffer for use
Hepatocyte washing buffer:	20mM Tris/HCl (pH7.4)
	150mM NaCl
	5mM glucose
	protease inhibitor cocktail
Hepatocyte homogenisation buffer:	20mM Tris/HCl (pH7.4)
	2mM EDTA
	2mM EGTA
	protease inhibitor cocktail
P9 cell washing buffer:	145mM NaCl
	6mM KCl
	10mM glucose
	10mM Hepes (pH 7.4)
	protease inhibitor cocktail

P9 cell homogenisation buffer:	20mM Tris/HCl (pH7.4)
	1mM EDTA
	250mM sucrose
	protease inhibitor cocktail
PDE assay buffer:	20mM Tris/HCl (pH 7.4)
	10mM $MgCl_2$
cAMP binding assay buffer:	50mM Tris/HCl (pH7.4)
	4mM EDTA

Buffer for preparation of cAMP binding protein :

250mM Sucrose

25mM KCl

5mM $MgSO_4$

50mM Tris/HCl (pH 7.4)

Charcoal solution buffer: cAMP binding assay buffer containing

2% (w/v) charcoal (Norit GSX)

1% (w/v) BSA

Adenylyl cyclase ATP regeneration x2	Creatine Phosphate (7.4mg/ml)
	Creatine kinase (0.2mg/ml)
	BSA (0.8mg/ml)
	ATP (0.9mg/ml)
	in x2 incubation medium of
	25mM triethanolamine hydrochloride
	(pH7.4 with KOH)
	1mM EDTA

10mM theophylline

5mM magnesium sulphate

DTT 1mM

COS-7 cell washing buffer: 10mM Triethanolamine, pH7.2

150mM KCl

COS-7 cell homogenisation buffer: (KHEM complete buffer)

50mM KCl

50mM Hepes, pH7.4

10mM EGTA

1.92mM $MgCl_2$

1mM DTT

protease inhibitor cocktail

20µg/ml cytochalasin B

KHEM imcomplete buffer: As KHEM complete buffer but not containing protease inhibitor and cytochalasin B.

RNA gel running buffer: (0.5x): 45mM Tris/HCl, pH8

45mM Boric acid

1mM EDTA

Immunoprecipitation buffer: 1% Triton X-100

10mM EDTA

100mM $NaH_2PO_4.2H_2O$

50mM Hepes, pH7.2

protease inhibitor cocktail

SDS/PAGE resolving (8% gel):	30% acrylamide/bisacrylamide mix (29:1) 1.5M Tris/HCl, pH 8.8 10% (w/v)SDS 10% (w/v)ammonium persulphate0.06% (v/v) TEMED to a final volume of 30ml.
SDS/PAGE stacking (8% gel):	30% acrylamide/bisacrylamide mix (29:1) 1.0M Tris/HCl (pH 6.8) 10% SDS 10% ammonium persulfate 0.1%TEMED to a final volume 8ml
SDS/PAGE gel running buffer:	0.2M Glycine 0.25mM Tris 0.1% (w/v) SDS
Western blotting buffer:	0.2M Glycine 25mM Tris 0.1% (w/v) SDS 20% Methanol
Laemmli buffer:	0.02M Tris/HCl pH 6.8 20% (v/v) Glycerol 2% (w/v) SDS 2mM EDTA 0.02% Bromophenol blue add 5% of β-mecaptoethanol before use

Tris buffered saline: 20mM Tris/HCl, pH7.5

0.5M NaCl

Cell transfection medium: Appropriate medium supplemented with

10% (v/v)Nu serum

100μM Chloriqine

Nuserum: a synthetic serum replacement obtained from Collaborative Biomedical Products, Catalogue No. 55000.

2.3 Methods

2.3.1 Hepatocye preparation

2.3.1.1 Hepatocyte isolation

Hepatocytes were prepared by a modification of the method of Berry and Friend (1969). The buffer used throughout the liver perfusion and the subsequent hepatocyte incubations was Krebs bicarbonate buffer as detailed previously (see 2.2). Male Sprague-Dawley rats weighing 225-250g were anaesthetised by intraperitoneal (i.p.) injection with sagatal (0.5ml). After about 10 minutes, the unconscious rats were used for perfusion as detailed below.

The abdomen was opened by a longitudinal incision, the viscera displaced and the inferior vena cava and hepatic portal vein exposed. The inferior vena cava was cannulated with a N°.15 gauge needle containing anti-coagulant(100mM EDTA). The hepatic portal vein was immediately cannulated with a 1.1 mm needle and perfused with Ca^{2+} free Krebs bicarbonate buffer containing 5mM glucose at a rate of 30ml/minute. The thoracic cavity was opened and the inferior vena cava ligatured just below the right atrium to prevent back flow of the blood from the heart.

The buffer entered the liver through the hepatic portal vein and flowed to waste via the inferior vena cava. Ca^{2+} free Krebs bicarbonate buffer was required to flush the blood from the liver and to break down Ca^{2+} dependent junctions between the cells. Approximately 250ml of this buffer was pumped

through the liver before changing to a collagenase-containing buffer to digest the liver (Krebs bicarbonate containing 0.2% BSA with 40mg collagenase). This buffer was re-circulated through the liver for 15-20 minutes until a suitable degree of digestion was obtained as indicated by a marked increase in leakiness and the appearance of blisters of buffer trapped beneath the surface of the liver. At this point the liver was removed to 60ml of Krebs bicarbonate buffer containing 5mM glucose, chopped with scissors and washed through nylon mesh (pore aperture 100μm) with a further 100ml of buffer.
Buffer were gased with 95%O_2:5%CO_2 before used.

2.3.1.2 Hepatocyte disruption by pressure

Washed hepatocytes were resuspended in freshly prepared homogenisation buffer, and 4ml (approximately $4x10^6$ cells/ml) of this cell suspension was placed in plastic vials. These were then placed in a pressure cell, which was pressurised to 50PSI for 10min with O_2 free N_2. Finally the vessel was rapidly depressurized and the cells used for homogenisation after this stage.

2.3.1.3 Preparation of a hepatocyte (crude) membrane fraction

Cells (treated with appropriate ligands for desired incubation period), were placed on ice and 0.7ml samples of cell suspension quenched by adding an equal volume of ice-cold 1mM $KHCO_3$ pH7.2. All further procedures were performed at 4°C. Quenched reaction samples were then centrifuged at 13,000rpm for 10min in a bench-top microcentrifuge, supernatant fluid was removed and the cells were resuspended in 300μl of 1mM-$KHCO_3$, pH 7.2. Following centrifugation on a bench-top microcentrifuge at 13,000rpm for 5 min at 4°C, the pellet was resuspended in 100μl of 1mM-$KHCO_3$, pH 7.2 to yield a crude membrane fraction. In all cases membranes were assayed within 2 hours of preparation.

2.3.1.4 Fractionation of hepatocytes

Following incubation of hepatocytes with appropriate ligands, reactions were stopped by adding an equal volume of ice-cold washing buffer. Cells were then centrifuged at 1000g for 2min at 4°C. The supernatant was aspirated and the cells were washed again by resuspension in the same washing buffer and centrifuged as before. After pressurisation, hepatocytes were homogenised gently in a glass vessel using a Teflon pestle and subsequently fractionated as described for P9 cells.

2.3.2 Attachment of hepatocytes to collagen plates

Hepatocytes isolated as described earlier (section 2.3.1.1) were plated out by adding 1ml of cell suspension to collagen-coated (0.3mg/ml) Petri dishes and placing them in an incubator for 40min until the cells had adhered to the plates but not spread. After this period, cells that had not adhered were aspirated and the plates were washed once with Krebs-Henseleit buffer (1ml/well) before the addition of hormones.

2.3.3 Assessing desensitization in immobilized cells

Hepatocytes were attached to collagen plates and incubated for 40 minutes to allow adherence. Medium was then aspirated from the immobilized hepatocytes before the addition of fresh medium (1ml) containing the indicated ligands or with medium alone to determine the control state. They were then incubated for 6min in this pre-incubation/desensitization period. Cells were then washed with Krebs buffer (0.9ml/well) to remove the ligand, and left for 6min at 37°C. Over this period, irrespective of the pre-incubation conditions, intracellular cyclic AMP levels stabilized to that seen for the cells at the start of the experiment under basal conditions. After this washing and stabilization period, glucagon desensitization was assessed by determining the ability of glucagon to

stimulate an increase in intracellular cyclic AMP. This was done by treating the cells with 10nM glucagon for 3min and subsequently harvesting them to determine the intracellular concentration of cyclic AMP.

2.3.4 Determination of ATP content of hepatocytes

The ATP content in isolated hepatocytes was determined by the luciferase method on a neutralized $HClO_4$ extract. Cells with ATP concentrations of 8.6 nmol/mg dry weight were judged to be viable, and were therefore used (Heyworth and Houslay, 1983).

2.3.5 Treatment of hepatocytes

2.3.5.1 Incubation of hepatocytes with ligands

Cells were centrifuged at 40g for 2 minutes then resuspended in the hepatocyte washing buffer; this washing procedure was repeated twice. The isolated hepatocytes were finally resuspended in 2% BSA/Krebs bicarbonate buffer with 5mM glucose. Cells were placed in plastic containers under an atmosphere of O_2/CO_2: 95%/5%, which was maintained by gassing every 10-15 minutes. The cell suspension was preincubated for 30 minutes at 37°C in a shaking water bath with sufficient shaking to prevent the cells settling. Most experiments entailed addition of 2ml of hepatocytes suspension to disposable vials containing the appropriate ligands. The vials were gassed with O_2/CO_2: 95%/5%, then sealed and placed in a shaking water bath at 37°C, for the desired time of incubation.

2.3.5.2 Treatment of hepatocyte membranes with alkaline phosphatase.

A hepatocyte membrane fraction was prepared and resuspended in the buffer as described previously (section 2.2). Then 100µl of this, containing 200µg membrane protein, was treated with 20 units of alkaline phosphatase

for 10min at 37°C. An equal volume of 4% PCA was added to stop the reaction. The extract was then used for assay of adenylyl cyclase activity.

2.3.6 Cell culture

2.3.6.1 Culture of P9 cells

Cells were grown at 37°C, in a 5% CO_2/95%O_2 humidified incubator in 75cm^2 cell culture flasks with DMEM (x1) as described earlier. Confluent cells were passaged by trypsinisation followed by centrifugation at 600rpm for 5min. The cell pellet was re-suspended in complete culture medium and seeded at a split ratio of 1:6 into new cell culture flasks.

2.3.6.2 Culture of CHO cells

For the culture of CHO cells, HAMS-F12 medium was supplemented by the addition of 1% penicillin/streptomycin and glutamine; 10% foetal calf serum was also included. Cells were grown in 75cm^2 flasks at 37°C in an atmosphere of 95% air/5% CO_2 in a humidified incubator. Confluence was attained within 3-4 days, and cells were routinely split 1:4 by trypsinisation.

2.3.6.3 Culture of NIH-3T3 cells

For the culture of NIH-3T3 cells, DMEM medium was supplemented by the addition of 1% penicillin/streptomycin and glutamine; 10% foetal calf serum was also included. Cells were grown in 75cm^2 flasks at 37°C in an atmosphere of 95% air/5% CO_2 in a humidified incubator. Confluence was attained within 3-4 days, and cells were routinely split 1:4 by trypsinisation.

2.3.7 Treatment of P9 cells

2.3.7.1 Preparation of P9 membrane and soluble fractions

Confluent P9 cells were washed and then incubated on ice in the presence of 1.5ml (per flask) homogenisation buffer containing fresh protease

inhibitors. Cells were scraped off the plate into microfuge tubes and spun for 5min in a bench-top microcentrifuge to remove cell debris and hormone medium. 300µl of homogenisation buffer was added per tube and the pellet re-suspended and homogenised using a 1ml syringe within 30 up/down strokes. This was recognised as total homogenate. Lysed cells were spun at 1,000g for 10mins at 4 °C, to yield supernatant regarded as a post nuclear fraction and a pellet of nuclei and cell debris, unbroken cells and plasma membrane. This supernatant was then spun at 100,000g at 4°C for 1h, to yield supernatant regarded as cytosol and a pellet considered as a membrane fraction.

2.3.7.2 Actinomycin D treatment of cells

After removing cell culture medium, cells were incubated with 2% serum medium supplemented with actinomycin D alone at the required concentration (1µg/ml) for 45minutes before any agonist challenge. Incubation with other hormones for the appropriate times was then performed, still in the presence of actinomycin D.

2.3.7.3 Bacterial toxin treatment of cells

After removing cell culture medium, cells were incubated in serum-free medium (containing IBMX 1mM and BSA 1%) supplemented with cholera toxin at the indicated concentration for the stated times.

2.3.7.4 Longterm hormone treatment of cells

After cells nearly 90% confluent in DMEM (x1) culture medium, cells were changed into 2% serum medium supplemented with forskolin and cholera toxin at the concentration of 100µM and 1µg/ml respectively for various time until 24 hours.

2.3.8 Thermal denaturation of adenylyl cyclase

A washed membrane fraction was prepared as described in the method above (section 2.3.1.3) and the pellet fraction was resuspended in 1mM $KHCO_3$ (pH 7.2) buffer. Sample were incubated at 50°C in a shaking water bath, aliquots (100µl) were then removed into ice-cold microfuge tubes every 30 second over a period of 10min. 10µl of samples were then assayed for adenylyl cyclase activity (section 2.3.10) with stimulation by forskolin.

2.3.9 Cyclic AMP binding assay.

2.3.9.1 Assay principle

The measurement of cyclic AMP concentrations was carried out as outlined by Kishihara *et al.*, (1993). The binding protein utilised in the assay is cyclic AMP-dependent protein kinase (PKA) which has binding sites for cyclic AMP. A crude preparation of adrenal glands was made (Brown *et al.*, 1972), which is a rich source for this enzyme (scetion 2.3.9.2). Upon stimulation of the experimental cells, in the presence of the desired effectors for a fixed time, the cells reaction were stopped by adding 2% final concentration of PCA. After neutralising the sample, the unknown cyclic AMP extraction were incubated with [^{3}H]-cyclic AMP (5- and 8- position of the adenine ring) and cyclic AMP binding protein, allowing competition of labelled and unlabelled cyclic AMP for a limited number of binding sites on the binding protein. A standard curve was obtained by incubating known concentrations of unlabelled cyclic AMP with fixed amounts of binding protein and radioactive cyclic AMP. Thus, the unknown cyclic AMP concentrations can be determined in samples by comparison with the standard curve. After incubation, activated charcoal was added to the sample to absorb unbound cyclic nucleotide from the solution. The charcoal (Norit Gsx) with the bound cyclic nucleotide was removed by a brief centrifuging step, and a proportion of radioactivity in the sample was ascertained.

2.3.9.2 Preparation of cyclic AMP binding protein.

Bovine adrenal glands (20-30), obtained from the abattoir were transported to the laboratory on ice. At 4°C, the fat was removed from the outside of glands and they were then hemisected revealing the pale coloured inner medulla and the darker outer cortex. The medulla was removed and discarded, retaining the cortex. One volume of tissue was homogenised in one and a half volumes of buffer (250mM sucrose / 25mM KCl / 5mM $MgSO_4$ / 50mM Tris/HCl, pH7.4) in a Waring blender. The homogenate was filtered through muslin and then centrifuged at 15 000rpm on MSE 18 for 15 minutes at 4°C. The supernatant was decanted through filter paper, and the eluate was aliquotted into 1ml fractions and stored at -20°C for up to 3 months. Separate samples were thawed and diluted for each assay.

2.3.9.3 Assessing intracellular cAMP concentrations

(A) For hepatocyte cell suspensions, after the appropriate incubation period, 100µl of ligand treated sample was placed into a microfuge tube containing 100µl of 4% PCA (samples were assayed in triplicate for each condition). The precipitated protein was pelleted by centrifugation in a bench-top microcentrifuge for 2 minutes. Universal indicator was added to the supernatant and the solution was then neutralised by addition of 2M KOH / 0.5M triethanolamine (TEA). Samples were then pelleted in a microfuge for 3 minutes, and 50µl of the supernatant was removed for assay of intracellular cyclic AMP (section 2.3.9.4) using a cyclic AMP binding protein prepared as described previously (section 2.3.9.2)

(B) Incubations of cultured cells with ligands (volumes added were not more than 2% of total incubation sample volume) were stopped by aspirating the medium from the multiple-well plates in which cells were grown and adding 250µl of 2% PCA to terminate the reaction. Cells were incubated on ice for 2-3 minutes to allow lysis of the membrane, and the extraction of the

cyclic nucleotides. Cells were then scraped and pelleted by centrifugation in a bench-top microcentrifuge for 2 minutes. Neutralisation of samples was then carried out as described in section (A).

2.3.9.4 Assay procedure

As mentioned previously, a standard curve was prepared for each assay, using cyclic AMP amounts between 0.0625 and 16 pmols. This part of the assay was carried out in duplicate, whilst the unknown samples were assayed in triplicate.

In each tube, assays were performed by adding 50µl of supernatant from the unknown samples to 50µl of cAMP binding assay buffer (50mM Tris/5mM EDTA pH7.4). 100µl of 5, 8-^{3}H-adenosine 3'5' cyclic monophosphate (diluted in Tris/EDTA buffer to give approximately 500,000cpm/ml) was added to the sample. For standards, cyclic AMP solutions containing 50µl of known cAMP amounts (from 0.0625pmole to 16pmole) were added to 50µl of cAMP binding assay buffer, together with 100µl of tritiated cAMP. 100µl of binding protein was added to the samples and tubes were vortexed and incubated at 4°C for 2-3 hours. After this time, 250µl charcoal solution was added, and tubes were immediately vortexed and centrifuged in a microfuge at 12,000g_{av} for 5 minutes. A 300µl aliquot was removed from the resulting supernatant, added to 2ml Ecoscint and counted and a curve fitting program used to determine pmols cAMP relative to the standard curve.

2.3.10 Adenylyl cyclase assay

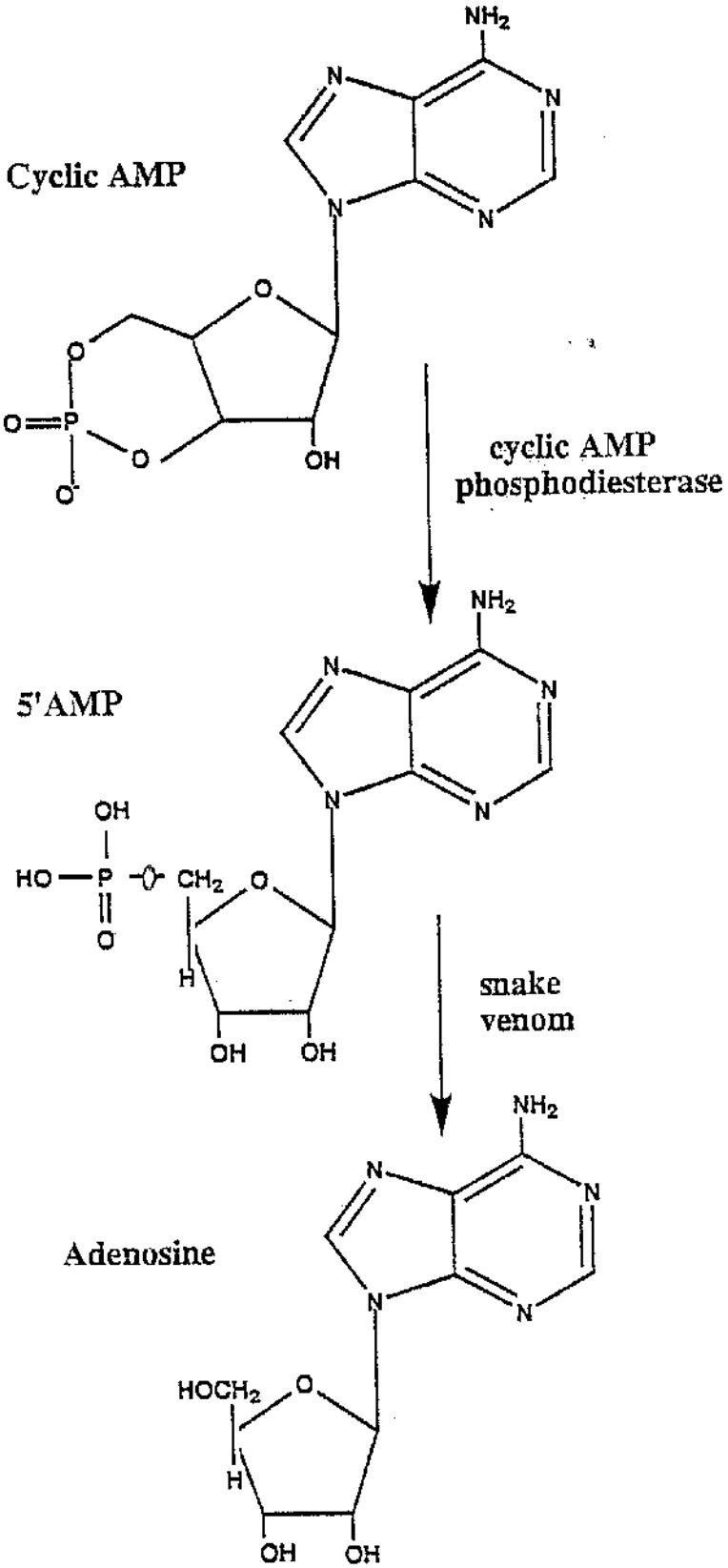
Adenylyl cyclase activity was determined by a modification of an assay described previously by Houslay *et al.*, 1976. Briefly, this involved using an assay mixture containing 10µl of membranes (30-40µg/µl protein), 50µl of buffer/ATP regenerating system (section 2.2), ligands present at the concentrations required and a final volume made up to 100µl with water. These were then incubated at 30°C for 10 minutes before an equal volume of 4% PCA was added to stop the reaction. The samples were centrifuged, the supernatants were neutralised with TEA/HCl pH7.4 and the cyclic AMP content was measured as described previously (section 2.3.9)

2.3.11 Cyclic AMP phosphodiesterase assay.

2.3.11.1 Assay principle

Cyclic AMP phosphodiesterase activity was assayed by a modification of the two-step procedure of Thompson and Appleman (1971) and Rutten *et.al.* (1973) as previously described by Marchmont and Houslay (1980b). The principle of the assay is outlined in **Figure 2.1**. Briefly, [^{3}H]-cyclic nucleotide (8 position of the adenine ring) is hydrolysed to form labelled nucleotide mono-phosphate. The nucleotide mono-phosphate ring is then converted to the corresponding labelled nucleoside by incubation with snake venom which has 5'-nucleotidase activity. The conditions are such that complete conversion takes place within the incubation time. Unhydrolysed cyclic nucleotide is separated from the nucleoside by batch binding of the mixture to Dowex-1-chloride. This binds the charged nucleotides, but not the uncharged nucleoside

Figure 2.1 The principle of the cyclic AMP PDE assay


Cyclic AMP
cyclic AMP
phosphodiesterase
5'AMP
snake
venom
Adenosine

2.3.11.2 Preparation of Dowex-Cl

400g Dowex was added to 4L of 1M NaOH and stirred gently for 15min. This was left to settle, and the Dowex was washed in distilled water until a pH of approximately 7 attained.

4L of 1M HCl was then added and stirred for 15min, Dowex resin was washed in distilled H_2O until the pH was approximately 3. Dowex was stored as 1:1 (v/v) slurry solution in H_2O at 4°C, and used as 1:1:1 (Dowex:ethanol:H_2O) slurry in the PDE assay.

2.3.11.3 Assay procedure

The enzymes sample (approximately 150µg protein/25µl) was added to 20mM Tris/HCl, pH7.4, 5mM $MgCl_2$ (25µl) containing the required phosphodiesterase effectors at the desired concentration, at 4°C. The reaction was started with the addition of 2µM cAMP containing 0.15µCi cAMP (50µl), mixing the tubes by vortexing, and incubating for 10 minutes at 30°C. After this time the tubes were placed in a boiling water bath for 2 minutes to terminate the phosphodiesterase activity. The samples were then allowed to cool on ice. 25µl snake venom (1mg/ml) was added to the tubes and they were incubated for 10mins in a 30°C waterbath. The tubes were then placed on ice, and freshly prepared slurry of Dowex:ethanol:water (1:1:1) (400µl) was added. During this addition, the Dowex was stirred gently to ensure that a homogeneous suspension was being added. The tubes were vortexed twice over a 15 minute period, and then spun in a benchtop centrifuge (Jouan) at 12 $000g_{av}$ to sediment out the Dowex resin. An aliquot of 150µl supernatant was removed and mixed with 2ml Ecoscint, before being counted in a liquid scintillation counter for 1 minute per sample.

All assays were conducted at 30°C. PDE inhibitors were dissolved in DMSO at a stock concentration and subsequently diluted in 20mM Tris/HCl

(pH 7.4) to provide a range of drug concentrations. Routine cyclic AMP-PDE activity assays use a cyclic AMP substrate concentration of 1μM.

2.3.12 Bradford assay

The protein concentration in the membrane and cytosolic fractions was determined by the Bradford (1976) method using BSA as a standard.

2.3.13 Lactate dehydrogenase (LDH) assay

The assay was used to provide a measure of synaptosomal integrity. The level of LDH was ascertained by measuring the rate of oxidation of NADH. NADH absorbs at 340nm and therefore the rate of decrease of optical density at 340nm absorbance is a measure of enzyme activity.

Free LDH was measured first, the Triton (2% v/v final) was used to burst open any intact cells and total LDH measured. The difference between the total and the free LDH levels gives the occluded LDH in the cells under investigation. Each reaction comprised 1.35ml Tris/HCl (0.15M), 50μl sodium pyruvate (10mM) and 50μl tissue sample in a final volume of 1.5ml where 50μl β-NADH (2mM) is added as substrate. A_{340} was determined before and after addition of LDH.

$$\% \text{ Cells broken} = \frac{\text{Free LDH} \times 100}{\text{Total LDH}}$$

2.3.14 Inducing diabetes in rats

Rats were subjected to i.p.(intraperitoneal) injection with 80mg/kg (each rat weighed 200-250g) streptozotocin (dissolved in 0.3ml citrate buffer which contained 0.1M sodium citrate and 0.1M citric acid pH 4.5). A diabetic state ensued after 3-7 days. Animals were judged to be diabetic with

assessment of glucose levels in urine and blood, was done as described by Gawler *et al.*, 1987.

2.3.15 Dissection of the rat brain

Animals were killed rapidly by decapitation and the fur covering the skull was removed. After dissecting the covering of the brain, the cavity was opened by peeling the two halves of the bone back off the brain. The intact brain could then be gently removed with a round ended spatula.

In the now empty brain cavity, it was possible to identify the olfactory bulb (at front of snout, possibly covered by membrane), the optic chiasima which is white neural tissue and the pituitary gland. With the front of the brain facing away, the cerebellum could be gently teased off with a spatula. The medulla is underneath this and could be cut away with scissors. To open up the cortex, a scalpel was gently inserted between the two lobes of the cortex to peel it open; this exposes the frontal and parietal cortex, the globus pallidus, the caudate putamen and the hippocampus.

The different regions were immediately weighed and used for the isolation of RNA.

2.3.16 RNA extraction

2.3.16.1 RNA extraction by Tri-reagent

This method is available to isolate RNA from tissue, cells in suspension and monolayer. All procedures are under sterile conditions.

A small tissue sample was homogenised in Tri-reagent(T-R) (1ml per 50-100mg tissue) using a sterilized glass homogeniser. The homogenate was left at room temperature for 5min and 1ml fractions transferred to microfuge tubes. Cell membranes/high MW DNA/polysaccharide were spun down at 12,000g for 10mins at 4°C and the supernatant transferred to a new eppendorf tube.

RNA was then separated from DNA and protein by the following phase separation:

0.2ml RNAse-free chloroform was added per 1ml of T-R, vortexed for 15 seconds and then stored at room temperature for 3 mins. Following centrifugation at 12,000g for 15 mins at 4°C, most of the upper aqueous phase was transferred to new eppendorfs.

RNA was then precipitated from this phase by adding isopropanol(propan-2-ol): 0.5 ml per 1ml of T-R used initially. Samples were left at room temperature for 5-10 mins and centrifuged at 12,000g for 10mins at 4°C. RNA pelleted on side and bottom of tube.

The supernatant was removed and 1ml of 75% ethanol was added to wash the RNA. Samples can be stored at this stage at 4°C for a week or -20°C for 1 year. Ethanol was removed by spinning at 7500g for 5mins at 4°C and the RNA pellet dried under vacuum for 5-10 mins. The RNA was resolubilised in (1%) DEPC treated water by pipetting a few times and incubating for 10-15 mins at 55°C-60°C.

2.3.16.2 Determining RNA concentration

The concentration of the RNA was determined by measuring the A_{260} of an aliquot of the final preparation. A 1/100 dilution of the resuspended RNA was used to read the absorbance at 260nm against a dH_2O blank, and the RNA concentration calculated as follows:

A solution of RNA with $A_{260}=1$ contains approximately 40µg of RNA per ml, therefore multiply the reading by the dilution factor and by 40 to give the concentration per ml; the total amount of RNA can then be determined by multiplying this figure by the volume of the RNA. Thus, for example, an A_{260} reading of 0.213 gives:

RNA 0.213 x 100 (dilution) x 40 (factor) = 852µg/ml= 0.852µg/µl

This then allows dilution of the RNA to the required working concentration.

2.3.17 First strand cDNA synthesis

Sterile microfuge tubes and pipette tips were used throughout the whole procedure.

5µg of RNA was added to a sterile 0.5ml microfuge tube with DEPC treated water to a total volume of 20µl. This was then heated to 65°C in a PCR machine for 10 mins.and the sample chilled on ice.

To a second, sterile 0.5ml microcentrifuge tube, the following components were added:

11µl of " Bulk 1st strand reaction mix"

1µl of "DTT" solution

1µl of "Not I-d(T) $_{18}$" (0.2µg/µl) primer

After gently pipetting up and down and following a brief spin, 20µl of heat-denatured RNA from first tube was added to this mix and incubated at 37°C for 1 hour. The resultant first strand cDNA synthesised using the RNA as a template was then stored at -70°C.

2.3.18 PCR (polymerase chain reaction)

The following components were combined in a 0.5ml microcentrifuge tube with dNTP (0.2mM), $MgCl_2$ (25mM); Taq polymerase (5units/µl); 5' primer (25pmole); 3' primer (25pmole) and 1µg of template DNA to a total volume of 50µl with DEPC treated H_2O. The reaction mix was overlayed with 40µl of mineral oil and used in a PCR machine programmed for approximately 40 cycles of denaturation/annealing and extension. For example a PCR programme may employ 40 cycles of the following conditions

Denaturation	94°C	1min
Annealing	50°C	2min
Extension	72°C	3min

Annealing temperatures, however, are dependent on the true melting temperatures of the individual primers, see individual experiments for exact conditions used.

2.3.19 Purificaton of oligonucleotides

2.3.19.1 Precipitation of oligonucleotides

Synthesised oligonucleotides were supplied as NH_4OH solutions and had to be purified before use. This was:

Oligonucleotide solution	300µl
3M sodium acetate (pH 4.6)	30µl
70% absolute ethanol	1000µl

This was incubated with 3M sodium acetate pH4.6 and 70% ethanol at -70°C for at least 15 mins. Samples were then spun in a microcentrifuge at 12,000g for 15mins. The supernatant was removed and discarded, leaving the pellet visible in the bottom of the tube. The pellet was then washed with 1000µl of 70% ethanol and spun as above, after which it was vacuum dried for 15 mins. and then resuspended in 30µl of sterile dH_2O.

2.3.19.2 Quantitation of oligonucleotides

The principles of oligonucleotide concentration measurements are the same as for RNA except that here a solution of oligonucleotides with an A_{260}=1 contains approximately 33µg of RNA per ml, therefore multiply the reading by the dilution factor and then by 33 to give the concentration per ml.

2.3.20 DNA purification

After the PCR reactions were complete the mineral oil was removed and the sample extracted with an equal volume of chloroform to remove residual oil. A small fraction of the sample (5µl) was removed and retained whilst the remainder was digested with the required restriction enzymes

according to the manufacturers' instructions (normally digest samples overnight at 37°C).

After digestion the fragment was cleaned to remove small digestion products by running the entire reaction mixture on a 1.5% low melting agarose gel.

The band of interest was then excised from the gel and purified from the agarose by using Promega DNA purification systems as follows.

The agarose slice containing the DNA fragment was melted by heating at 70°C for 2min, then 1ml of the purification resin was added and the solution vortexed for 15seconds. The resin was then applied to a minicolumn connected to a 2ml plastic syringe and washed by running 2ml of 80% isopropyl alcohol through the syringe. After discarding the syringe, the minicolumn was transferred to a 1.5ml microfuge tube and spun for 20 seconds.

The minicolumn was placed in a fresh microcentrifuge tube and 30-50μl of dH_2O (preheated to 70°C) was added to the minicolumn. After incubation at room temperature for 1min the DNA was collected by spinning the tube in a microfuge for 5 second.

DNA prepared in this manner was then used in ligations.

2.3.21 Antibody preparation

2.3.21.1 Antibody production

Polyclonal PDE antisera used for immunoblotting were raised in New Zealand White rabbits against synthetic peptides. These were synthesised with a cysteine at the C-terminus in order to facilitate conjugation to keyhole limpet haemocyanin (KLH), as described by Lerner (1981). Antiserum was normally added to KLH-Sepharose and mixed overnight at 4°C before being centrifuged briefly to pellet the sepharose. The supernatant was collected and dialysed overnight against 20mM Tris/HCl pH 8/1.4M NaCl at 4°C. In each

instance the sera from rabbits were used with up to 3 different bleeds with essentially identical results being obtained. Peptide used are listed below:

Antiserum Code	Peptide used	PDE epitope		Antiserum name
1992	TPGRWGSGGDPA	RD1	598-610	PDE4A
652/653	ATEDKSSLIDT	DPD	508-517	PDE4B
Anti4D	Sequence unavailable	C-terminal mPDE4D		PDE4D

2.3.21.2 Preparation of anti-serum

10-20ml blood was obtained from the ear artery of rabbits and left overnight at 4°C to allow clot formation and contraction. The serum was removed and centrifuged at 1000g for 5min. The supernatant was retained and 0.05% sodium azide added. This was then split into small aliquots (eg. 100µl) and stored at -80 °C. The test of anti-serum was carried out by ELISA.

2.3.21.3 ELISA

This method was used to detect the most effective antibody dilution. Antigen was diluted with PBS (1:100) and used to coat the sensitized plastic plate overnight. The plate was then incubated in PBS buffer (containing 0.1% BSA, 0.1% Tween-20) for 2 hours and washed with PBS buffer 3 times. Addition of first antibody (50µl/well) at various dilutions for 2 hours was followed by washing in PBS 3 times. The second antibody (HRP-anti-rabbit IgG 1:1000 dilution with PBS) was added for 30mins. After washing as before, the plate was developed with H_2O_2 in O-phenylenediamine (OPD) containing buffer for 5min. The amount of test antibody was measured by assessing the amount of coloured end-product by reading A 492nm. From this value the most effective antibody dilution could be determined.

2.3.22 Gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was carried out by the method of Laemmli (1970).

2.3.22.1 Resolving gel preparation

8% (w/v) acrylamide gels were prepared using mixtures as detailed in the section 2.2. Solutions were poured between 180x160mm glass plates separated by 1.5mm spacers and allowed to set at room temperature.

2.3.22.2 Stacking gel preparation

Stacking gels were prepared as detailed in the section 2.2 and the solution layered on top of the resolving gel and allowed to set around a 10-well teflon comb. Gels were run at 8mA/gel overnight or 50mA/gel for 4-5hours with cooling.

2.3.23 Western blotting

Proteins were transferred from the SDS gel to nitrocellulose at 1Amp setting for 2 hours in a transblot apparatus (Hoefer Instruments). The 'blotting buffer' used was described previously in the section 2.2.

The nitrocellulose was then incubated in 20mM Tris/HCl pH7.5 with 500mM NaCl (i.e. TBS) and 5% dried milk powder for 2 hours at room temperature. This stage blocks any non-specific protein binding. After 2 hours the 5% dried milk powder/TBS was poured off and the nitrocellulose washed briefly with distilled water before washing twice with TBS containing 0.2% Nonident P40 and twice with TBS, each for 5 minutes. The nitrocellulose was then immunoblotted with TBS containing 1% dried milk powder and a 1:250 dilution of the appropriate antisera for 2 hours.

After immunoblotting, the nitrocellulose was washed as before and incubated for 2 hours in TBS with 1% dried milk powder plus 10µl of anti-

rabbit peroxidase linked IgG or ^{125}I-labelled anti-(rabbit IgG). With antirabbit peroxidase linked IgG, the Amersham enhanced chemiluminescence (ECL) Western blotting visualization protocol was used to detect the bands, whilst ^{125}I-labelled bands were visualised by autoradiography or phosphoimaging. Quantification of the relative amounts of antigen present in each of the fractions was done in a variety of ways, as described (Bushfield et al 1990), in order to identify situations where a linear relationship held between the amount of antigen present and the magnitude of the signal detected. The densitometric scanning of the ECL experiments was done using both photosensitive Amersham film and X-ray film with various exposure times and a range of applied protein concentrations so as to identify a linear detection range. Confirmation was also obtained in selected instances using ^{125}-I labelled anti-rabbit IgG with excision of the labelled bands identified by auto-radiography, for counting, as described previously by Bushfield *et al.*, (1990); Tang *et al.*, (1993).

2.3.24 COS cell transfection

2.3.24.1 DEAE Dextran COS cell transfection

The SV40-transformed monkey kidney cell line (COS-7) was cultured at 37°C in an atmosphere of 5% CO_2/95% air in a complete growth medium containing DMEM (x1) and 10% (v/v) penicillin/streptomycin (100units/ml) supplemented with 10% (v/v) foetal calf serum.

Confluent flasks were split to 1/3 confluence by plating in 75cm^2 flask/10cm plates, and left overnight. DNA for transfection (5µg) was diluted to 250µl in 10mMTris/HCl, 1mM EDTA buffer and 200µl of DEAE dextran was added. This DNA/dextran mix was then left at room temperature for 15min. Medium was aspirated from the COS cells and 10ml transfection medium added. The DNA/DEAE dextran mix was added drop wise onto the cells and mixed by swirling the flask. Cells were incubated for 3-4 hours at

37°C in an atmosphere of 95% air/5% CO_2. Medium was then aspirated and 5ml of 10% DMSO in PBS added for 2min at room temperature (the longer DMSO stays in contact with cells, the more DNA is taken up but, as damage is also caused to the cells, then more cells will die). Cells were washed with PBS and 10ml of DMEM (x1) containing 10% (v/v) serum was added to the plates before they were incubated at 37°C for 72 hours. Maximum expression should occur during this time.

Mock-transfected cells were treated as above but received no DNA.

2.3.24.2 Preparation of cell extracts

After cells were transfected as described above, they were prepared for homogenization as follows: the growth medium was removed and the cells incubated in complete KHEM for 45min at 4°C. Cells were then washed with 5ml of 10mM triethanolamine/0.15M KCl at 4°C for 10min, the TEA/KCl was aspirated and cells washed with 5ml of KHEM imcomplete buffer. Finally cells were washed with 1ml complete KHEM for 2min and then drained of buffer. Cells were scraped into a final volume of 100µl per plate and this cell suspension was homogenised by 20 down strokes in a glass dounce homogeniser. Subcellular fractionation was performed as described for P9 cells (section 2.3.7.1).

2.3.25 Immunoprecipitation of PDE activity

Solubilized membrane fractions, homogenate and cytosol (500µg of starting material) fractions were used as sources of material for the specific immunoprecipitation of PDE activity. Antiserum was added to each sample at a dilution of 1:10 to 1:100 and the samples briefly vortexed before being left overnight at 4°C in order to allow conjugation to occur. Pansorbin (10%) was then added and the resultant suspension gently mixed at 4°C for 2h. The preparations were then centrifuged at 14000g for 2min and the pellets were

resuspended in the immunoprecipitation buffer before being recentrifuged as before. The resultant pellets were then washed twice more. The final pellet was resuspended in PDE assay buffer (section 2.2) and assayed (section 2.3.11) immediately.

Chapter 3

Insulin and vasopressin elicit inhibition of cholera-toxin-stimulated adenylyl cyclase activity in both hepatocytes and the P9 immortalized hepatocyte cell line through an action involving protein kinase C

Introduction

The adenylyl cyclase system is responsible for generating the intracellular second messenger cyclic AMP. It is now recognised that this activity is expressed by a large multi–gene family whose regulatory characteristics would seem to be remarkably different (Cooper *et al.*, 1994; Jacobowitz *et al.*, 1993; Chen and Iyengar, 1993; Yoshimura and Cooper, 1993). For example, there are distinct isoforms whose activity can be regulated by Ca^{2+}/CaM, phorbol esters and G–protein βγ subunits. All species reported on to date, however, appear to be activated by the diterpene forskolin which acts directly upon the catalytic unit of the enzyme (Seamon *et al.*, 1981) and also by the G–protein G_s, which mediates not only the effect of stimulatory hormones but also the action of cholera toxin (Birnbaumer *et al.*, 1990; Gilman, 1987; Gierschik and Jakobs, 1990). Thus, occupied receptors interact with G_s causing it to bind GTP and dissociate to yield a GTP–bound α–subunit which serves to activate adenylyl cyclase (Birnbaumer *et al.*, 1990; Gilman, 1987; Gierschik and Jakobs, 1990). Cholera toxin achieves adenylyl cyclase activation after a characteristic lag phase (Houslay and Elliott, 1979) which is considered (Houslay and Elliott, 1981; Orlandi *et al.*, 1993) to reflect toxin entry into the cell by endocytosis with subsequent processing to deliver the activated cholera toxin α-subunit to the cytosol surface of the plasma membrane. This activated species then elicits the NAD^+–dependent ADP-ribosylation of $G_{s\alpha}$(Birnbaumer *et al.*, 1990; Gilman, 1987; Gierschik and Jakobs, 1990) allowing it to dissociate in a GTP–bound state. However, ADP–ribosylated $G_{s\alpha}$is unable to hydrolyse GTP and undergo inactivation. Thus the entire pool of G_s becomes chronically activated by attaining the fully activated GTP-bound state, leading to the constitutive activation of adenylyl cyclase (Birnbaumer *et al.*, 1990; Gilman, 1987; Gierschik and Jakobs, 1990).

Hepatocytes have an adenylyl cyclase system that is able to be potently activated by the hormone glucagon (Johnson *et al.*, 1972; Heyworth and Houslay, 1983; Murphy *et al.*, 1987; Refnes *et al.*, 1989). However, in intact cells, this activation is rapidly followed by a profound desensitization effect which occurs independently of any increase in intracellular cyclic AMP concentrations (Heyworth and Houslay, 1983; Murphy *et al.*, 1987; Refnes *et al.*, 1989; Murphy and Houslay, 1988). This effect is in itself transient with resensitization attaining near completion some 20min after hormonal addition (Houslay, 1991). The nature of this desensitization appears to take the form of an uncoupling of the ability of the glucagon receptor to stimulate G_s with no inhibition being evident of either the catalytic activity of adenylyl cyclase or G_s-adenylyl cyclase coupling (Heyworth and Houslay, 1983; Murphy *et al.*, 1987; Houslay, 1991; Houslay, 1994). Such a process appears to be mediated through the action of protein kinase C (Murphy *et al.*, 1987; Houslay, 1991; Houslay, 1994; Savage *et al.*, 1995), which itself becomes activated in hepatocytes in response to glucagon (Pittner and Fain, 1991). Thus desensitization can be mimicked by hormones such as vasopressin and angiotensin as well as the tumour promoting phorbol ester PMA, all of which activate protein kinase C in hepatocytes (Houslay, 1991; Houslay, 1994; Savage *et al.*, 1995; Tang and Houslay, 1992) and whose abilities to elicit desensitization can be blocked by protein kinase C inhibitors (Savage *et al.*, 1995).

It has previously been shown that cholera toxin activates hepatocyte adenylyl cyclase activity after a characteristic lag time of ~15 min. (Houslay and Elliott, 1979; Houslay and Elliott, 1981). Additionally it was noted that the maximal degree of activation that was achieved by cholera toxin could be attenuated by the presence of the hormones glucagon and insulin (Heyworth *et al.*, 1984). These hormones neither affected the lag time for the action of

cholera toxin nor did they alter the degree of ADP–ribosylation of G_s (Heyworth *et al.*, 1984).

In this study I have shown that a profound inhibitory action on the ability of cholera toxin to stimulate hepatocyte adenylyl cyclase can be achieved through either the activation of protein kinase C or the inhibition of protein phosphatase activity (Zeng and Houslay, 1995). Such inhibition appears to be regulated through the phosphorylation of a membrane protein. This target may be provided by a specific form of adenylyl cyclase which can be phosphorylated by particular isoforms of PKC. Such inhibition could be demonstrated in hepatocytes and the immortalised hepatocyte P9 cell line but it was not apparent in either NIH-3T3 fibroblasts or CHO cells.

Results

Cholera toxin is known to cause the NAD^+ dependent ADP-ribosylation of the $G_{s\alpha}$-subunit. This ADP-ribosylation, occurring in the presence of GTP, causes the dissociation of G_s to release a free $G_{s\alpha}$-subunit,which is trapped in its active GTP-bound form. This species caused the constitutive activation of adenylyl cyclase activity at a range of cholera-toxin concentrations **(Figure 3.1)** after a well-defined lag period of around 10-15min in hepatocytes **(Figure 3.2)**. However, the magnitude of this stimulatory effect of cholera toxin was shown to be profoundly attenuated if either glucagon or insulin was added to hepatocytes along with cholera toxin **(Figure 3.3)** without any change in the lag time for onset of activation of adenylyl cyclase.

In addition to this, we showed here that both the hormone vasopressin and the tumour-promoting phorbol ester PMA can also attenuate cholera-toxin-stimulated adenylyl cyclase activity **(Table 3.1)** without affecting the lag time for activation by this toxin.

This inhibitory effect on adenylyl cyclase elicited by both insulin and vasopressin could be blocked by challenging the hepatocytes with either of the selective PKC inhibitors, staurosporine or chelerythrine (Nixon *et al.*, 1992; Herbert *et al.*, 1990; Gordge and Ryves, 1994) **(Table 3.1; Figure 3.4).** These compounds had, however, no apparent effect on the magnitude of activation of adenylyl cyclase by cholera toxin achieved in the absence of these hormones **(Table 3.1).**

In contrast with this, exposure of cells to the protein phosphatase inhibitor okadaic acid elicited a profound decreased in the cholera-toxin-stimulated adenylyl cyclase activity **(Figure 3.5).**

Treatment of membranes with alkaline phosphatase **(Table 3.1)** has been used to achieve the dephosphorylation of phosphorylated cellular proteins (Savage *et al.*, 1995; Pears *et al.*, 1992). Diminished cholera-toxin-stimulated

adenylyl cyclase activity was observed in membranes from cells which had been challenged with PMA, vasopressin or insulin (**Table 3.1**). In each of these instances, however, membrane treatment with alkaline phosphatase increased adenylyl cyclase activity up to a level approaching that seen in membranes from cells which had only been challenged with cholera toxin (**Figure 3.6**).

Interestingly, when incubated together, the ability of insulin, PMA and vasopressin to inhibit cholera toxin stimulated adenylyl cyclase was not additive (**Figure 3.7**). Furthermore, I have shown here that treatment of hepatocytes with the phorbol ester PMA did not inhibit the activity of either the catalytic unit of adenylyl cyclase (assayed by monitoring both basal activity and that amplified by forskolin (**Figure 3.8 (a)**) or G_s-stimulated adenylyl cyclase when monitored with NaF (**Figure 3.8 (b)**).

An immortalized hepatocyte cell line, generated using SV40 DNA (Livingstone *et al.*, 1994) was shown to express G-protein-regulated adenylyl cyclase activity and a functional high-affinity insulin response (Livingston *et al.*, 1994). Here I demonstrated that the adenylyl cyclase activity in such cells could be markedly stimulated by cholera toxin (**Figure 3.9**) and that this activity could be inhibited by either PMA or insulin treatment (**Figure 3.10**) in a similar fashion to that seen with native hepatocytes. Such inhibitory effects were observed when adenylyl cyclase activity was assessed, either directly in membranes isolated from such cells or, *in situ*, by assessing cAMP accumulation in the presence of the phosphodiesterase inhibitor IBMX (**Table 3.2**).

Cholera toxin was also able to achieve a marked stimulation of adenylyl cyclase activity in both CHO (**Figure 3.11; Table 3.3**) and NIH-3T3 cells (**Figure 3.12; Table 3.3**). However, in contrast with expriments done with hepatocyte and P9 cells, this activity was not attenuated by treatment with the phorbol ester PMA (**Figure 3.13; Table 3.3**). Indeed, treatment of NIH-3T3 cells with PMA led to an increase in cholera-toxin-stimulated adenylyl

cyclase activity when assayed either in membranes isolated from such cells or, *in situ*, by assessing cAMP accumulation in the presence of the phosphodiesterase inhibitor IBMX (**Figure 3.14; Table 3.3**). This was not observed in the native CHO cells (**Table 3.3**). Meanwhile, I also investigated the action of PMA in CHO cells which had been transfected so as to overexpress various PKC isoforms. In no instance, however, did I observe any inhibition by phorbol ester. Interestingly though, we noted that PMA mediated an increase in the cholera-toxin-stimulated adenylyl cyclase activity of CHO cells transfected so as to overexpress PKC-γ (**Table 3.3**). These observations appeared to be independent of whether adenylyl cyclase activity was gauged by direct membrane assay or through determining intracellular cyclic AMP accumulation in the presence of a phosphodiesterase inhibitor.

The thermostability of adenylyl cyclase in hepatocytes and CHO cells was carried out as described in methods section 2.3.8. Results are shown as % activity plots for the decay of adenylyl cyclase activity in enzyme fractions incubated at different temperatures. **Figure 3.15** shows denaturation of adenylyl cyclase activity with increasing temperature. One component of the activity was fully denatured at 40°C in CHO cells whereas, in hepatocytes, similar denaturation of adenylyl cyclase is observed up to 50°C (**Figure 3.15**). From the level of thermostable activity remaining, it would seem that in hepatocytes more adenylyl cyclase activity appears to be temperature sensitive whilst in CHO cells, more adenylyl cyclase appears temperature insensitive (**Figure 3.15**). When enzyme fractions were incubated at elevated temperatures (50°C) for both hepatocyte and CHO cells, the decay of adenylyl cyclase activity in hepatocytes was observed as two single exponentials, as indicated by the linear semi-logarithmic plots of activity against time (**Figure 3.16**). This was consistent with at least two enzyme activities being analysed. The same profile for CHO cells also contained two single exponentials supporting the presence of at least two types of adenylyl cyclase activity being

present (**Figure 3.16**). However, this did contrast with hepatocytes where the linear semi-logarithmic plots were different indicating that different enzymes may contribute to adenylyl cyclase activity in those cells. Meanwhile, assay of the thermostability of adenylyl cyclase activity in P9 cells showed a similar profile of thermostability to that indicated in hepatocytes (**Figure 3.17**), implying similar adenylyl cyclase activity to be expressed in both cells.

In order to see if there were indeed different adenylyl cyclase isoforms in hepatocyte, P9, NIH3T3 and CHO cells, RT-PCR was carried out with sense and antisense primers. These were able to detect type I, II, IV, V and VI adenylyl cyclase isoforms specifically. Under the appropriate reaction condition (see individual figure legends), PCR products were analysed by running in 2% minigel agarose gel and visualising products with ethidium bromide under UV light. Results showed that there was neither type I (**Figure 3.18 (a)**) nor type II adenylyl cyclase (**Figure 3.18(b)**) in any of these cells; there was type IV adenylyl cyclase in hepatocytes and CHO cells but not in the P9 and NIH-3T3 cells (**Figure 3.18 (c)**). As for the type V adenylyl cyclase, this was found in both hepatocyte and P9 cells but not in the CHO and NIH-3T3 cells (**Figure 3.18 (d)**). Whilst results showed that type VI adenylyl cyclase was expressed in the hepatocyte, P9 and NIH-3T3 cells, it was not found in the CHO cells (**Figure 3.18 (e)**). The use of adenylyl cyclase-specific primers to analyse PCR products observed within different tissues, therefore, indicated that certain types of adenylyl cyclase are tissue specific. Here we demonstrated the presence of type V and VI adenylyl cyclase in hepatocyte and P9 cells. It is likely that type V could provide the target membrane protein for phosphorylation by particular isoforms of PKC (see section 1.3.4.3), as it is not expressed in the CHO and NIH-3T3 cells (**Figure 3.18 (d); (e)**), where protein kinase C-mediated attenuation of adenylyl cyclase activity was not detectable.

These results also show the thermal denatuation profiles to be an oversimplification, as on the basis of transcript analysis more than the predicated two forms of adenylyl cyclase were shown to exist in hepatocytes, i.e. at least types IV, V and VI occur. Similarly, in CHO cells thermal denaturation showed **(Figure 3.16)** two forms of adenylyl cyclase to exist, yet by PCR studies, we were only able to detect type IV adenylyl cyclase. Of course other isoforms of adenylyl cyclase may occur in these cells which we have not looked at, either due to lack of available tools (e.g. for type III adenylyl cyclase) or because they remain, as yet unidentified isoforms.

Figure 3.1 Dose dependence of cholera toxin-stimulated cAMP response in hepatocytes

Hepatocytes were treated with increasing concentrations of cholera toxin in the presence of 1mM IBMX for 60min at 37°C. Intracellular cAMP was determined as described in methods section (2.3.9.3). Results are means±S.E. for n=3 experiments using different cell preparations from different animals.

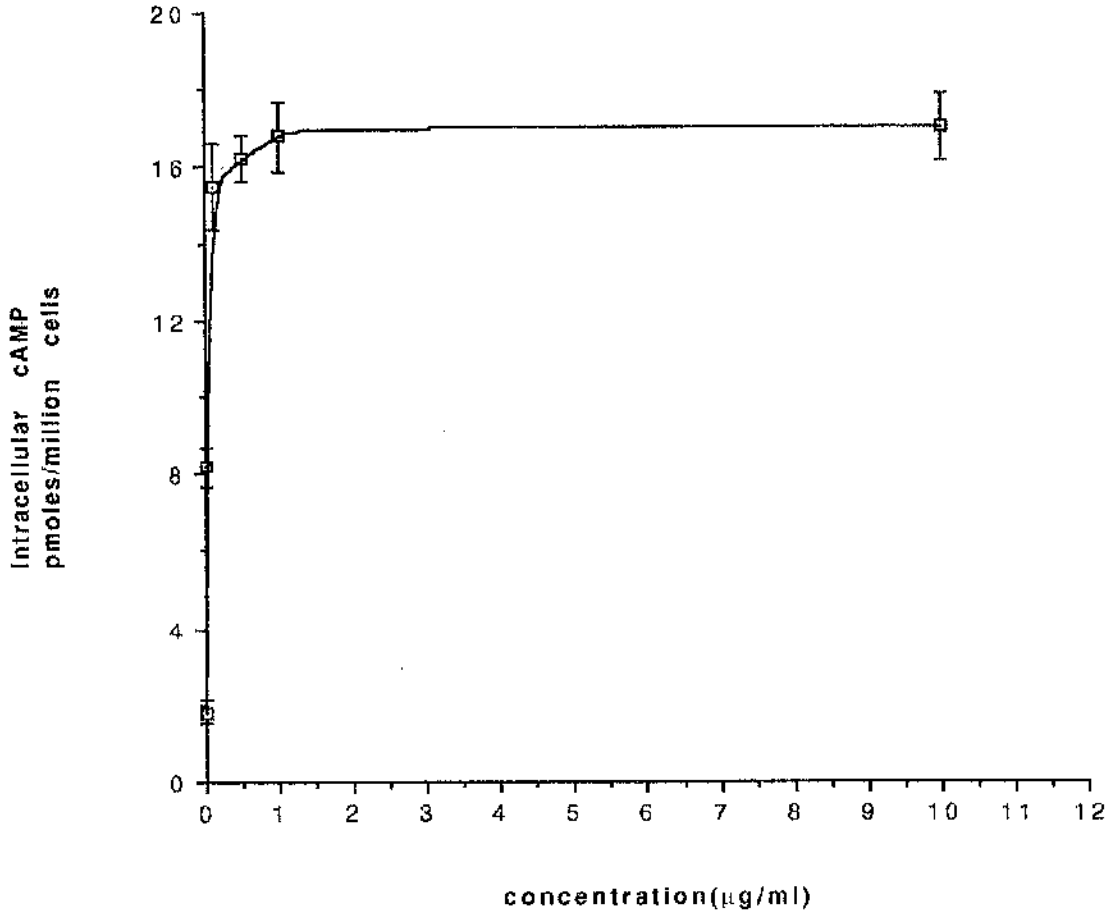

Intracellular cAMP
pmoles/million cells
20
16
12
8
4
0
0
1
2
3
4
5
6
7
8
9
10
11
12
concentration(μg/ml)

Figure 3.2 Time dependence of cholera toxin-stimulated adenylyl cyclase response in hepatocytes

Hepatocytes were treated with 1μg/ml of cholera toxin at different times over a period of 60min at 37°C. At the indicated times, adenylyl cyclase activity was determined as described in methods section (2.3.10). Results are means±S.E. for n=5 experiments using different cell preparations from different animals.

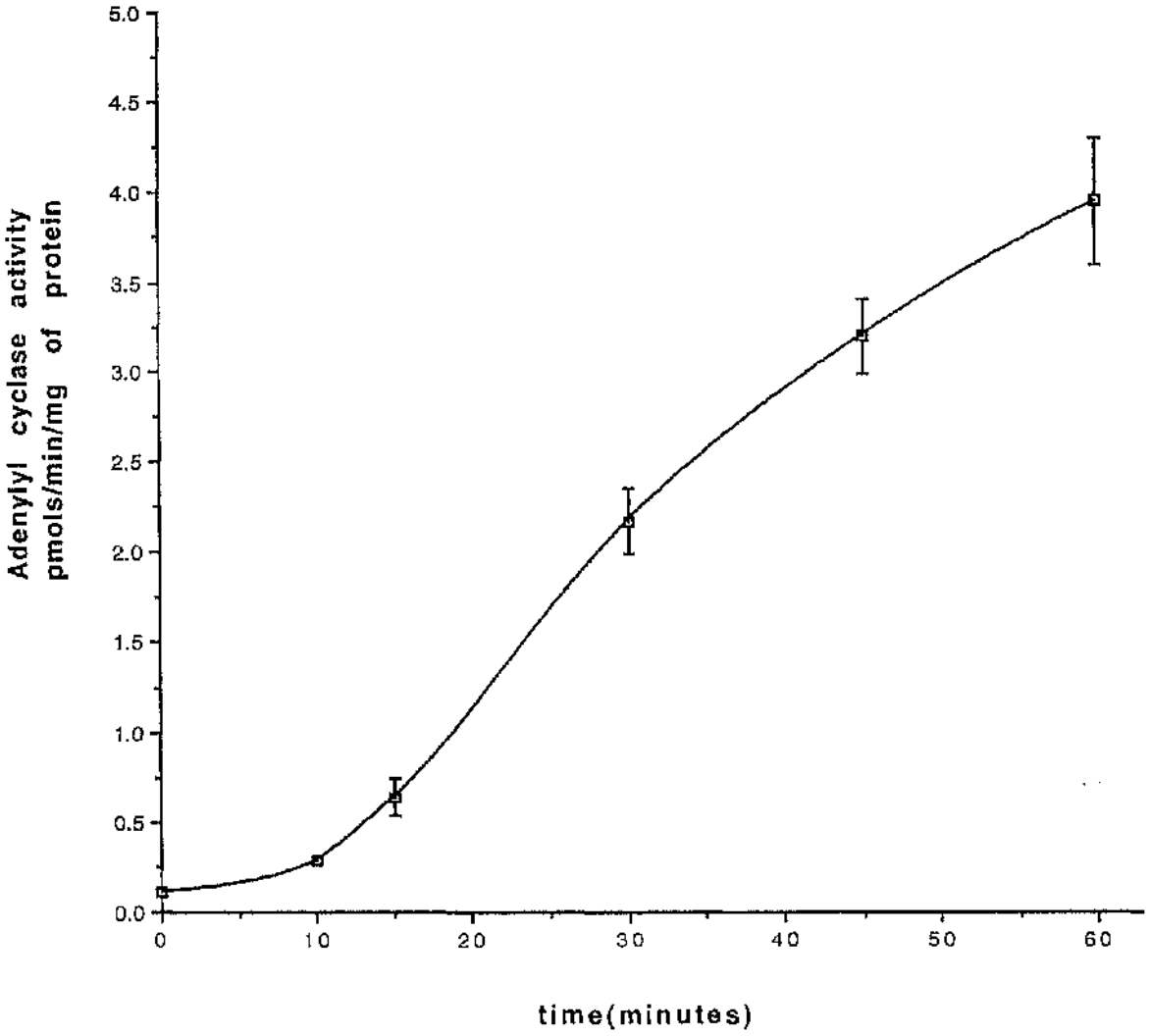

Adenylyl cyclase activity
pmols/min/mg of protein
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
10
20
30
40
50
60
time(minutes)

Figure 3.3 The presence of insulin and glucagon attenuates the ability of cholera toxin to activate adenylyl cyclase in intact hepatocytes

Hepatocytes were treated at 37°C with 1μg/ml cholera toxin (a) in the absence of any other ligands, (b) in the presence of 10nM-insulin or (c) in the presence of 10nM-glucagon over 60min. At the time points shown, cells were harvested and a washed membrane fraction was taken to assess basal adenylyl cyclase activity see methods section (2.3.10). Results are means±S.E. for n=2 experiments using different cell preparations from different animals.

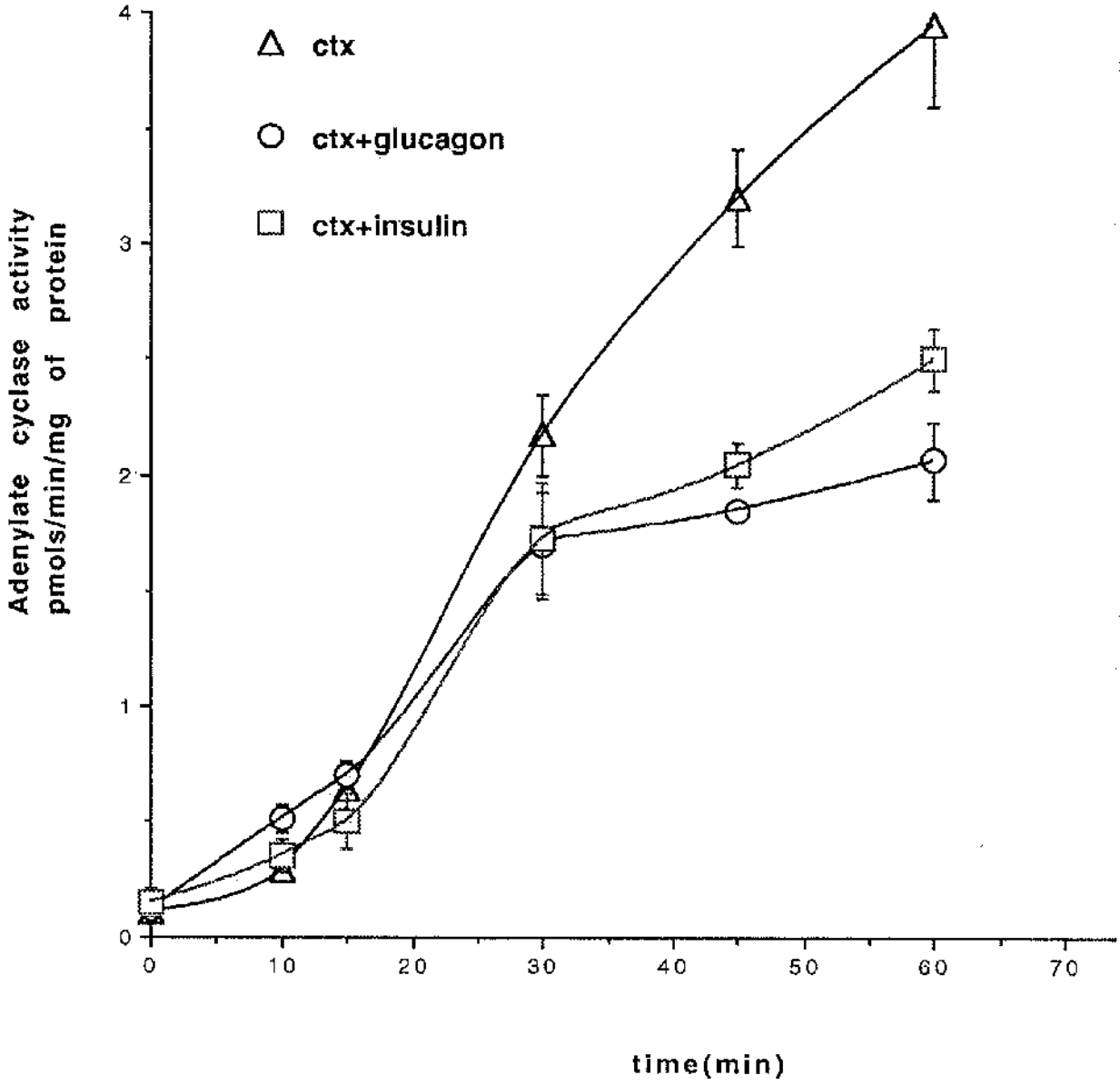

ctx
ctx+glucagon
ctx+insulin
Adenylate cyclase activity
pmols/min/mg of protein
time(min)
0
1
2
3
4
0
10
20
30
40
50
60
70

Figure 3.4 PKC inhibitors can blocked the vasopressin and insulin mediated attenuation of cholera toxin stimulated adenylyl cyclase activity in hepatocytes

Hepatocytes were incubated for 1h with cholera toxin (1μg/ml), harvested, and a washed membrane fraction was then prepared and taken to assess adenylyl cyclase activity as described in methods section (2.3.10). Where indicated, other ligands were added together with cholera toxin in the hepatocyte incubation; vasopressin was added at a final concentrations of 10nM, PMA was added at 100nM and staurosporine and chelerythrine were added at final concentrations of 100nM and 10μM respectively.

Results are means±S.E. for n=3 experiments using different cell preparations from different animals.

In experimental sets the cholera toxin stimulated adenylyl cyclase value (7.6 pmols/min/mg of protein) was taken as the native stimulation regarded as 100%.

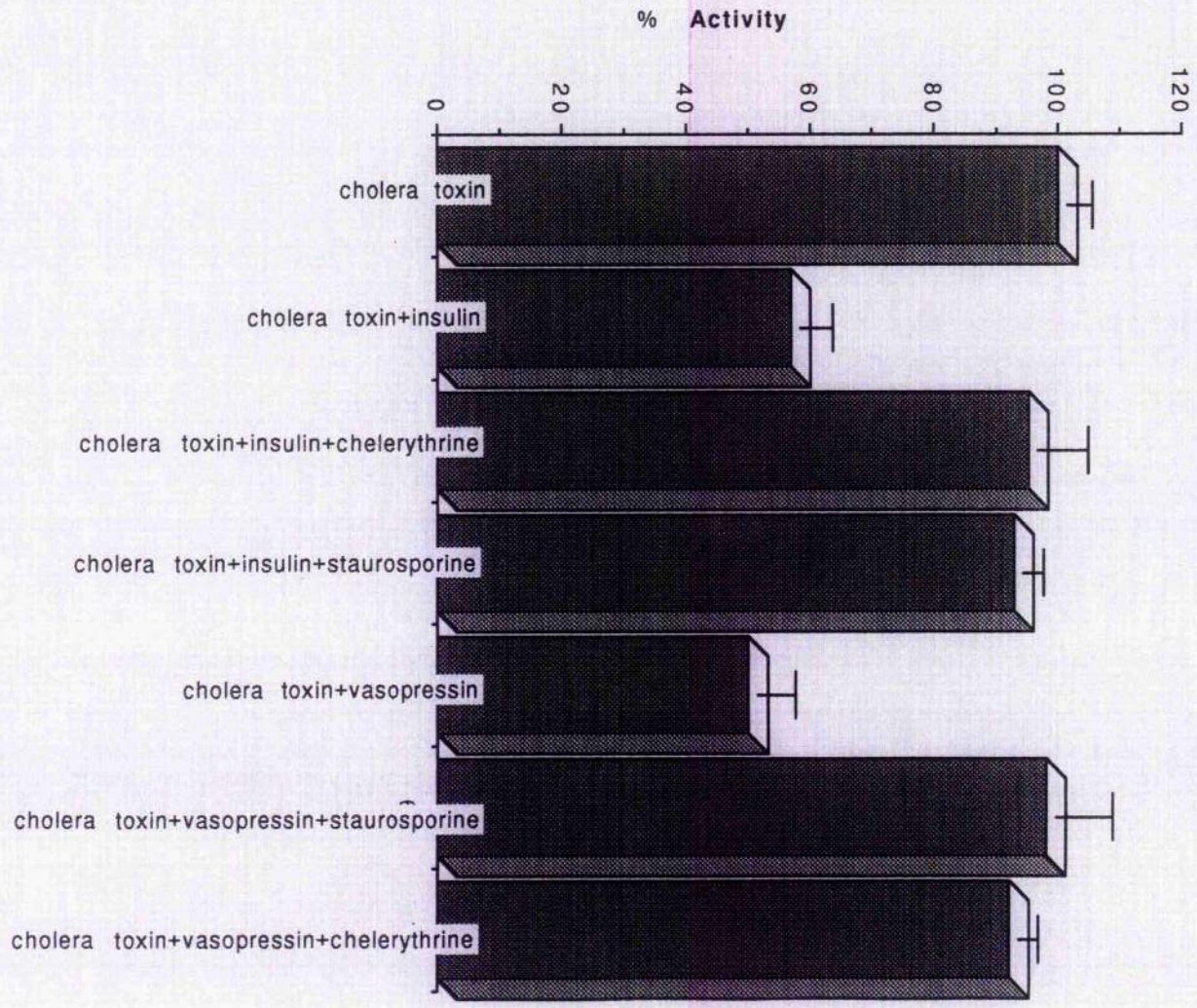

% Activity
0
20
40
60
80
100
120
cholera toxin
cholera toxin+insulin
cholera toxin+insulin+chelerythrine
cholera toxin+insulin+staurosporine
cholera toxin+vasopressin
cholera toxin+vasopressin+staurosporine
cholera toxin+vasopressin+chelerythrine

Figure 3.5 Okadaic acid mimics PKC's ability to attenuate cholera-toxin stimulated adenylyl cyclase activity in hepatocytes

Hepatocytes were treated at 37°C with cholera toxin (1μg/ml) in the absence of any other ligand or in the presence of 100nM okadaic acid, harvested and a washed membrane fraction was then prepared and taken to assess adenylyl cyclase activity as described in methods section (2.3.10). Results are means±S.E. Similar data were obtained in two other experiments from different animals.

In experimental sets the cholera toxin stimulated adenylyl cyclase value (9.2 pmols/min/mg of protein) was taken as the native stimulation regarded as 100%.

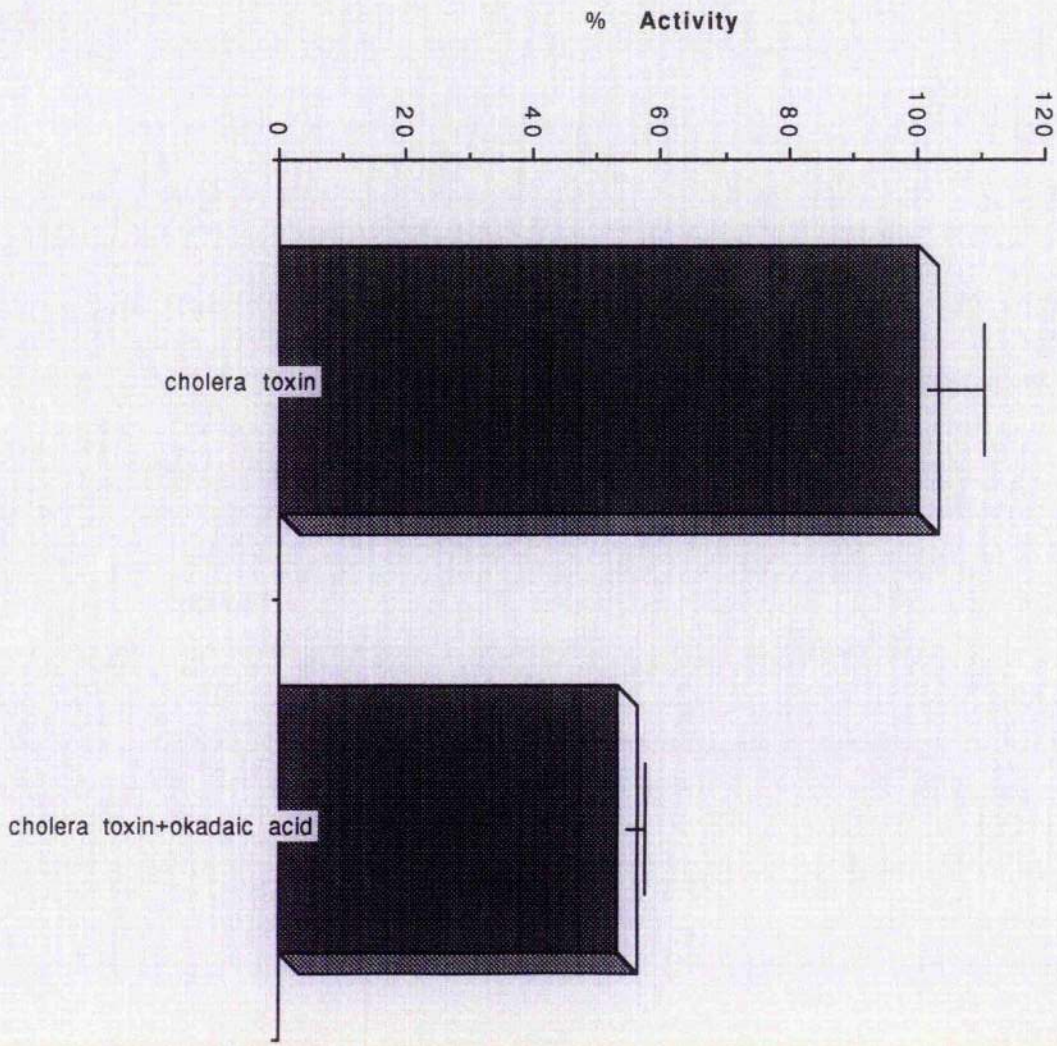

% Activity
0
20
40
60
80
100
120
cholera toxin
cholera toxin+okadaic acid

Figure 3.6 Alkaline phosphatase reversed the PKC attenuation of cholera-toxin stimulated adenylyl cyclase activity in hepatocytes

Hepatocytes were treated at 37°C with cholera toxin (1μg/ml) in the presence of various ligands like vasopressin (10nM), insulin (10nM) and PMA (100nM) for 60 mins. In some instances the isolated membrane fraction was subsequently treated with alkaline phosphatase (1unit/μl) as described in the method section (2.3.5.2). The adenylyl cyclase activity was then assessed by methods section (2.3.10). Results are means±S.E. Similar data were obtained in three other experiments from different animals.
In experimental sets the cholera toxin stimulated adenylyl cyclase value (5.8 pmols/min/mg of protein) was taken as the native stimulation regarded as 100%.

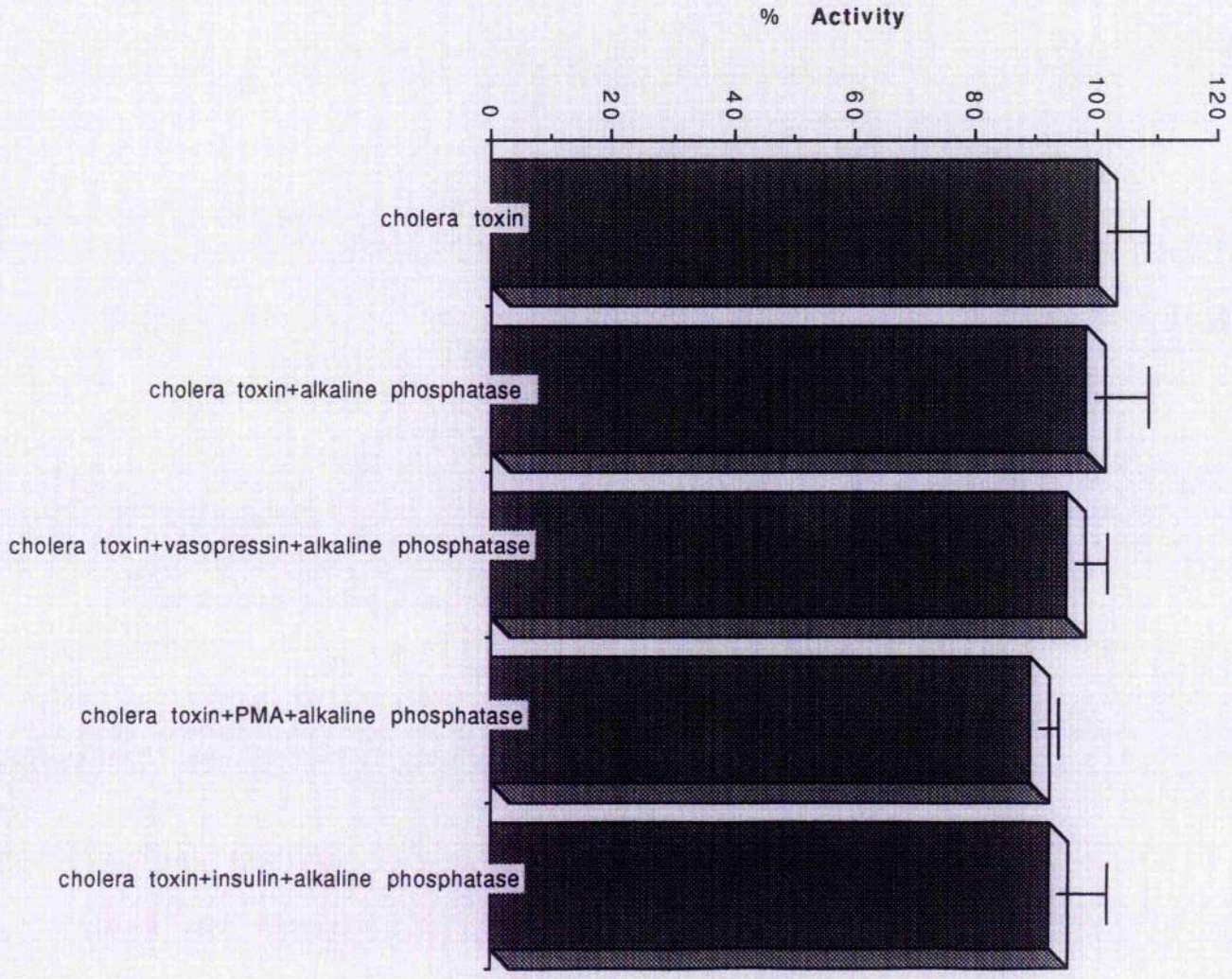

% Activity
0
20
40
60
80
100
120
cholera toxin
cholera toxin+alkaline phosphatase
cholera toxin+vasopressin+alkaline phosphatase
cholera toxin+PMA+alkaline phosphatase
cholera toxin+insulin+alkaline phosphatase

Figure 3.7 Insulin, PMA and vasopressin were not additive in their ability to inhibit cholera-toxin stimulate adenylyl cyclase activity in hepatocytes

Hepatocytes were treated at 37°C with cholera toxin (1μg/ml) in the presence of other indicated ligands like insulin (10nM), vasopressin (10nM) and PMA (100nM) for 60min. The isolated membrane fraction was then obtained for use in assessing adenylyl cyclase activity by methods section (2.3.10). Results are means±S.E. Similar results were obtained in an other experiments.

In experimental sets the cholera toxin stimulated adenylyl cyclase value (6.5 pmols/min/mg of protein) was taken as the native stimulation regarded as 100%.

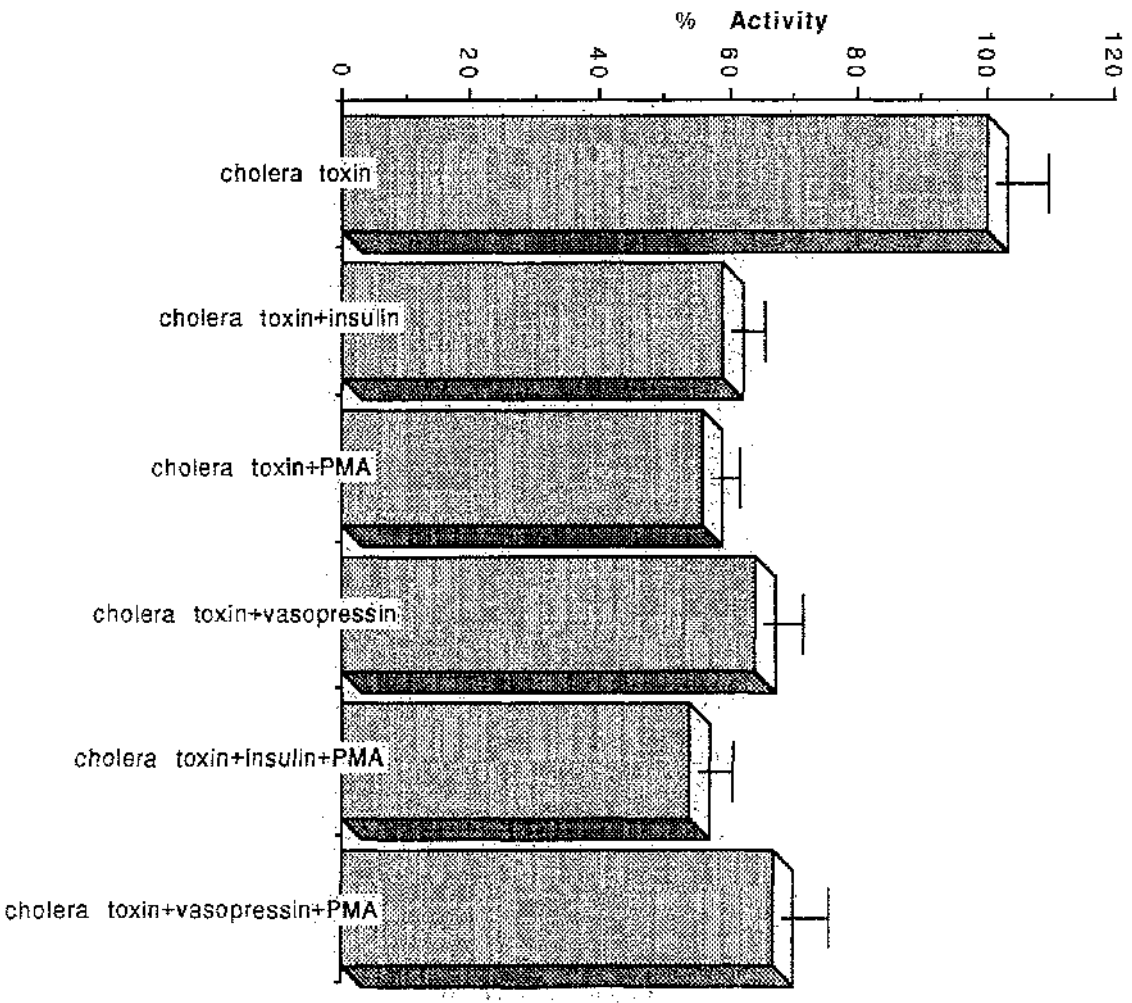

% Activity
0
20
40
60
80
100
120
cholera toxin
cholera toxin+insulin
cholera toxin+PMA
cholera toxin+vasopressin
cholera toxin+insulin+PMA
cholera toxin+vasopressin+PMA

Figure 3.8 (a) PMA treatment of hepatocytes did not affect the catalytic unit of adenylyl cyclase

Hepatocytes were incubated alone at 37°C for 1h or in the presence of PMA (100nM). As the indicated times, a membrane sample was obtained for use in assessing basal or forskolin (10μM) stimulated adenylyl cyclase activity by methods section (2.3.10). Similar results were obtained in another experiment.

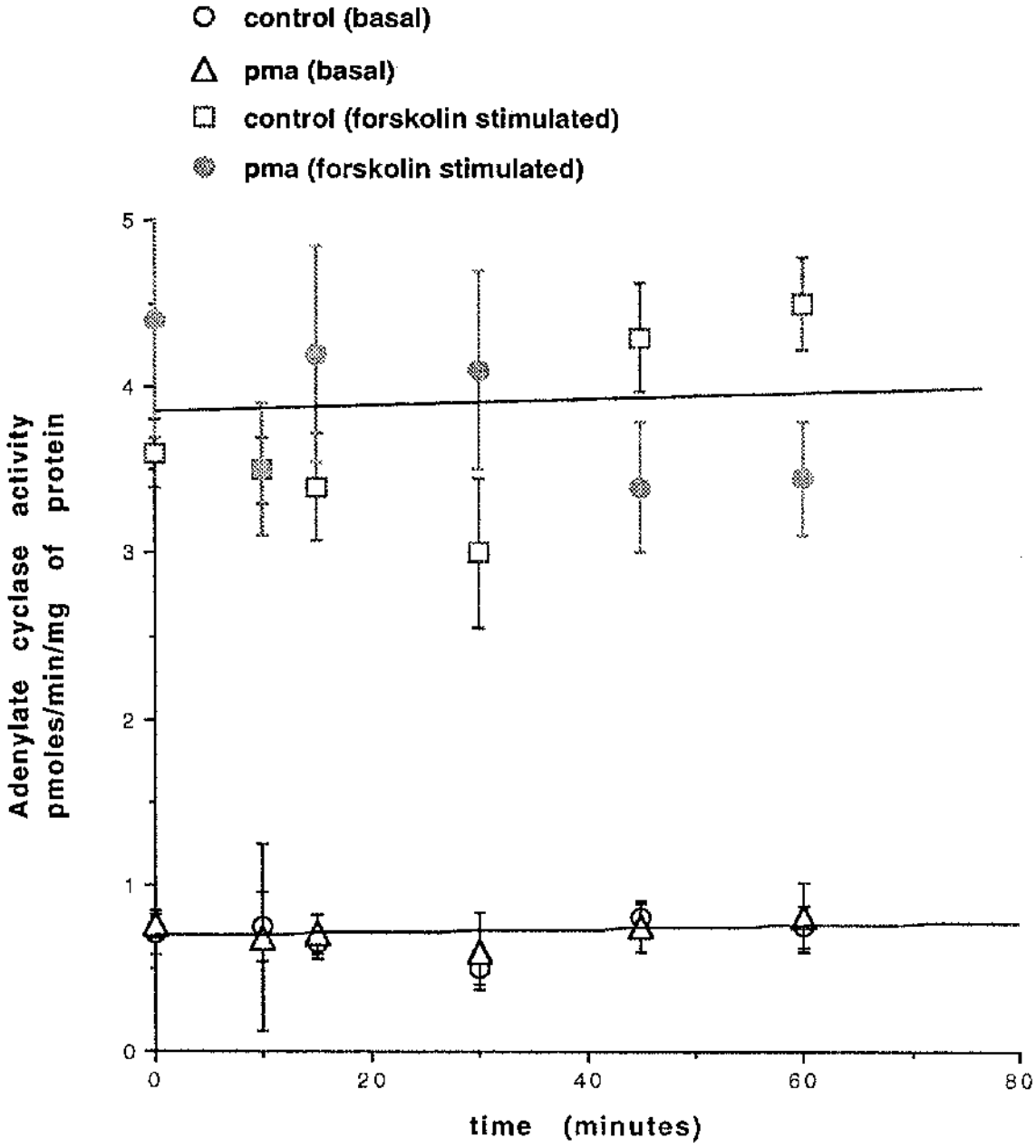

control (basal)
pma (basal)
control (forskolin stimulated)
pma (forskolin stimulated)
Adenylate cyclase activity
pmoles/min/mg of protein
0
1
2
3
4
5
0
20
40
60
80
time (minutes)

Figure 3.8 (b) PMA treatment of hepatocytes did not affect Gs-stimulated adenylyl cyclase activity

Hepatocytes were incubated alone at 37°C or in the presence of PMA (100nM) for over a period of 1h. As the indicated times, a membrane sample was obtained for use in assessing basal or NaF (15mM) stimulated adenylyl cyclase activity by methods section (2.3.10). Similar results were obtained in another experiment.

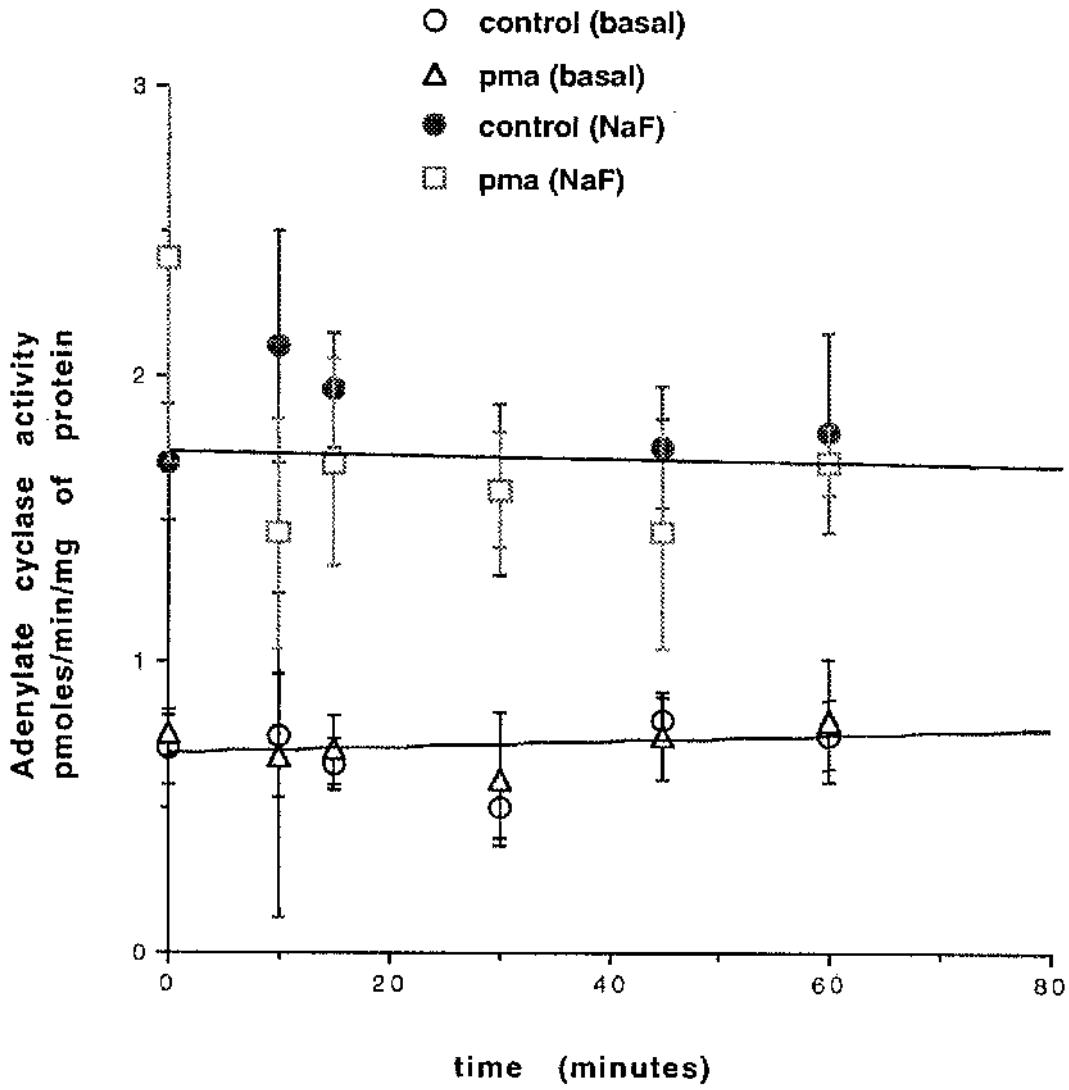

control (basal)
pma (basal)
control (NaF)
pma (NaF)
Adenylate cyclase activity
pmoles/min/mg of protein
3
2
1
0
0
20
40
60
80
time (minutes)

Figure 3.9 Time dependence of cholera toxin-stimulated cAMP response in immortalized hepatocyte P9 cells

P9 cells were treated with 1μg/ml of cholera toxin in the presence of 1mM IBMX at different times over a period of 60min at 37°C. After the indicated time, intracellular cAMP was determined as described in methods section (2.3.9.3). Results are means±S.E. for n=3 experiments using different cell preparations showing similar data.

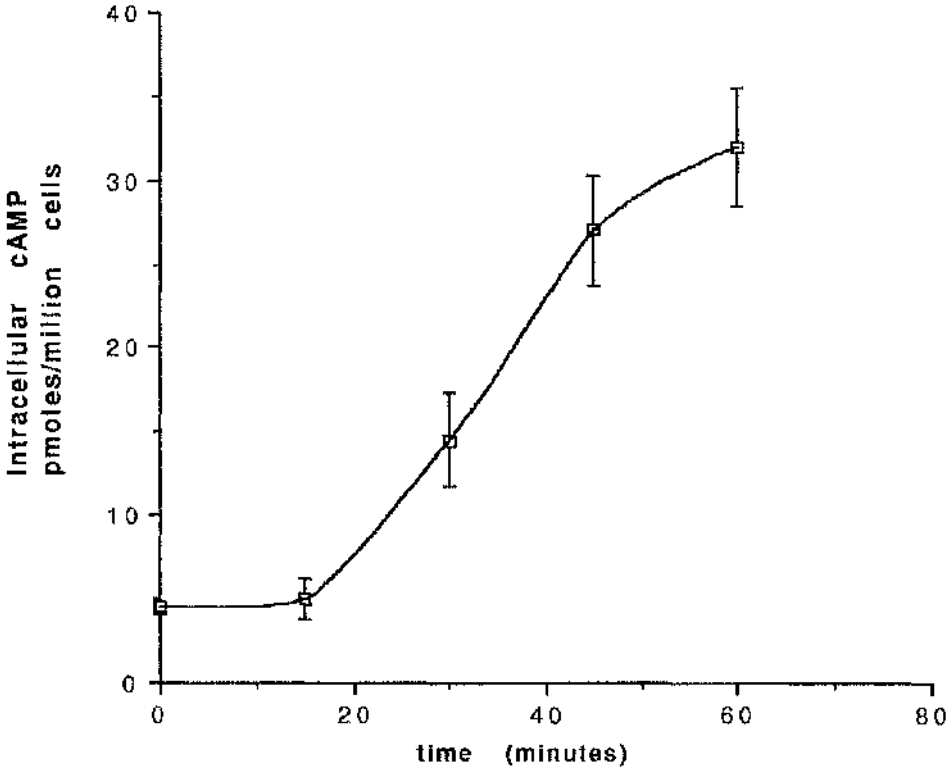

Intracellular cAMP
pmoles/million cells
40
30
20
10
0
0
20
40
60
80
time (minutes)

Figure 3.10 Inhibition of cholera toxin stimulated adenylyl cyclase activity by insulin and PMA in P9 cells

Immortalised hepatocyte P9 cells were incubated with cholera toxin (1μg/ml) for the indicated times together with the cyclic AMP phosphodiesterase inhibitor IBMX (1mM). cAMP amount was assessed by methods section (2.3.9.3). Data is shown for treatment with either cholera toxin alone or in combination with either insulin (10nM) or PMA (100nM) in the hepatocyte incubation. Results are means±S.E. This shows typical data of experiments that were done on three separate occasions. Intracellular cyclic AMP was expressed as pmol cyclic AMP/10^6 cells.

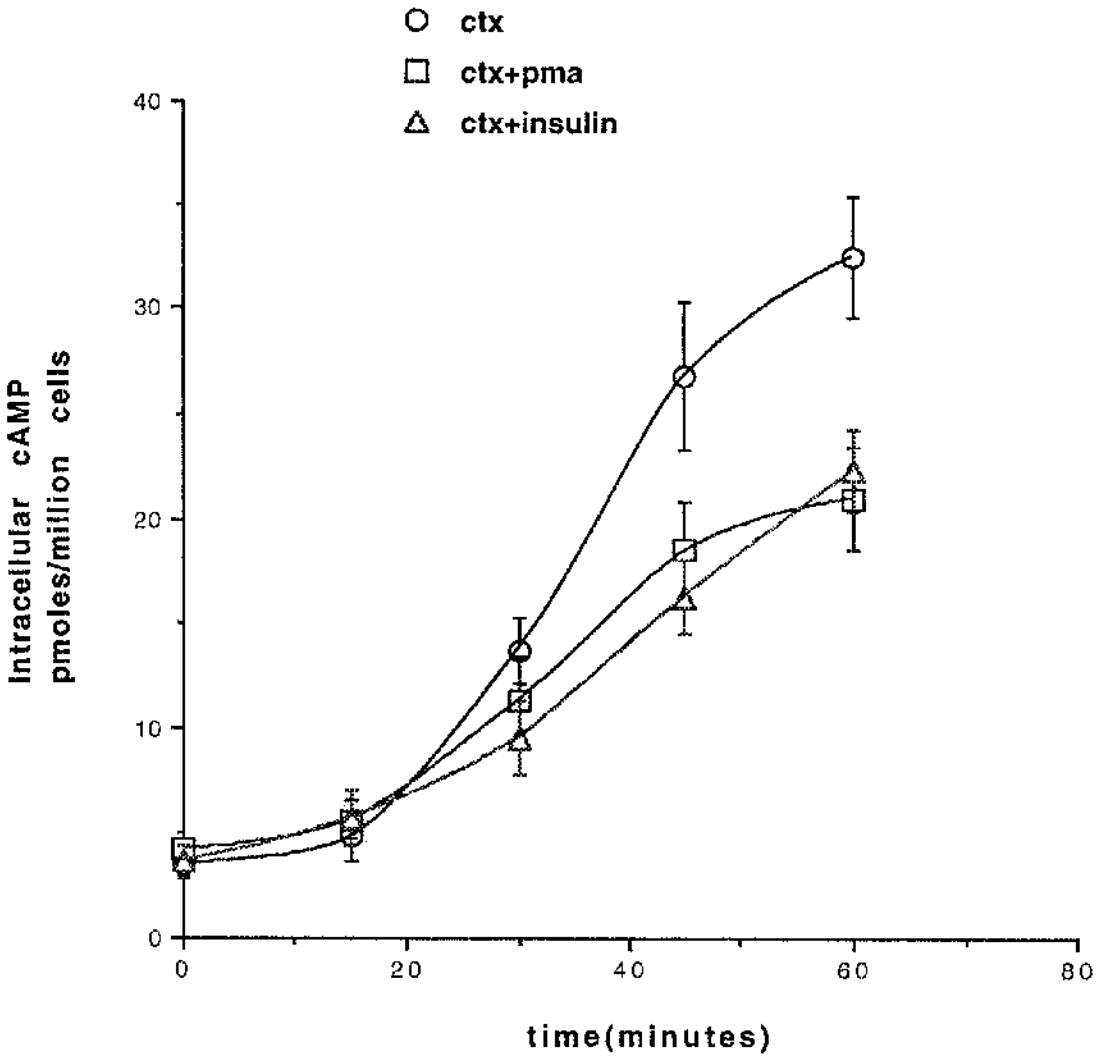

ctx
ctx+pma
ctx+insulin
Intracellular cAMP
pmoles/million cells
40
30
20
10
0
0
20
40
60
80
time(minutes)

Figure 3.11 Time dependence of the cholera toxin-stimulated intracellular cAMP response in CHO-HIR cells

CHO-HIR cells were treated with 1μg/ml of cholera toxin in the presence of the phosphodiesterase inhibitor IBMX (1mM) at different times over a period of 2h at 37°C. After the indicated times, intracellular cAMP was determined as described in methods section (2.3.9.3). Results are means±S.E. for n=5 experiments using different cell preparations showing similar results.

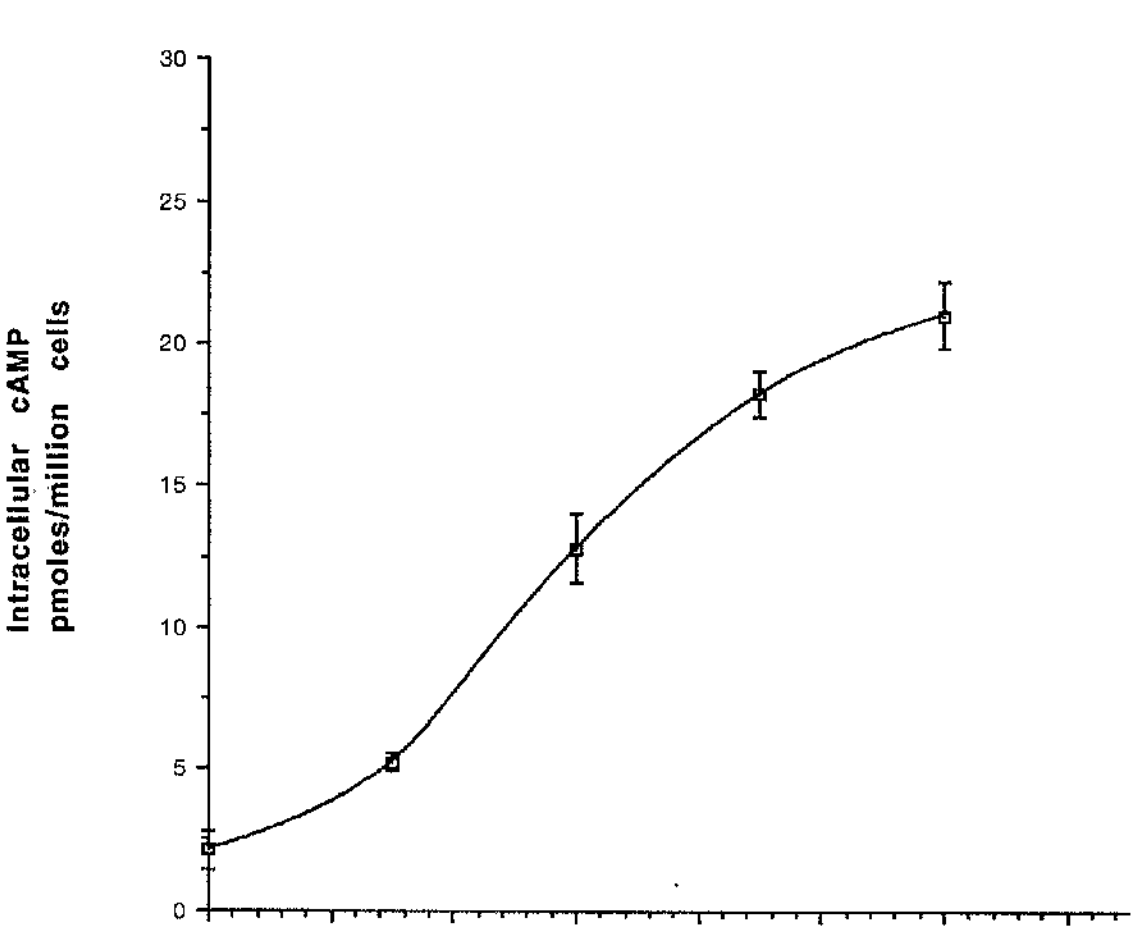

Intracellular cAMP
pmoles/million cells
30
25
20
15
10
5
0
0
20
40
60
80
100
120
140
time(minutes)

Figure 3.12 Time dependence of the cholera toxin-stimulated intracellular cAMP response in NIH-3T3 cells

NIH-3T3 cells were treated with 1μg/ml of cholera toxin in the presence of PDE inhibitor 1mM IBMX at different times over a period of 90min at 37°C. After the indicated times, intracellular cAMP was determined as described in methods section (2.3.9.3). Results are mean±S.E. for n=3 experiments using different cell preparations showing similar results.

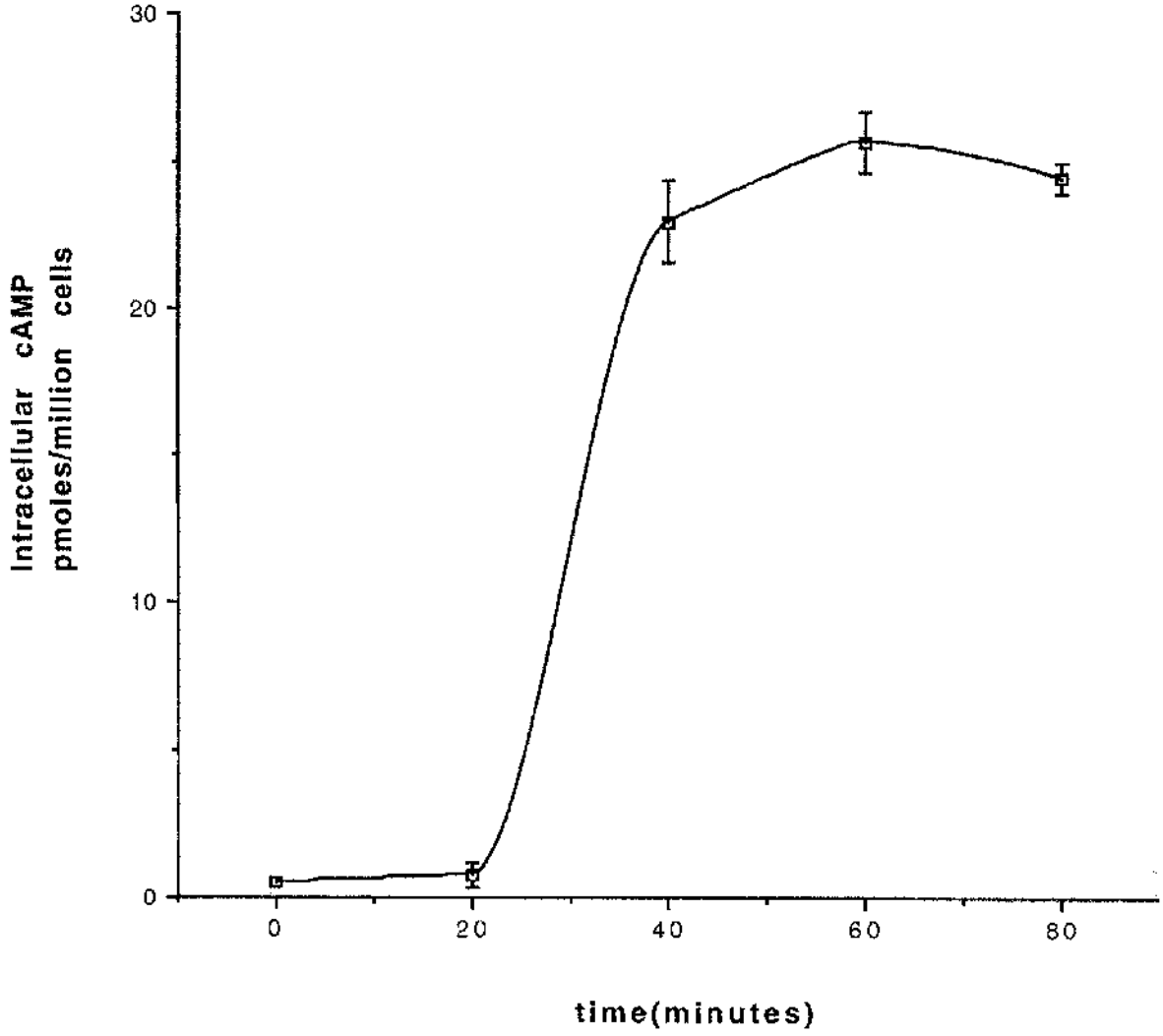

Intracellular cAMP
pmoles/million cells
30
20
10
0
0
20
40
60
80
time(minutes)

Figure 3.13 Cholera toxin actived adenylate cyclase activity in CHO cells in a manner which is resistant to inhibition by PMA

CHO cells were incubated for 2h with cholera toxin (1μg/ml) in the presence of the phosphodiesterase inhibitor IBMX (1mM). They were then harvested, and the intracellular concentration of cAMP was determined as described in methods section (2.3.9.3). Experiments were done with cholera toxin either alone or together with PMA added to the incubations at 100nM final concentration. Result are means±S.E. Data are typical of experiments done on three separate cell preparations.

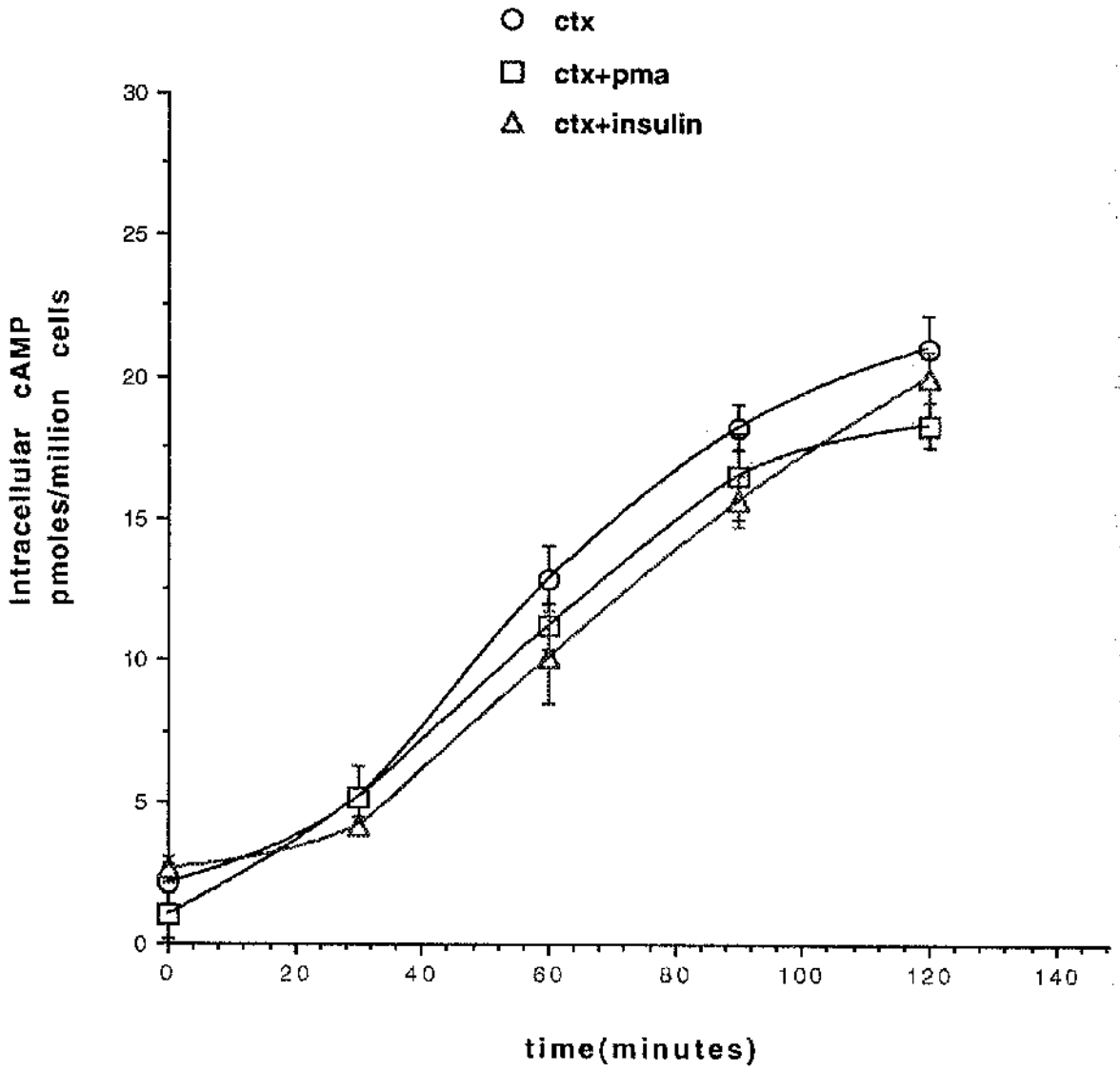

ctx
ctx+pma
ctx+insulin
Intracellular cAMP
pmoles/million cells
30
25
20
15
10
5
0
0
20
40
60
80
100
120
140
time(minutes)

Figure 3.14 PMA enhanced cholera-toxin-stimulated adenylyl cyclase in NIH-3T3 cells

NIH-3T3 cells were incubated for 2h with cholera toxin (1µg/ml) in the presence of the phosphodiesterase inhibitor IBMX (1mM). They were then harvested, and the intracellular concentration of cAMP was determined as described in methods section (2.3.9.3). Experiments were done with cholera toxin either alone or together with PMA added to the incubations at 100nM final concentration. Results are means±S.E. Data are typical of experiments done on three separate cell preparations.

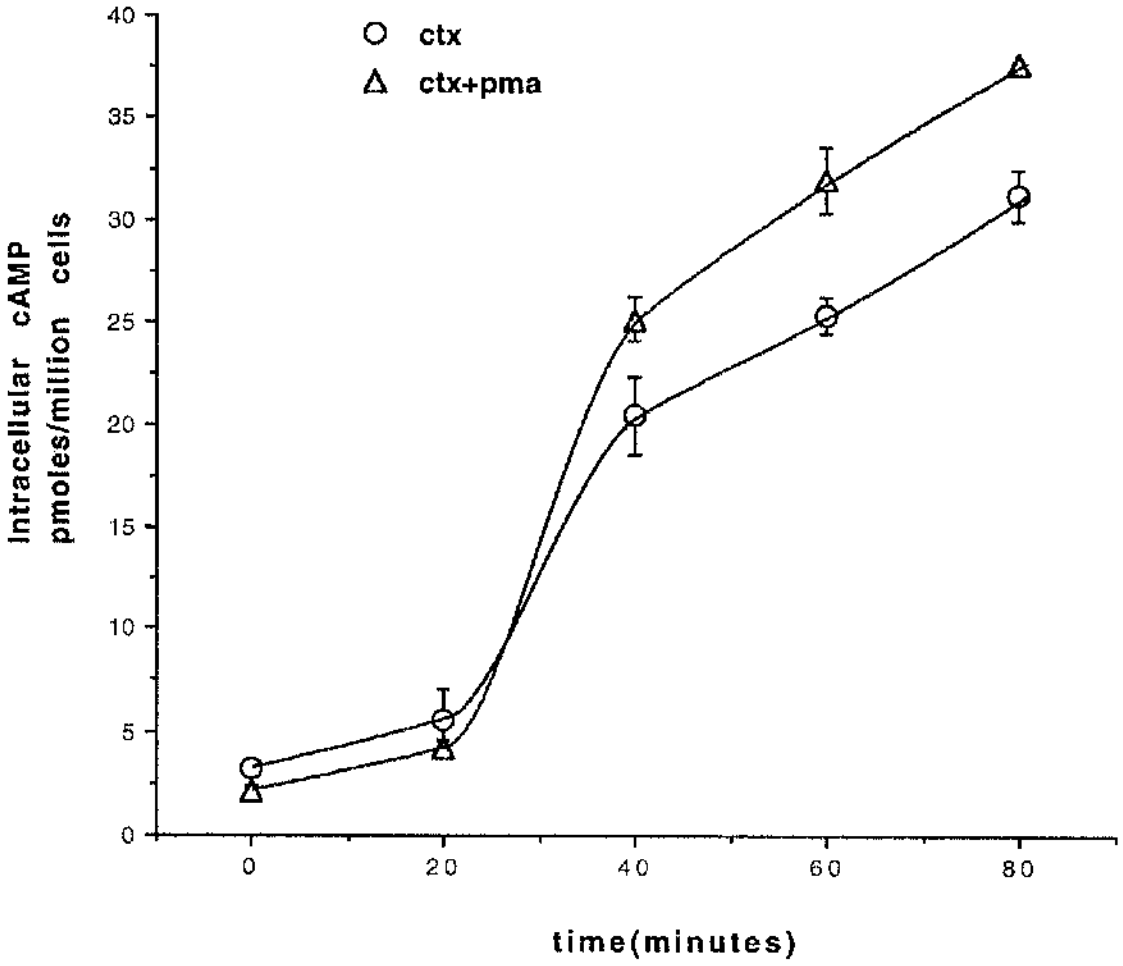

ctx
ctx+pma
Intracellular cAMP
pmoles/million cells
0
5
10
15
20
25
30
35
40
0
20
40
60
80
time(minutes)

Figure 3.15 Thermal denaturation of adenylyl cyclase activities in CHO cells and hepatocytes

Washed membrane fractions were obtained as described in methods section (2.3.1.3) from hepatocyte and CHO cells. Membranes were incubated at increasing temperatures for 10min. At the indicated temperatures, samples were taken for assay of adenylyl cyclase activity by methods section (2.3.8). Data are typical of experiments done on two other separate preparations.

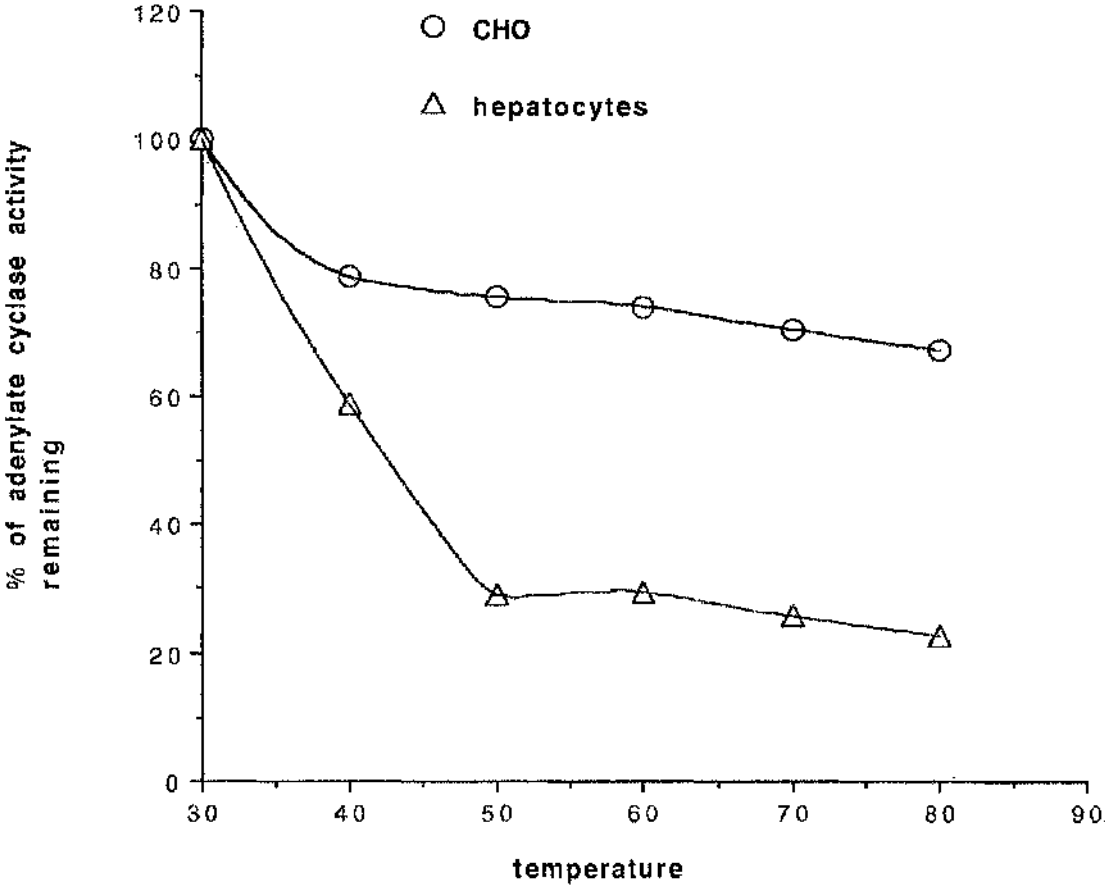

CHO
hepatocytes
% of adenylate cyclase activity remaining
120
100
80
60
40
20
0
30
40
50
60
70
80
90
temperature

Figure 3.16 Thermostability of adenylyl cyclase activities in CHO cells and hepatocytes

Washed membrane fraction were obtained as described in methods section (2.3.1.3) from hepatocyte and CHO cells. Membranes were incubated at the elevated temperature 50°C for various times up to 10min. At the indicated times, samples were taken for assessing adenylyl cyclase activity by methods section (2.3.10). The plots shown are typical of ones performed at least three times using different cell preparations.

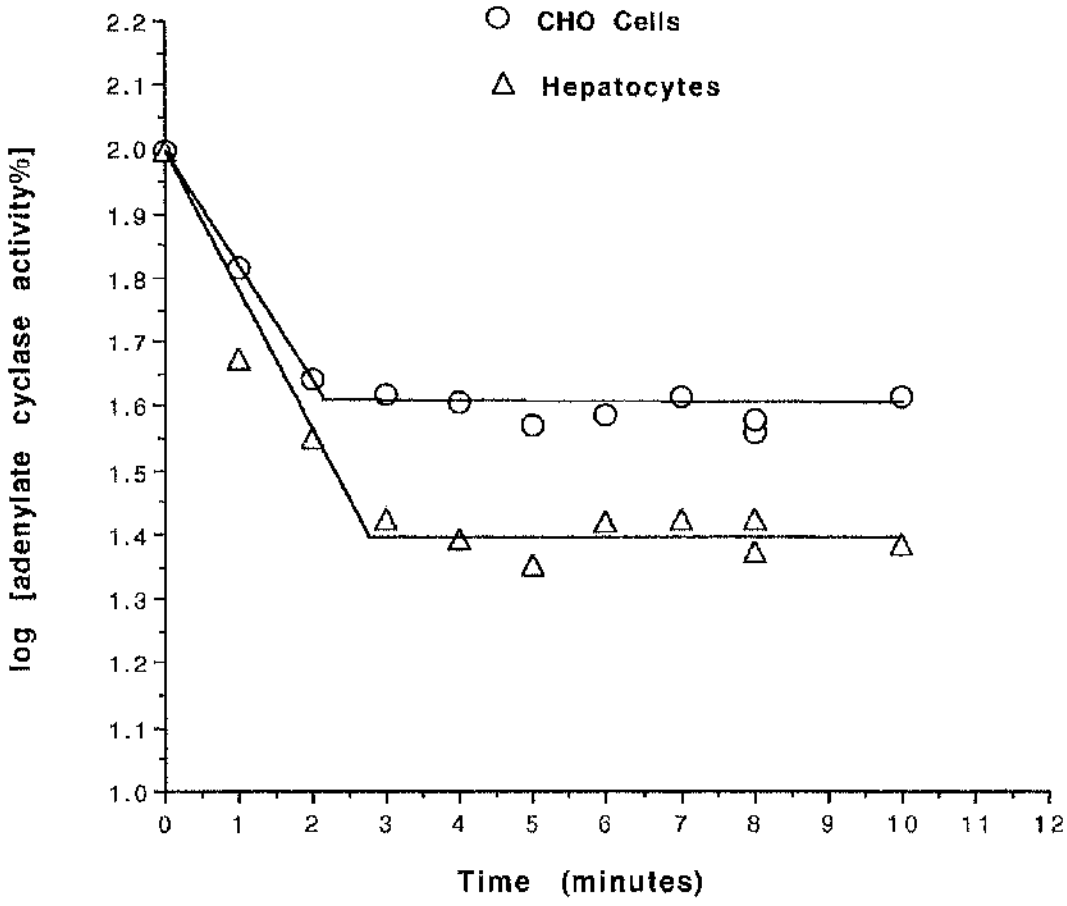

CHO Cells
Hepatocytes
log [adenylate cyclase activity%]
2.2
2.1
2.0
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0
1
2
3
4
5
6
7
8
9
10
11
12
Time (minutes)

Figure 3.17 Thermostability of adenylyl cyclase activities in P9 cells

Washed membrane fractions were obtained as described in methods section (2.3.7.1) from P9 cells. Membranes were incubated at the elevated temperature 50°C for various times up to 10min. At the indicated time, samples were taken for assay of adenylyl cyclase activity by methods section (2.3.10). The plots shown are typical of ones performed at least three times using different cell preparations.

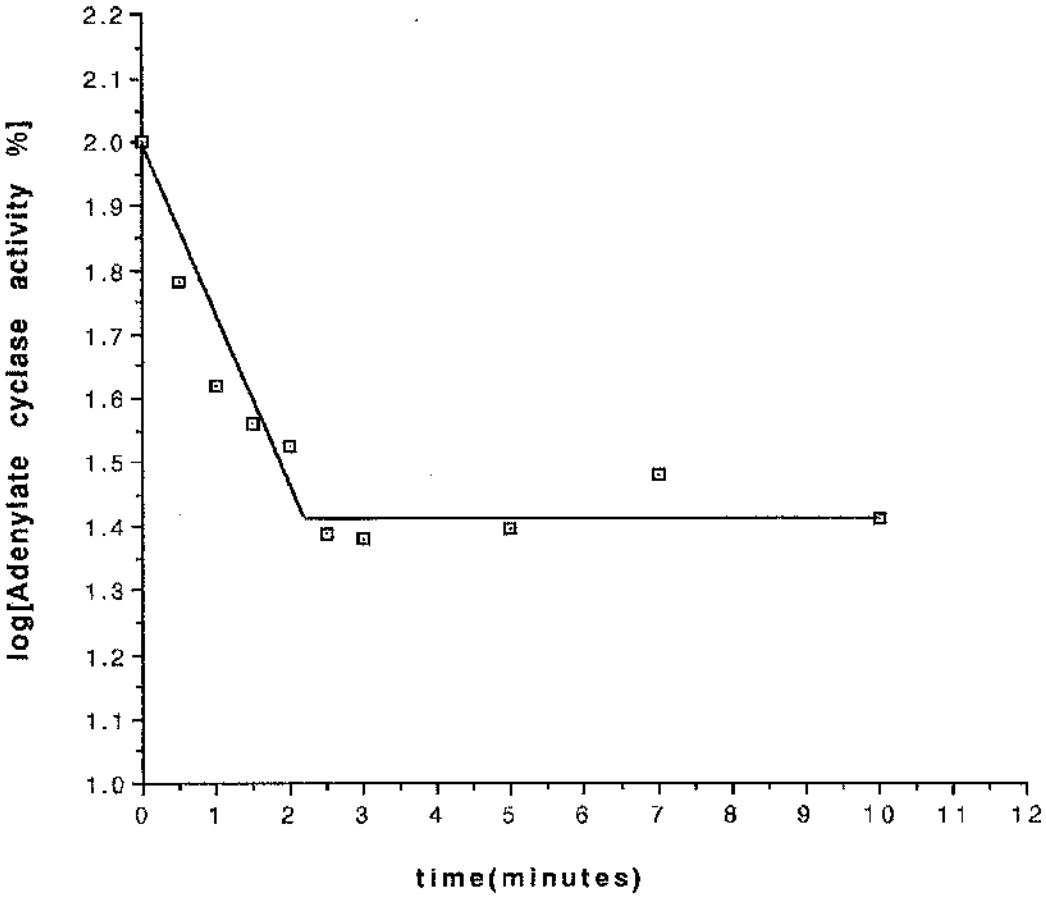

log[Adenylate cyclase activity %]
2.2
2.1
2.0
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0
1
2
3
4
5
6
7
8
9
10
11
12
time(minutes)

Figure 3.18 (a) RT-PCR analyses of type-I adenylyl cyclase transcripts in different cells

This was done as described in methods (section 2.3.18) using the degenerate primers designed (Sense; GATCCTGCTC TCCGGGCTCA. Antisense; CTTCTCAGCA GCCGGTGGAC T) to detect transcripts selectively for the type-I adenylyl cyclase in a species independent fashion. PCR reaction conditons employed here utilized 1 cycle at 95°C for 5min, 60°C for 1min and 72°C for 1min, followed by 30 cycles at 95°C for 30 seconds, 60°C for 1min and 72°C for 1min, then 1 cycle at 95°C for 30 seconds, 60°C for 1min and 72°C for 5min. This reaction condition allowed the detection of a 830bp fragment for type I adenylyl cyclase.

Analyses were performed on rat hepatocytes (H), immortalised hepatocytes (P9), CHO cells (C) and NIH-3T3 cells (N). Marker (M), plasmid control (P).

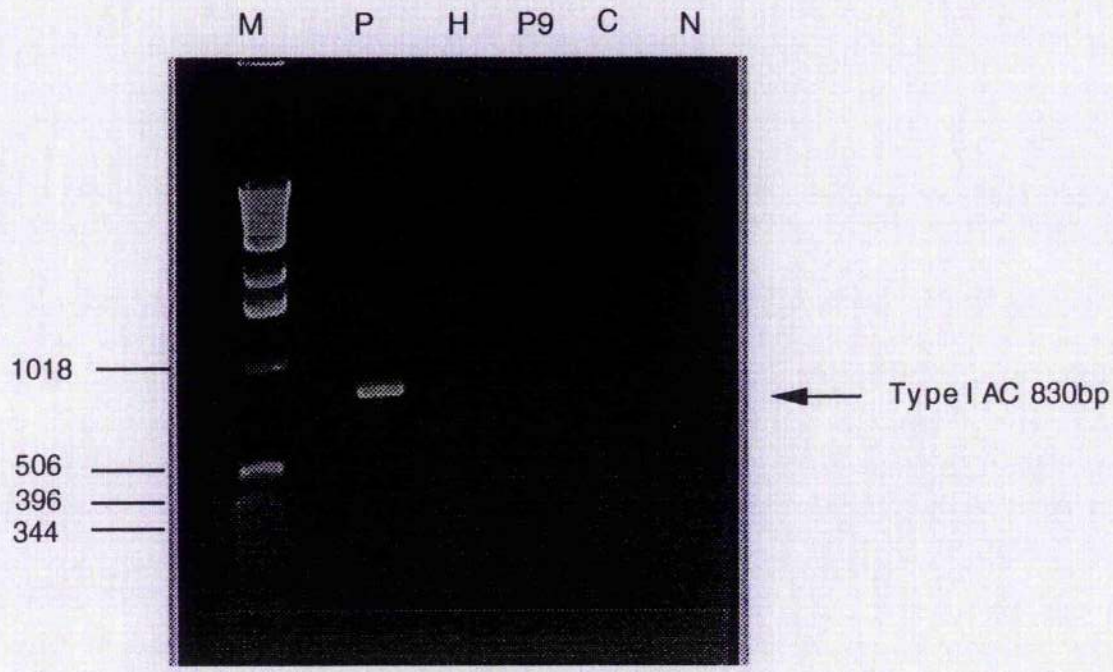

M
P
H
P9
C
N
1018
506
396
344
Type I AC 830bp

Figure 3.18 (b) RT-PCR analyses of type-II adenylyl cyclase transcripts in different cells

This was done as described in methods (section 2.3.18) using the degenerate primers designed (Sense; CACGGATCTCCCTCACAATCGTCA. Antisense; TGCGAGTGGG TATCAACCAT GGGC) to detect transcripts selectively for the type-II adenylyl cyclase in a species independent fashion. PCR reaction conditons employed here utilized 1 cycle at 95°C for 5min, 60°C for 1min and 72°C for 1min, followed by 30 cycles at 95°C for 30 seconds, 60°C for 1min and 72°C for 1min, then 1 cycle at 95°C for 30 seconds, 60°C for 1min and 72°C for 5min. This reaction condition allowed the detection of a 980bp fragment for type II adenylyl cyclase.

Analyses were performed on rat hepatocytes (H), immortalised hepatocytes (P9), CHO cells (C) and NIH-3T3 cells (N). Marker (M), plasmid control (P).

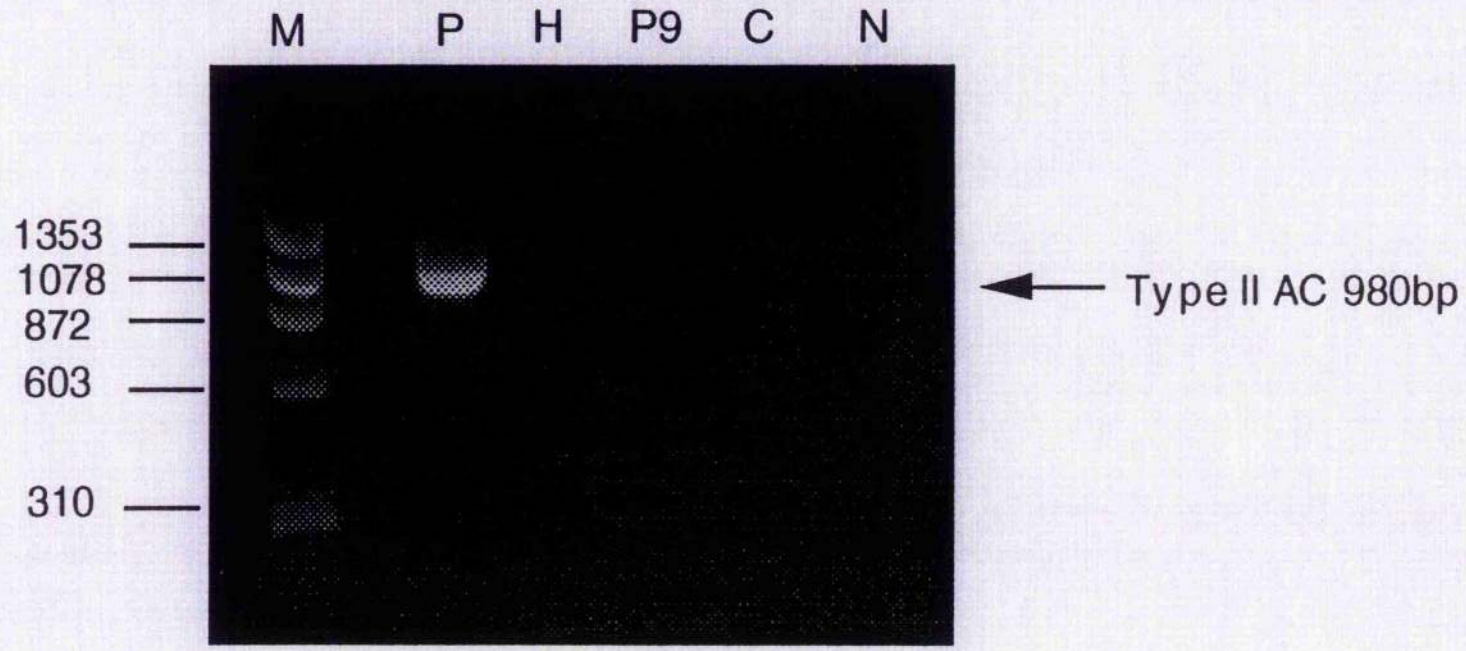

M
P
H
P9
C
N
1353
1078
872
603
310
Type II AC 980bp

Figure 3.18 (c) RT-PCR analyses of type-IV adenylyl cyclase transcripts in different cells and type V adenylyl cyclase transcripts in hepatocytes and P9 cells

This was done as described in methods (section 2.3.18) using the degenerate primers designed (Sense; CAGGAGCACC TCCTCTTGTC TAT. Antisense; GTTGTTCGAT GACCTGGAAG AAC) to detect transcripts selectively for the type-IV adenylyl cyclase in a species independent fashion. PCR reaction conditons employed here utilized 1 cycle at 95°C for 5min, 60°C for 1min and 72°C for 1min, followed by 30 cycles at 95°C for 30 seconds, 60°C for 1min and 72°C for 1min, then 1 cycle at 95°C for 30 seconds, 60°C for 1min and 72°C for 5min. This reaction condition allowed the detection of a 966bp fragment for type IV adenylyl cyclase.

For the type V adenylyl cyclase, an arrow indicating the 861 bp fragment is shown only in hepatocytes and P9 cells. For PCR reaction condition see next Figure (3.18 (d)).

Analyses were performed on rat hepatocytes (H), immortalised hepatocytes (P9), CHO cells (C) and NIH-3T3 cells (N). Marker (M), plasmid control (P).

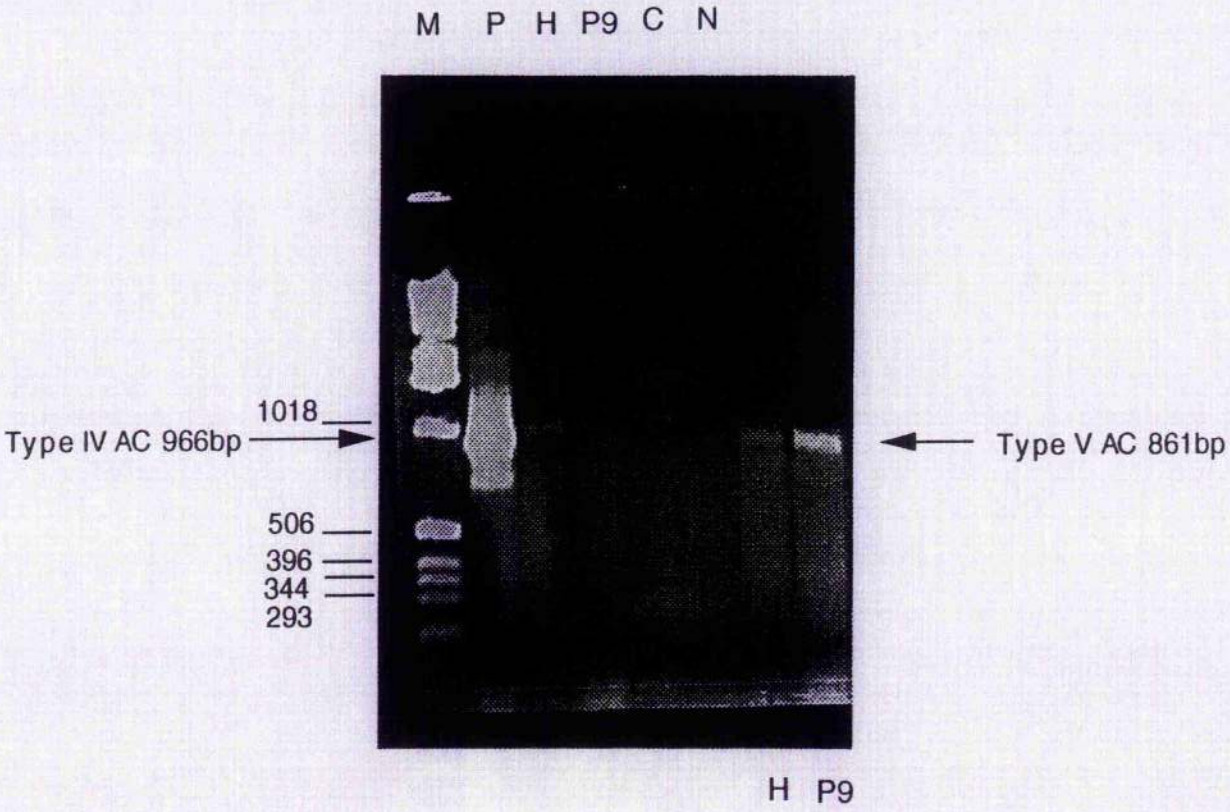

M P H P9 C N
1018
Type IV AC 966bp
506
396
344
293
Type V AC 861bp
H P9

Figure 3.18 (d) RT-PCR analyses of type-V adenylyl cyclase transcripts in CHO and NIH-3T3 cells

This was done as described in methods (section 2.3.18) using the degenerate primers designed (Sense; CTACAACCAC TTGGGTGGCA AC. Antisense; ATACCGTAAC CGCCACTGGT C) to detect transcripts selectively for the type-V adenylyl cyclase in a species independent fashion. PCR reaction conditons employed here utilized 1 cycle at 95°C for 5min, 60°C for 1min and 72°C for 1min, followed by 30 cycles at 95°C for 30 seconds, 60°C for 1min and 72°C for 1min, then 1 cycle at 95°C for 30 seconds, 60°C for 1min and 72°C for 5min. This reaction condition allowed the detection of a 861bp fragment for type V adenylyl cyclase.

Analyses were performed on rat hepatocytes (H), immortalised hepatocytes (P9), CHO cells (C) and NIH-3T3 cells (N). Marker (M).

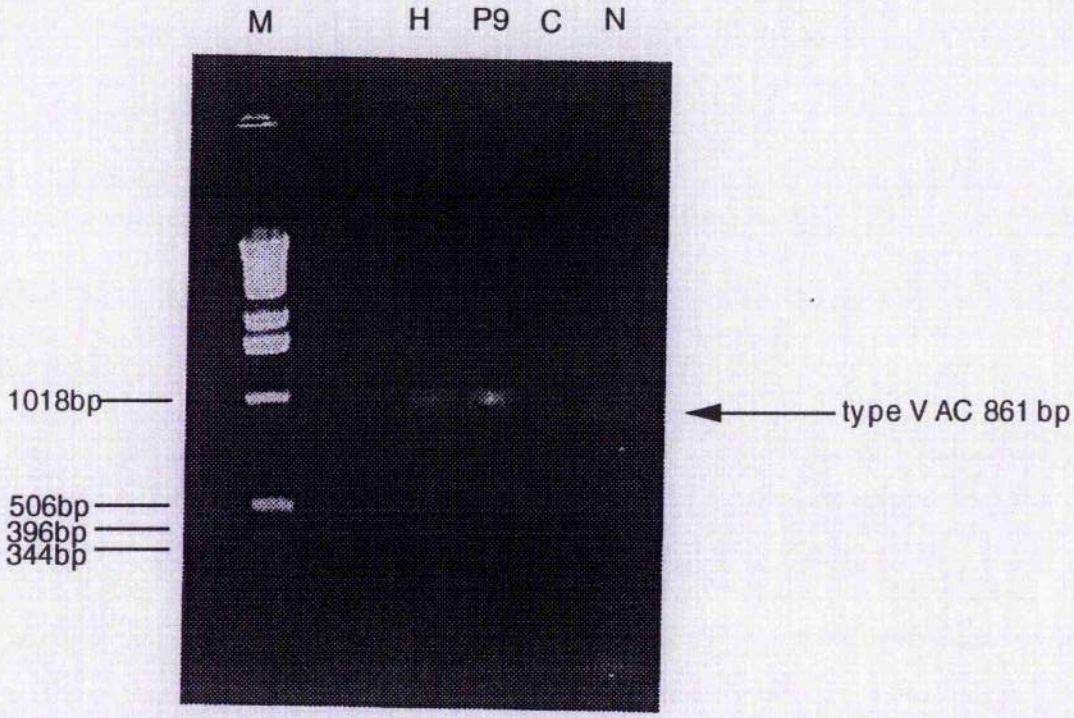

M
H
P9
C
N
1018bp
506bp
396bp
344bp
type V AC 861 bp

Figure 3.18 (e) RT-PCR analyses of type-VI adenylyl cyclase transcripts in different cells

This was done as described in methods (section 2.3.18) using the degenerate primers designed (Sense; CGGAAAGTAGACCCTCGTTTCGGA. Antisense; TGCTTAGCGT CCATGGCTTG GC) to detect transcripts selectively for the type-VI adenylyl cyclase in a species independent fashion. PCR reaction conditons employed here utilized 1 cycle at 95°C for 5min, 60°C for 1min and 72°C for 1min, followed by 30 cycles at 95°C for 30 seconds, 60°C for 1min and 72°C for 1min, then 1 cycle at 95°C for 30 seconds, 60°C for 1min and 72°C for 5min. This reaction condition allowed the detection of a 637bp fragment for type VI adenylyl cyclase.

Analyses were performed on rat hepatocytes (H), immortalised hepatocytes (P9), CHO cells (C) and NIH-3T3 cells (N). Marker (M), plasmid control (P).

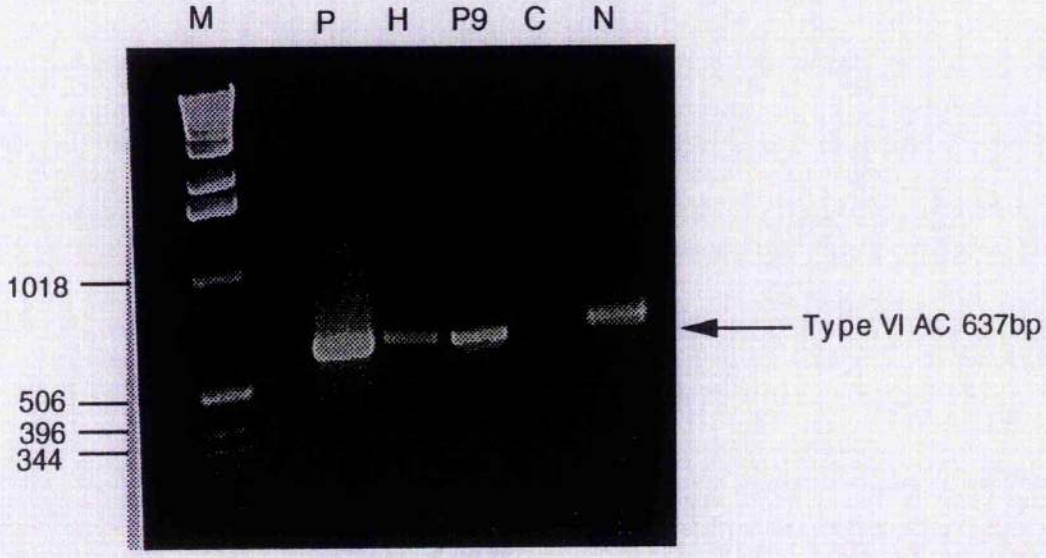

M
P
H
P9
C
N
1018
506
396
344
Type VI AC 637bp

Table 3.1 Cholera-toxin-stimulated adenylyl cycylase activity in hepatocyte membranes

Hepatocytes were incubated for 1h with cholera toxin (1µg/ml), harvested, and a washed membrane fraction was then prepared and taken to assess adenylyl cyclase activity as described in the methods section (2.3.10). Where indicated, other ligands were added together with cholera toxin in the hepatocyte incubation. Vasopressin, insulin, PMA, staurosporine and chelerythrine were added at final concentrations of 10nM, 10nM, 100nM, 100nM and 10µM respectly. In some instances the isolated membrane fraction was subsequently treated with alkaline phosphatase (1unit/µl) as described in methods section (2.3.5.2). Data were presented relative to that activity seen in membranes from cells treated with cholera toxin alone (100%). Results are means±S.E. for n=3 experiments using different cell preparation from different animals

Treatment	activity %
cholera toxin	(100)
cholera toxin + vasopressin	47 ± 8
cholera toxin + PMA	62 ± 7
cholera toxin + insulin	55 ± 4
cholrea toxin+staurosporine	92±5
cholera toxin+chelerythrine	93±6
cholera toxin, then membrane alkaline phosphatase treated	97±6
cholera toxin (60min) then cell PMA treatment (15min)	63±10

Table 3.2 Cholera toxin-stimulated adenylyl cyclase activity in P9 cells.

The effect of cholera toxin (1μg/ml; 60min) on P9 cell adenylyl cyclase function was assessed either by measuring adenylyl cyclase activity in an isolated membrane fraction (membrane) or by incubating P9 cells in the presence of the cyclic AMP phosphodiesterase inhibitor IBMX (1mM) and measuring the intracellular concentration of cyclic AMP (intracellular) as described in the methods section (2.3.9). Resting cells had an intracellular cyclic AMP concentration of 1.5-2.6 pmol of cyclic AMP/10^6 cells, which was typically raised to 28-37pmol/10^6 cells after 1h treatment of the intact cells with cholera toxin (1μg/ml) (range; n=3). Membrane adenylyl cyclase activity in resting cells was 2.1±0.4 pmol of cyclic AMP/min per mg of membrane protein. This rose to 9.8±0.6 pmol/min per mg of membrane protein after cholera-toxin treatment. Where indicated, other ligands were added together with cholera toxin in the P9 cell incubation, with insulin and vasopressin at final concentrations of 10nM and PMA at 100nM. Experiments were done five times with different cell preparations, and results are given as mean±S.D. Shown are changes in cholera-toxin-stimulated adenylyl cyclase activity relative to that found in cells not simultaneously challenged with ligands (100%).

Treatment	Adenylyl cyclase activity in membranes	Intracellular cAMP levels
cholera toxin	(100)	(100)
cholera toxin + vasopressin	57±6	48±5
cholera toxin + PMA	59±8	68±4
cholera toxin + insulin	63±6	61±8

Table 3.3 Cholera toxin-stimulated adenylyl cyclase activity in CHO and NIH-3T3 cells.

Cells were incubated for 2h with cholera toxin (1μg/ml) in the presence of the phosphodiesterase inhibitor IBMX (1mM). They were then harvested and the intracellular cyclic AMP concentration determined as described in methods section (2.3.9). The concentration of cyclic AMP was given in pmol cyclic AMP/10^6 cells for cells treated either with toxin alone or with PMA added to the incubations at 100nM. An indication of the effect of PMA on the cholera toxin stimulated accumulation of cyclic AMP is given by expressing the levels of cyclic AMP accruing with PMA and cholera toxin added together as a percentage of those found when cholera toxin was added alone. The various CHO cell lines are denoted as k, wild-type CHO cells; T, transfected so as to overexpress the human insulin receptor and otherwise with Greek letters indicating the particular isoform of PKC that these cells have been transfected to overexpress. All CHO cells overexpressing PKC isoforms also overexpressed the human insulin receptor (Chin *et al.*, 1993). NIH-3T3 cells were wild-type cells. Experiments were done 3 times with different cell preparations and errors given as means ±S.D.

cell line	cAMP accumulation			adenylyl cyclase activity		
	-PMA	+PMA	(%)	-PMA	+PMA	(%)
CHO-k	24±2.4	22.8±1	95	4.7±0.2	4.5±0.3	96
CHO-T	22±1.5	23.4±2.1	106	-	-	-
CHO-α	23.6±1.5	21.6±1.2	92	4.8±0.3	4.6±0.5	96
CHO-βII	25.2±0.8	31.8±2.5	126	-	-	-
CHO-ε	30.1±0.9	30.2±0.6	100	-	-	-
CHO-γ	21.1±1.2	28.5±1.5	135	4.5±0.1	6.5±0.2	144
NIH-3T3	25.4±1	32±1.6	126	5.4±0.2	7.1±0.3	132

Table 3.4 Summary of adenylyl cyclase expression in different cell lines

Adenylyl cyclase isotype	Hepatocyte	P9	NIH3T3	CHO
I	-	-	-	-
II	-	-	-	-
IV	+	-	-	+
V	+	+	-	-
VI	+	+	+	-

N.B. + stands for signal expressed in cells, - stands for signal not expressed in cells.

Discussion

These experiments showed that treatment of intact hepatocytes with cholera toxin led to the constitutive activation of adenylyl cyclase activity after a well-defined lag period **(Figure 3.2)**. This lag time was also observed in other cell lines **(Figure 3.11; Figure 3.12.)**. The molecular basis of this lag period remains to be defined, although it can be altered by changing temperature, cholera-toxin concentration and membrane fluidity (Van, 1977; Vaughan and Moss, 1978; Houslay and Elliott, 1979). The lag time may, in part, be connected with a requirement for cholera toxin to undergo endocytosis (Houslay and Elliott, 1981) before it can act on G_s at the cytosolic surface of the plasma membrane. The magnitude of this stimulatory effect of cholera toxin was shown to be profoundly attenuated if either glucagon or insulin was added to hepatocytes along with cholera toxin **(Figure 3.3)**. Such inhibitory effects were observed when adenylyl cyclase activity was assessed either in membranes isolated from such cells or, *in situ*, by assessing cAMP accumulation in the presence of the phosphodiesterase inhibitor IBMX. These hormones did not affect the lag time for the action of cholera toxin nor did they alter the degree of ADP–ribosylation of G_s (Irvine and Houslay, 1988), suggesting that either the functioning of the catalytic unit of adenylyl cyclase was attenuated or its ability to be stimulated by G_s was diminished.

Glucagon is known to generate a number of intracellular signals in hepatocytes (Houslay, 1991 and 1994). These include the well-established elevation of intracellular Ca^{2+} levels (Blackmore and Exton, 1986; Mauger and Claret, 1985; Mine *et al.*, 1988) and also stimulation of both phosphatidyl-choline (Pittner and Fain, 1991) and inositol phosphate metabolism (Wakelam *et al.*, 1986; Whipps *et al.*, 1987; Williamson *et al.*, 1986), which elicit the production of diacylglycerol (Pittner and Fain, 1991; Blackmore and Exton, 1986; Boeckino *et al.*, 1985) and thus the activation of protein kinase C (Tang

and Houslay, 1992). Glucagon clearly can not be exerting its inhibitory effect on the ability of cholera toxin to activate adenylyl cyclase through any elevation of intracellular cyclic AMP levels as cholera toxin itself achieves just that (Houslay and Elliott, 1979 and 1981; Heyworth *et al.*, 1984). This suggested that such an action of glucagon might be mediated through protein kinase C activation. Thus, in order to ascertain whether protein kinase C activation might exert an inhibitory effect on cholera toxin-stimulated adenylyl cyclase activity, We incubated hepatocytes with either the phorbol ester PMA or with vasopressin; agents which have been shown to activate protein kinase C in hepatocytes (Nixon *et al.*, 1992; Nishizuka, 1989; Parker *et al.*, 1992; Creba *et al.*, 1983). In both instances we observed inhibition of the cholera toxin response (**Table 3.1**) although this attenuation was not additive (**Figure 3.7**). However, inhibition of cholera toxin stimulated-adenylyl cyclase activity by the above ligands may occur independently, although the quantitative contributions were not additive. It has been shown that PMA exerts its effects through a well-established, direct action upon the regulatory site of protein kinase C (Nixon *et al.*, 1992; Nishizuka, 1989; Parker *et al.*, 1992) where it mimics the endogenous activator, DAG. In contrast, vasopressin stimulates inositol phospholipid metabolism in hepatocytes (Creba *et al.*, 1983) to produce DAG (Pittner and Fain, 1991; Blackmore and Exton, 1986; Bocckino *et al.*, 1985) which leads to the activation of protein kinase C (Pittner and Fain, 1991; Tang and Houslay, 1992). The different pathways by which these compounds act suggested their ability to inhibited cholera toxin-stimulated adenylyl cyclase was through a common final action which is mediated by protein kinase C. The non-additive effect of these ligands is consistent with this. Confirmation was gived by the inclusion of the compounds staurosporine and chelerythrine, which can serve as protein kinase inhibitors with some selectivity in their action as regards the inhibition of protein kinase C (Nixon *et al.*, 1992; Herbert *et al.*, 1990; Gordge and Ryves, 1994), which ablated the inhibitory action of

vasopressin **(Figure 3.4).** Interestingly, however, they also served to ablate the inhibitory action of insulin **(Figure 3.4)**, implying that insulin also exerted effects on adenylyl cyclase activity through the action of protein kinase C. In this regard, it has been suggested that insulin might activate protein kinase C activity in various cells (Faresc *et al.*, 1992). However, it is also possible that members of the protein kinase cascade known to form one branch of the pleiotropic network which comprises the insulin receptor signalling system (White and Kahn, 1994), may transduce the inhibitory effect of insulin noted here and that such a species could be inhibited by these compounds.

It has previously been suggested that, under resting conditions in hepatocytes, there is an active phosphorylation/dephosphorylation cycle involving protein kinase C action (Bushfield *et al.*, 1991; Houslay, 1991). As such the level of phosphorylation of any protein which provides a target for protein kinase C can be profoundly amplified by the action of the protein phosphatase inhibitor okadaic acid (Tachibana *et al.*, 1981; Cohen *et al.*, 1990; Bialojan and Takai, 1988; Haystead *et al.*, 1989). This compound can therefore serve as a novel tool for the study of protein phosphorylation in intact cells and like phorbol esters, okadaic acid acts as a tumour promoter, presumably by increasing the phosphorylation of key regulatory proteins. However, unlike phorbol esters, okadaic acid exerts its effect by inhibiting protein phosphatases 1 and 2A (Bialojan and Takai, 1988) rather than by activation of protein kinase C. Thus exposure of hepatocytes to okadaic acid has been shown to mimic the effect of protein kinase C action in eliciting the phosphorylation of the inhibitory G-protein $G_{i\alpha 2}$ at the protein kinase C phosphorylation ('C') site, ser^{144} (Morris *et al.*, 1994). Okadaic acid also appears able to mimic protein kinase C-mediated glucagon desensitization of adenylyl cyclase activity in hepatocytes (Savage *et al.*, 1995). In contrast, under such basal conditions then no phosphorylation of the inhibitory G-protein $G_{i\alpha 2}$ at the protein kinase A phosphorylation ('AN') site ser^{207} was elicited by okadaic acid (Morris *et al.*,

1994). Consistent with the contention that protein kinase C effects an inhibitory action on cholera toxin-stimulated adenylyl cyclase activity in hepatocytes, the observation here of a profound attenuation of cholera toxin to stimulate adenylyl cyclase activity by okadaic acid in hepatocytes **(Figure 3.5)** would be consistent with PKC mediation of this response.

Such data imply that protein kinase C action can attenuate cholera toxin-stimulated adenylyl cyclase activity. This is intriguing as we also have shown that treatment of hepatocytes with the phorbol ester PMA, for example, did not inhibit the activity of either the catalytic unit of adenylyl cyclase, assessed by monitoring both basal activity and that amplified by forskolin **(Figure 3.8 (a))**, or G_s-stimulated adenylyl cyclase when monitored using NaF^- **(Figure 3.8 (b))**. However, in such experiments hepatocytes were challenged with agents which activated protein kinase C under very different conditions from those prevailing in the cells which have been exposed to cholera toxin. For, in the presence of cholera toxin then not only do intracellular levels of cyclic AMP rise above the threshold for activation of protein kinase A (Houslay, 1991; Heyworth *et al.,* 1983) but adenylyl cyclase is subject to chronic stimulation by $G_{s\alpha}$. It may be that either one or both of these conditions is a pre-requisite for protein kinase C to be able to modify a component of the G_s-adenylyl cyclase system and thus attenuate cholera toxin-stimulated adenylyl cyclase activity. The possibility that chronic elevation of intracellular cyclic AMP levels may allow for this novel action of protein kinase C might be inferred from an earlier study by Murphy and Houslay,(1988). In that case, it has been shown that when hepatocytes were exposed to glucagon in the presence of the phosphodiesterase inhibitor IBMX then a stable, profound inhibition of membrane adenylyl cyclase activity ensued rather than a transient inhibition as seen using glucagon alone (Murphy and Houslay, 1988; Houslay, 1991; Houslay, 1994). Indeed, in the presence of both glucagon and IBMX then the levels of cyclic AMP achieved were comparable to those observed in

hepatocytes treated solely with cholera toxin (Murphy and Houslay, 1988). Such data might be taken to imply that elevated intracellular cyclic AMP levels in hepatocytes allow protein kinase C to act at a site which can lead to the attenuation of G_s-stimulated adenylyl cyclase activity.

That an attenuated cholera toxin-stimulated adenylyl cyclase activity can be observed in an isolated hepatocyte membrane preparation might imply that a component of this system is stably phosphorylated. Consistent with this, treatment of membranes with alkaline phosphatase has been used to achieve the dephosphorylation of phosphorylated cellular proteins (Savage *et al.*, 1995; Pears *et al.*, 1992). Such a treatment did not affect the adenylyl cyclase activity of membranes obtained from hepatocytes which had been treated with cholera toxin alone **(Table 3.1)**. However, it did increase the cholera toxin-stimulated adenylyl cyclase activity seen in membranes from cells which had also been treated with either PMA or vasopressin or insulin **(Table 3.1; Figure 3.6)**. In each of these instances such a treatment increased adenylyl cyclase activity up to a level which indicated that it had been able to reverse the inhibitory effect exerted by these various agents. This is consistent with the notion that such an inhibitory process occurred as a result of the phosphorylation of a membrane protein. In this regard, we had previously concluded that inhibition was not due to any impedance in the mechanism of cholera toxin action *per se* (Irvine and Houslay, 1988). For internalisation did not appear to be attenuated, as lag times for the onset of activation were not delayed (Irvine and Houslay, 1988) and no change in the ability of cholera toxin to elicit the ADP ribosylation of α-G_s was evident (Irvine and Houslay, 1988). This conclusion is also supported by the demonstration here **(Table 3.1)** that PMA was capable of causing a similar degree of inhibition of cholera toxin-stimulated adenylyl cyclase activity when it was added to intact hepatocytes at a time (60min) when cholera toxin had already achieved near maximal stimulation of this system rather than at the start of the incubation with the toxin.

An immortalised hepatocyte cell line generated using SV40 DNA (Livingstone *et al.*, 1994) was also investigated for a similar mediated action of PKC. This P9 cell line was shown (see section 1.3.6.2) to express G-protein regulated adenylyl cyclase activity and a functional, high affinity insulin response. Here we demonstrated that the adenylyl cyclase activity in such cells could be markedly stimulated by cholera toxin **(Figure 3.9)** and that this activity could be inhibited by either PMA or insulin treatment **(Figure 3.10)** in a similar fashion to that seen with native hepatocytes. This was true of adenylyl cyclase activity assessed either in membranes isolated from cells or, *in situ*, by assessing cAMP accumulation in the presence of the phosphodiesterase inhibitor IBMX **(Table 3.2)**. This provided further evidence that P9 cells should provide a useful cell line for analysing various receptor regulated functions which are representative of native hepatocytes. Intriguingly, the observation that vasopressin could elicit an inhibition of cholera toxin-stimulated adenylyl cyclase activity which was comparable with that seen in native hepatocytes **(Table 3.1)**, is to our knowledge the first evidence of an immortalized hepatocyte cell line to have functional vasopressin receptors.

As with experiments done on hepatocytes and P9 cells, cholera toxin achieved a marked stimulation of adenylyl cyclase activity in both CHO and NIH-3T3 cells **(Figure 3.11; Figure 3.12)**. However, this activity was not attenuated by treatment with the phorbol ester PMA when adenylyl cyclase activity was assessed either in membranes isolated from such cells or, *in situ*, by assessing cAMP accumulation in the presence of the phosphodiesterase inhibitor IBMX **(Table 3.3; Figure 3.13; Figure 3.14)**, implying a fundamental difference in susceptibility of these systems. One possible explanation for this might be that levels of protein kinase C were limiting in such cells or that a specific isoform of protein kinase C may be required. However, this would appear to be unlikely, as PMA failed to inhibit cholera-toxin-stimulated adenylyl cyclase activity in CHO cells which had been

transfected so as to overexpress various protein kinase C isoforms **(Table 3.3)** (Chin *et al.*, 1993), representative of both the Ca^{2+}-dependent and independent families (Nishizuka, 1992; Asaoka *et al.*, 1992; Parker *et al.*, 1989; Ohno *et al.*, 1990). The insensitivity to phorbol ester action in these cells may thus be due to the expression of different target proteins for the action of protein kinase C.

As indicated above, either $G_{s\alpha}$ or the catalytic unit of adenylyl cyclase could form two possible targets for the action of protein kinase C. In this regard, $G_{s\alpha}$ has been shown to be a single gene product whose sequence is highly conserved between species (Birnbaumer *et al.*, 1990; Gilman, 1987; Gierschik *et al.*, 1990; Kaziro, 1990). In contrast, adenylyl cyclase forms part of a large multi-gene family where differential expression in various cells is well established (Krupinski *et al.*, 1992; Krupinski *et al.*, 1989; Cooper *et al.*, 1994) **(Table 3.4)**, and differential susceptibility to phorbol esters has been noted, with enhancement of the activity of certain, but not all, isoforms of adenylyl cyclase occurring (Yoshimura and Cooper, 1993; Chen and Iyengar, 1993; Jacobowitz *et al.*, 1993; Kawbe *et al.*, 1994). On this basis, the prejudice would be that the the differences in susceptibility to PMA action in these cells were due to the expression of different forms of adenylyl cyclase. RT-PCR **(Figure 3.18 (a)-(e))** and thermostability studies of adenylyl cyclase **(Figure 3.16; Figure 3.17)** comfirmed that there are different levels of adenylyl cyclase activities expressed in these cells. A particular species of adenylyl cyclase, namely type V or VI, may provide a major fraction of the activity in hepatocytes and P9 cells whilst supplying the target to be modified by protein kinase C. With this in mind it has been shown that the type-V isoform of adenylyl cyclase *in vitro* can be stimulated by phosphorylation mediated by PKC (Kawabe *et al.*, 1994), and that PKC isoforms α and δ specifically mediated this effect (Jacobowitz *et al.*, 1993). The type V adenylyl cyclase isoforms was not expressed in NIH-3T3 and CHO cells **(Figure 18 (d; e)**

and **Table 3.4**) and indeed we were unable to detect a protein kinase C-mediated phosphorylation of adenylyl cyclase in these cells.

Certainly there is a precedent for the notion that adenylyl cyclase may be phosphorylated by PKC, albeit in studies indicating that phorbol ester treatment can enhance the activity of certain, but not all, isoforms of adenylyl cyclase (Yoshimura and Cooper, 1993; Chen and Iyengar, 1993; Jacobowitz *et al.*, 1993; Kawbe *et al.*, 1994). Whilst in certain of these isoforms the effect is due to the removal of G_i-mediated inhibition, in other instances it is thought to be due to the modification of the catalytic unit (Chen and Iyengar, 1993; Jacobowitz *et al.*, 1993). In this regard we noted that treatment of NIH-3T3 cells with PMA led to a recognisable increase in the cholera toxin-stimulated adenylyl cyclase activity (**Table 3.3; Figure 3.14**). Additionally, in CHO cells we observed a PMA-mediated increase in the cholera toxin-stimulated adenylyl cyclase activity of cells transfected so as to overexpress PKC-γ (**Table 3.3**). This may indicate a specificity for the action of particular protein kinase C isoforms in being able to modify a particular adenylyl cyclase isoform in CHO cells. It does not appear to relate to differences in the relative magnitudes of protein kinase C activity in these transfected cell lines. For, compared to total protein kinase C levels seen in wild type CHO cells, Chin *et al.*, 1993 have shown that the PKC βI transfectants exhibited the highest levels of PKC expression, with levels 18–fold higher, followed by PKC-ε transfectants, with levels 10–fold higher, and then the PKC-α and PKC-γ transfectants, with levels 5–fold higher. More dramatic elevations of activity would therefore be expected in CHO PKC-β cells. This is clearly not the case (**Table 3.3**). We also noted an increase in the cholera toxin stimulated adenylyl cyclase activity of cells transfected so as to overexpress CHO-ε (**Table 3.3**). However, whilst such an increase could be due to the modification of adenylyl cyclase activity caused by the basal activity of the

enlarged protein kinase C pool, there are other possibilities which include altered expression of either adenylyl cyclase or G_s.

These experiments showed that in both hepatocytes and the immortalised hepatocyte P9 cell line attenuation of cholera toxin-stimulated adenylyl cyclase activity can be achieved by either the activation of protein kinase C (achieved either directly by PMA, or by the hormones glucagon, insulin and vasopressin) or the inhibition of protein phosphatase activity. Such an inhibitory effect can be reversed by alkaline phosphatase treatment of membranes, consistent with a phosphorylation event. That insulin and vasopressin can exert such an inhibitory action on cholera-toxin-stimulated adenylyl cyclase in the P9 immortalized hepatocyte cell, indicates that these cells can provide a useful model of a hormonally responsive system. The study thus identified a novel point of cross-talk between the cyclic AMP and the phospholipid and tyrosine kinase signalling pathways involving protein kinase C, which highlights and extends the range of actions of this protein kinase in being able to modulate signal transduction processes.

Chapter 4

Induction of specific cyclic AMP phosphodiesterase PDE4 splice variants by forskolin and cholera toxin-treatment of the P9 immortalised hepatocyte cell line

(Basic PDE4 transcripts profile (PCR) is in chapter 5)

Introduction

Control of intracellular cAMP concentration is critical to the regulation of many cellular responses to extracellular stimuli. This is achieved through regulation of both its synthesis, by adenylyl cyclase, and its degradation by cyclic nucleotide phosphodiesterases (PDEs). PDE activity in cells is contributed by a large and diverse group of enzymes that catalyze the hydrolytic cleavage of cAMP's 3' phosphoester bond to form 5'AMP which cannot activate protein kinase A (PKA). Although most of the known cyclic nucleotide PDEs share a highly conserved 270-amino acid catalytic domain, they vary widely with respect to their kinetic characteristics, substrate specificity, cellular distribution and sensitivity to various endogenous and synthetic activators and inhibitors (Beavo, 1988). At least seven classes of PDE isozymes exist, some of which contain multiple subtypes (Conti *et al.*, 1991; Beavo *et al.*, 1994). All of these isozymes, as well as many of the subtypes, are encoded by distinct genes (Beavo *et al.*, 1994). Recently much interest has been expressed in the cyclic AMP specific PDE4 family where compounds able to exert selective inhibition of such enzymes can exert anti-depressant and anti-inflammatory actions (DiSanto and Heaslip, 1993 and 1994). Currently such PDE4 selective inhibitors are being considered for the treatment of asthma (Chan *et al.*, 1993; Torphy *et al.*, 1992; Peachell *et al.*, 1992; Torphy, 1988). Molecular cloning studies (Bolger, 1994; Conti *et al.*, 1991a) have, however, led to the realisation that there are four gene families (A, B, C and D) which can be mapped to three chromosomes in both man and mouse with PDE4A and PDE4C being located on human chromosome 19, PDE4B on chromosome 1 and PDE4D on chromosome 5 (Horton *et al.*, 1995; Milatovich *et al.*, 1994; Bolger *et al.*, 1994; Conti *et al.*, 1991a). However, further complexity is apparent as, in each instance, alternative splicing leads to the possible formation of a range of different protein products from each of these genes (Bolger *et al.*, 1994; Conti

et al., 1991a). The alternative splicing appears to take the form of 5'domain swops which leads to the production of enzymes with distinct N-terminal regions. The putative role of such regions is believed to relate to regulation of enzyme activity, stability (Shakur *et al.*, 1993) and intracellular targeting (Scotland and Houslay, 1995).

A common feature of the hormone-dependent cAMP regulation is that after an initial increase, the cyclic nucleotide concentration returns toward basal levels in spite of the continuous presence of hormone. This phenomenon involves the expression of active intracellular mechanisms terminating the hormonal stimulation, since extracellular dissociation of the hormone from the receptor might not be the primary cause of cessation of a stimulus (Dohlman *et al.*, 1991, Collins *et al.*, 1992). It is also thought to be the result of mechanisms that protect the cell from excessive stimulation (desensitization). Uncoupling of the hormone receptor complex from the Gsα component of adenylyl cyclase, receptor sequestration and down-regulation are some of the mechanisms causing the termination of the hormonal stimulation and/or desensitization (Benovic *et al.*, 1985; Hausdorff *et al.*, 1989; Clark *et al.*, 1989). Indeed, multiple phosphorylations of the receptor by cAMP-independent and cAMP-dependent protein kinase are a primary signal of the uncoupling of the receptor from the transduction system. (Dohlman *et al.*, 1991; Benovic *et al.*, 1986; Benovic *et al.*, 1987).

A decrease in cAMP synthesis is not the only mechanism which can cause an enhanced rate of return of cAMP to basal levels in the target cell even in the continous presence of hormone. The activation of cAMP phosphodiesterases, by PKA-mediated phosphorylation can also play an important role in terminating the hormonal stimulus and in inducing desensitization in the target cell (Corbin *et al.*, 1985; Gettys *et al.*, 1987). Indeed, several reports have provided evidence that the activity of specific PDE isoforms may be regulated by hormones or other stimuli (Conti *et al.*, 1981;

Conti *et al.*, 1982; Ball *et al.*, 1980; Narindrasorasak *et al.*, 1982). Additionally, long term up-regulation of PDE activity, that requires hours to develop, can also play a role. This may involve the activation of cAMP-dependent protein kinase and requires both protein and mRNA synthesis (Conti et al., 1991).

Streptozotocin-induced diabetic rats show elevated levels of glucagon and heightened glucagon-stimulated adenylyl cyclase activity in hepatocytes (Gawler *et al.*, 1987; Bushfield *et al.*, 1990b). As such, it is likely that hepatocyte cAMP levels in these cells will have been chronically elevated upon onset of diabetes. There have been reports that streptozotocin-induced diabetes caused changes in high-affinity PDE activities. In view of this and because little is known about the regulation of alternative splicing in the PDE4 enzyme family, we wished to determine the effect of chronic elevation of cAMP levels on hepatocyte PDE4 expression. Furthermore, as PDE inhibitors are finding use therapeutically and are likely to increase cAMP levels chronically in many cells, then we felt it could be of interest to determine any changes occurring in an important metabolic cell type, such as the hepatocyte. Given the problems in maintaining hepatocytes in prolonged culture, we decided to study the P9, SV40-DNA immortalised hepatocyte cell line which has been shown to exhibit many of the characteristics of native hepatocytes (Livingston *et al.*, 1994). As a prelude, however, we compared the PDE4 complement in these cells to native hepatocytes.

Results

P9 cells transformed by SV40 viral DNA retain a variety of differentiated enzyme activities characteristic of hepatocytes (Livingstone *et al.*, 1994). Compounds selective for effects on specific PDE isoforms (Beavo, 1990; Houslay and Kilgour, 1990; Manganiello *et al.*, 1990; Reeves and England, 1990; Nicholson *et al.*, 1991) were used to identify and gauge the magnitude of the activity of various PDE isoforms in this cell line, which were then compared to those expressed in hepatocytes. It is strikingly apparent from such studies that P9 cells have a much lower total PDE activity than hepatocytes. However, use of isoform selective PDE inhibitors shows both cell types to express PDE2, PDE3 and PDE4 activities (**Table 4.1**). No evidence for PDE1 was apparent (17.7±3.2pmoles/mg/min, n=3) when Ca^{2+}/CaM (50μM; 10units) was added to assays done in the presence of EGTA. i.e. PDE1 activity is defined as being activated by Ca^{2+} in the presence of the Ca^{2+}-binding protein calmodulin. The absence of detectable PDE1 activity in these cells may arise due to sufficient endogenous Ca^{2+} being carried over into the assay mixtures from the cell homogenates to stimulate any PDE1 enzyme in full. To obviate this, assays were performed in the presence of EGTA (1mM, 2mM or 5mM) with PDE activity being determined in both the presence and absence of (2mM, 5mM or 8mM respectively) Ca^{2+} (plus 10 units CaM). That no activation was elicited by Ca^{2+}/CaM under any of these conditions indicated the absence of any PDE1 activity from this cell type.

PDE3 species (cGMP-inhibited PDE) can be selectively inhibited by low concentrations of cilostimide, exhibiting IC_{50} values of 0.1-1μM when assayed at 1μM cAMP (Manganiello *et al.*, 1990; Charbonnear, 1990) in contrast with other PDEs which are either insensitive to cilostimide or show IC_{50} values that are typically some 500-fold higher (Manganiello *et al.*, 1990; Nicholson *et al.*, 1991). With assays performed at 1μM cAMP and 10μM cilostmide, which can

be expected to inhibit all of the PDE3 activity, we have shown **(Table 4.1)** that PDE3 activity in P9 cells is a small component compared to that in hepatocytes. Indeed, comparison of isoform activities showed that levels of PDE3 were dramatically reduced (62%) **(Table 4.1)** in P9 cells.

PDE4 species are cAMP-specific and can be inhibited selectively by rolipram (Conti and Swinnen, 1990; Bolger, 1994; Reeves and England, 1990; Shakur *et al.*, 1993; Wilson *et al.*, 1994; Sullivan *et al.*, 1994). They typically exhibit IC_{50} values of 0.1-1μM rolipram when assayed at 1μM cAMP, whereas other PDE species are either insensitive to rolipram or show IC_{50} values some 50-100 times higher (Conti and Swinnen, 1990; Reeves and England, 1990; Lobban *et al.*, 1994; Nicholson *et al.*, 1991). Thus assays carried out in the presence of 10μM rolipram, with 1μM cAMP as substrate, can be expected to yield complete, or near complete, inactivation of PDE4 activity. Using this approach, we were able to show that PDE4 isoforms constitute a major fraction of the total PDE activity in P9 cells **(Table 4.1)**, with activities remarkable similar to those in hepatocytes.

PDE2 activity is characterized by its activation by low concentrations of cGMP (Beavo, 1990; Houslay and Kilgour, 1990; Reeves and England, 1990; Pyne *et al.*, 1986). Here, however, low (1μM) concentration of cGMP showed only a slight activation of PDE activity, with the inclusion of the PDE2 selective inhibitor, EHNA (Podzuweit *et al.*, 1992; Michie *et al.*, 1996) at 10μM again causing only a slight attenuation. As with PDE3, comparison of isoform activities showed that levels of PDE2 were dramaticaly reduced (91%) in P9 cells.

PDE7 species specifically hydrolyse cAMP in a manner that is resistant to inhibition by the non-selective reversible PDE inhibitor, IBMX (Lavan *et al.*, 1989; Michaeli *et al.*, 1993; Weiss *et al.*, 1981). There is no satisfactory way of quantifying PDE7 activity. Nevertheless, one can reasonably calculate, on the basis of K_m (cAMP) and K_i (IBMX) values for various PDE species

(Houslay and Kilgour, 1990; Jin *et al.*, 1992; Nicholson *et al.*, 1991), that, in the absence of any PDE7 activity, P9 cells PDE activity can be predicted to be inhibited by about 90%-95% when assayed in the presence of 100μM IBMX and 1μM cAMP. That a value considerably lower that this was observed **(Table 4.1)** might indicate the presence of PDE7 activity.

To characterize further the PDE4 activity in P9 cells, the subcellular distribution of this activity type was determined. Cell homogenates were fractionated into a low speed pellet (P_1), a high speed pellet (P_2, includes membranes) and a high speed supernatant (cytosol). **Table 4.2** demonstrated that PDE4 activity was found in both cytosol and membrane (P_2 pellet) compartments from P9 cells, but with a high specific activity seen in the cytosol.

The level of rolipram-inhibited PDE4 activity was, however, dramatically increased in P9 cells when these cells were chronically challenged with the diterpene forskolin **(Figure 4.1)**. Forskolin serves to activate adenylyl cyclase by direct stimulation of the catalytic unit of the enzyme. This can be expected to increase cyclic AMP levels, depending upon the prevailing PDE activity. PDE activity in such forskolin treated cells began to increase after around 6h and was still increasing 24h after challenge with forskolin. At this point, PDE4 activity was ~4 fold higher than at the start. Over a similar time, the PDE4 activity of the control cells remained constant. Treatment of P9 cells with forskolin resulted in a nearly 3-fold increase in total homogenate PDE activity as compared with control. Adenylyl cyclase can also be stimulated by cholera toxin, which elicits the NAD^+-dependent ADP-ribosylation of Gs causing it to become constitutively activated (Gilman, 1987; Houslay and Elliott, 1979, 1981). Using cholera toxin I was able to demonstrate that rolipram-inhibited PDE4 activity rose ~8 fold to reach a specific activity of 23.7±1.8 pmol/min/mg **(Figure 4.2)** (n=3).

Assessing intracellular cAMP levels, we were able to show that chronic challenge with forskolin did increase intracellular cAMP levels **(Figure 4.3).** However, these reached a maximum after about 1h and then fell dramatically. We presume that this was due to enhanced PDE activity **(Figure 4.1).** Consistent with this, blockade of PDE activity using the non-specific inhibitor IBMX led to levels being chronically elevated **(Figure 4.3).** The slow decrease in cAMP levels with IBMX reflecting enzyme induction and the fact that this is a reversible inhibitor. Interestingly, rolipram potentiated forskolin stimulated cAMP levels initially almost as well as IBMX, perhaps indicating PDE4 species play a major role in cAMP metabolism (**Table 4.1**) under such conditions. However, after this initial rise then a marked fall occurred indicating the action of novelly induced of PDE species other than PDE4 in these cells. **Figure 4.4** shows that an almost identical intracellular cAMP profile was obtained employing cholera toxin to enhance adenylyl cyclase activity. Namely cyclic nucleotide levels peaked after 1h, with blockade of PDE activity (presumably mainly PDE4) potentiating this effect. Again, long term exposure to high nucleotide levels led to the apparent induction of another PDE species.

Both the action of forskolin and cholera toxin in increasing PDE4 activity showed a slow time-dependence with little or no change in activity being evident after 1h of challenge **(Figure 4.1 and Figure 4.2).** This suggested that the effects were due to the *de novo* synthesis of new enzyme. Consistent with this, we noted that both forskolin and cholera toxin were unable to increase rolipram-inhibited PDE4 activity after a challenge of 9h in cells which had been treated with the transcriptional inhibitor, actinomycin D **(Table 4.3).** This treatment not only completely blocked the ability of both forskolin and cholera toxin to increase PDE4 activity, it also depressed the levels of this activity below those of the control **(Table 4.3).** This might imply a relatively rapid turnover of members of the PDE4 family.

The breadth of splice variants arising from each of the four genes which encode PDE4 activity is not as yet fully appreciated. However, to date, all active protein products differ at the 5' regions of their mRNAs but not at the 3' regions of the mRNAs. Thus, in order to determine which PDE4 families are present in P9 cells we have designed generic PCR primers so as to be able to detect the presence of presumed active PDE4A, B, C and D family members **(Figure 4.5)**. Using such 'generic' primers, and appropriate positive controls, we were able to ascertain the presence of transcripts in P9 cells RNA for PDE-4A **(Figure 4.6 and Figure 5.2 in Chapter 5)** and PDE-4D **(Figure 4.7)** but not for PDE-4B or with a very low signal expressed **(Figure 4.8)** and PDE-4C **(Figure 4.9)**. Using the same approach with RNA from native hepatocytes we were, additionally, able to detect transcripts for PDE4B **(Figure 5.3)**.

The PDE4A gene in both rat and humans produces multiple splice variants which are expressed in a tissue specific fashion. In rat, the splice variants RD1, rPDE-6 and rPDE-39 have been characterised, although it is likely that other species occur in various specialised cells. These species all arise from 5' domain swops and thus specific probes can be generated. Using these we failed to identify RD1 in both P9 cells and hepatocytes **(Figure 5.6)**. This is perhaps not surprising as this species seems only to occur in the CNS. We also failed to detect rPDE-6 in P9 cells **(Figure 5.7)**. This surprised us as, although rPDE-6 has been characterised in the CNS, it is considered to have a much more general distribution. Indeed, rPDE-6 has been suggested to occur in liver on the basis of RNAase protection studies. However, only 50% of liver cells are of parenchymal origin (hepatocytes), suggesting that the source of 'liver' rPDE-6 may be Kupffer cells/macrophages or blood cells which commonly 'contaminate' extracts made from crude liver. Intriguingly, however, we obtained a positive response for probes generated against a very recently described PDE4A splice variant; rPDE-39 (Bolger *et al.*, 1995). This

species appears to have a very restricted redistribution with the only recognised good source to date being testis (Bolger *et al.*, 1996). Here, however, PCR detected rPDE39 to also be expressed in P9 cells **(Figure 5.8)**, with the conformation of protein expression of this splice variant in P9 cells being demonstrated by Western blotting **(Figure 4.10)**.

Two splice variants of the rat PDE4B gene have been recognised to date, namely PDE4B1 (DPD) and the so-called PDE4B2 ("pde4"). Again these differ at the 5'-end. It appears likely that at least PDE4B1 arises as a 5' truncated species as the start codon of the initiator methionine in the cloned cDNA does not have the characteristics reflecting the beginning of a full length protein. Consistent with this, much larger cognate species have been cloned from human beings (Beavo *et al.*, 1994). Not with standing this, PDE4B1 and PDE4B2 undoubtedly reflect two distinct PDE4 splice variants and, as such, diagnostic PCR primers can be designed to unique 5' regions. Using these we failed to detect the presence of either species in RNA from P9 cells **(Figure 5.9** and **Figure 5.10** respectively), although transcripts were apparent in hepatocytes.

The 4D family is also subject to complex multiple splicing, again at the 5'-end. Using specific primer pairs we were able to detect the presence of the PDE4D3 variant in both P9 cells **(Figure 4.11)** and hepatocytes **(Figure 5.12)**. Indeed, the signal for this variant was consistently greater (3 experiments) in hepatocytes compared to P9 cells. Transcript for PDE4D1 could also be detected in hepatocytes, although it was completely absent from P9 cells **(Figure 4.12)**. No satisfactory primers were available for PDE4D2 analysis. PCR analyses done on equivalent amounts of RNA from cells can provide useful qualitative information concerning transcript levels. We thus examined whether treatment of P9 cells with either forskolin or cholera toxin, for 24h, would affect the PCR signals obtained. In such experiments we observed that similar signals were obtained using primers designed to amplify up a region of the cytoskeletal protein actin. This was re-assurance that similar

levels of mRNA were present in our experiments, all of which were done using at least three different RNA preparations. For RNA preparations from cells with chronically elevated cAMP levels, we noted a decreased signal for PDE4A generic transcripts **(Figure 4.6)**, no change in the results for RD1, rPDE-6 and rPDE39 transcripts **(Table 4.4)**, nor did we observe any signal using generic PDE4C **(Table 4.4)**. However, for both forskolin and cholera toxin we observed an enhanced signal using the generic PDE4B primers **(Figure 4.8)**. Subsequent analysis using the two PDE4B specific splice variant primers failed to yield a signal with the PDE4B1 primers **(Table 4.4** and **Figure 4.13)** but yielded a clear signal for PDE4B2 transcripts **(Figure 4.14)** where none had been evident before. Analysis of PDE4D showed an apparent increase in the intensity of the signal for the generic primers using RNA from both forskolin and cholera toxin treated cells **(Figure 4.7)**. This appeared to be attributable to an enhanced transcript level of the '3.3' splice variant form of PDE4D **(Figure 4.11)**. Western blots were performed here to look for detectable changes in the protein level for PDE4D **(Figure 4.15)**. It showed the increased activity of PDE4D is due to PDE4D3 which codes for a 93kDa enzyme.

Other investigators have studied the effect of increased cAMP levels on PDE4 activities in monocyte (Torphy *et al.*, 1992) and macrophage cell lines (Torphy *et al.*, 1995) as well as in Sertoli cells (Sette *et al.*, 1994b). In contrast to the studies done here, in both of the blood cell lines PDE4A was up-regulated. However, such studies indicated that this was due to a particular splice variant, equivalent to rat rPDE-6. This form is not expressed in P9 cells which indicates that different promoters may control the expression of splice variants of the PDE4A gene. However, in both such studies the induction of a PDE4B product was noted which was cognate to the rat PDE4B2 gene product **(Table 4.4)**. This adds further support to the notion that the expression of one of the splice variants of the PDE4B gene is regulated by cAMP. Analysis

of PDE4D expression shows some differences (**Table 4.4**). In the U937 macrophage cell line (Torphy *et al.*, 1995) no indication of PDE4D activity ensued. However, in both monocytes (Torphy *et al.*, 1992) and Sertoli cells PDE4D (Sette *et al.*, 1994b) was induced, but as different species, namely PDE4D1 and PDE4D3, respectively. Our studies on P9 cells show that, as with Sertoli cells, elevation in cAMP levels induced the PDE4D3 form. This might indicate cell-specific differences in the cAMP-mediated regulation of PDE4D splice variants.

Figure 4.1 Time course of forskolin activated total PDE and PDE4 activity.

P9 cells were grown to nearly 90% confluence with 10% serum medium, then changed to 2% serum medium and incubated for different times up to 24h in the absence or presence of 100μM forskolin. At the end of the incubation, cells were rinsed twice, harvested and homogenized as described in method section 2.3.7.1. The PDE activity was determined at the time indicated, in the absence (regarded as total PDE activity) or presence of 10μM final concentration of rolipram (regarded as PDE4 activity) using the cell homogenates. Assays employed 1μM cAMP as substrate (2.3.11). Each point represents the mean±S.E. of at least three different experiments, each carried out in triplicate.

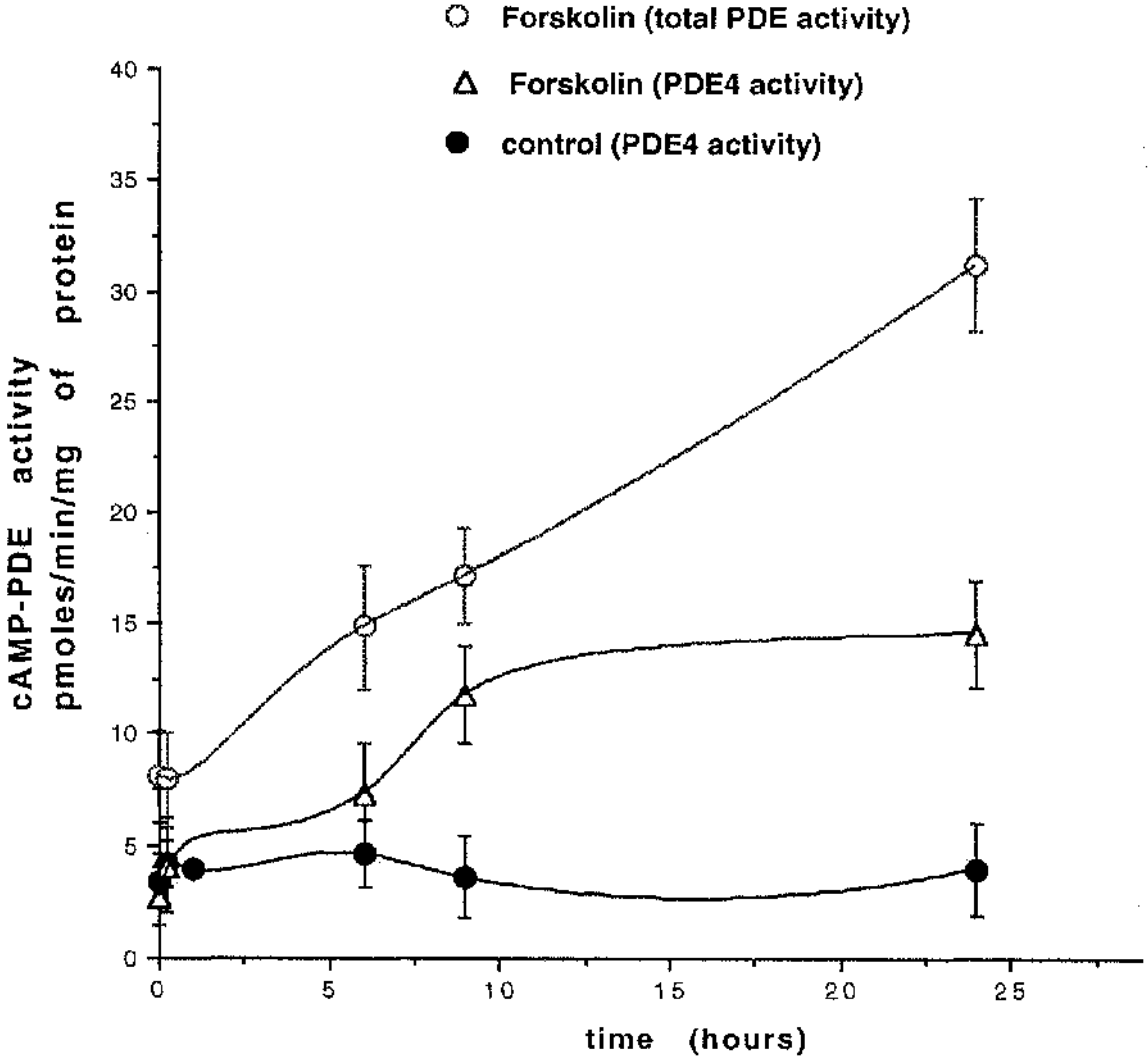

Forskolin (total PDE activity)
Forskolin (PDE4 activity)
control (PDE4 activity)
cAMP-PDE activity
pmoles/min/mg of protein
40
35
30
25
20
15
10
5
0
0
5
10
15
20
25
time (hours)

Figure 4.2 Time course of cholera toxin activated total PDE and PDE4 activity.

P9 cells were grown to nearly 90% confluence in 10% serum medium and then changed to 2% serum medium. They were then incubated for the indicated times up to 24h in the absence or presence of 1mg/ml cholera toxin. At the end of the incubation, cells were rinsed twice, harvested and homogenized as described in method section 2.3.7.1. The PDE activity was determined in the absence (regarded as total PDE activity) or presence of 10μM final concentration of rolipram (regarded as PDE4 activity) using the cell homogenates. Assays employed 1μM cAMP as substrate (for detail see method section 2.3.11). Each point represents the mean±S.E. of at least three different experiments, each carried out in triplicate.

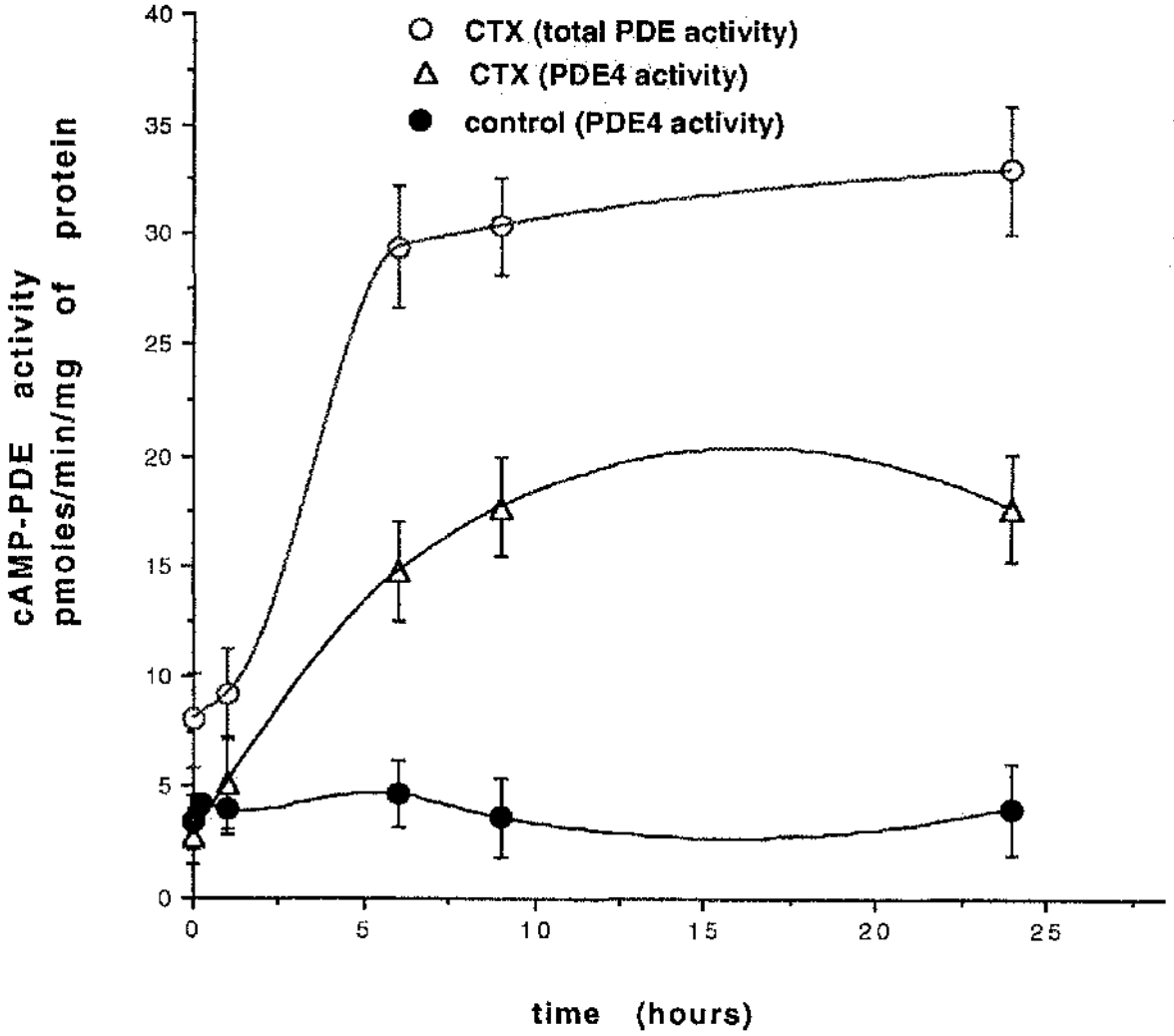

CTX (total PDE activity)
CTX (PDE4 activity)
control (PDE4 activity)
cAMP-PDE activity
pmoles/min/mg of protein
time (hours)
0
5
10
15
20
25
30
35
40

Figure 4.3 Time course of forskolin elevated intracellular cAMP in P9 cells.

P9 cells were grown to nearly 90% confluence in 10% serum medium in 75cm^2 flasks then transfered into 6-well plates with 2% serum medium. Cells were then incubated for different times up to 24h in the absence or presence of 100μM forskolin and in presence or absence of 10μM rolipram and 100μM IBMX. cAMP content was determined (see methods section 2.3.9.3) at the times indicated. Each point represents the mean±S.E. of at least three different experiments, each carried out in triplicate.

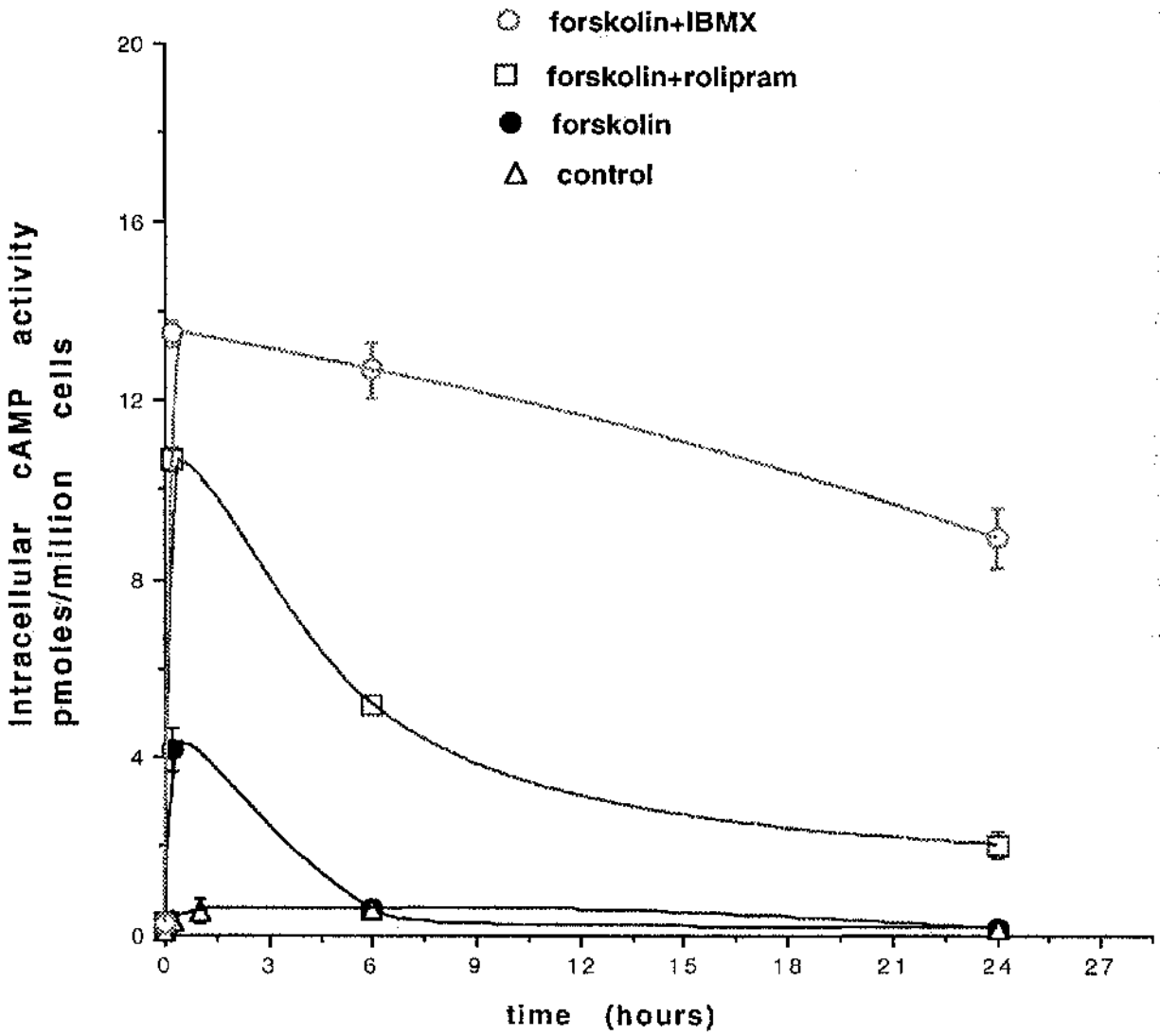

forskolin+IBMX
forskolin+rolipram
forskolin
control
Intracellular cAMP activity
pmoles/million cells
20
16
12
8
4
0
0
3
6
9
12
15
18
21
24
27
time (hours)

Figure 4.4 Time course of cholera toxin elevated intracellular cAMP in P9 cells.

P9 cells were grown to nearly 90% confluence in 10% serum medium in $75cm^2$ flasks then transfered into 6-well plates with 2% serum medium. Cells were then incubated for different times up to 24h in the absence or presence of 1mg/ml cholera toxin and in presence or absence of 10μM rolipram and 100μM IBMX. cAMP content was determined (see method section 2.3.9.3) at the times indicated. Each point represents the mean±S.E. of at least three different experiments, each carried out in triplicate.

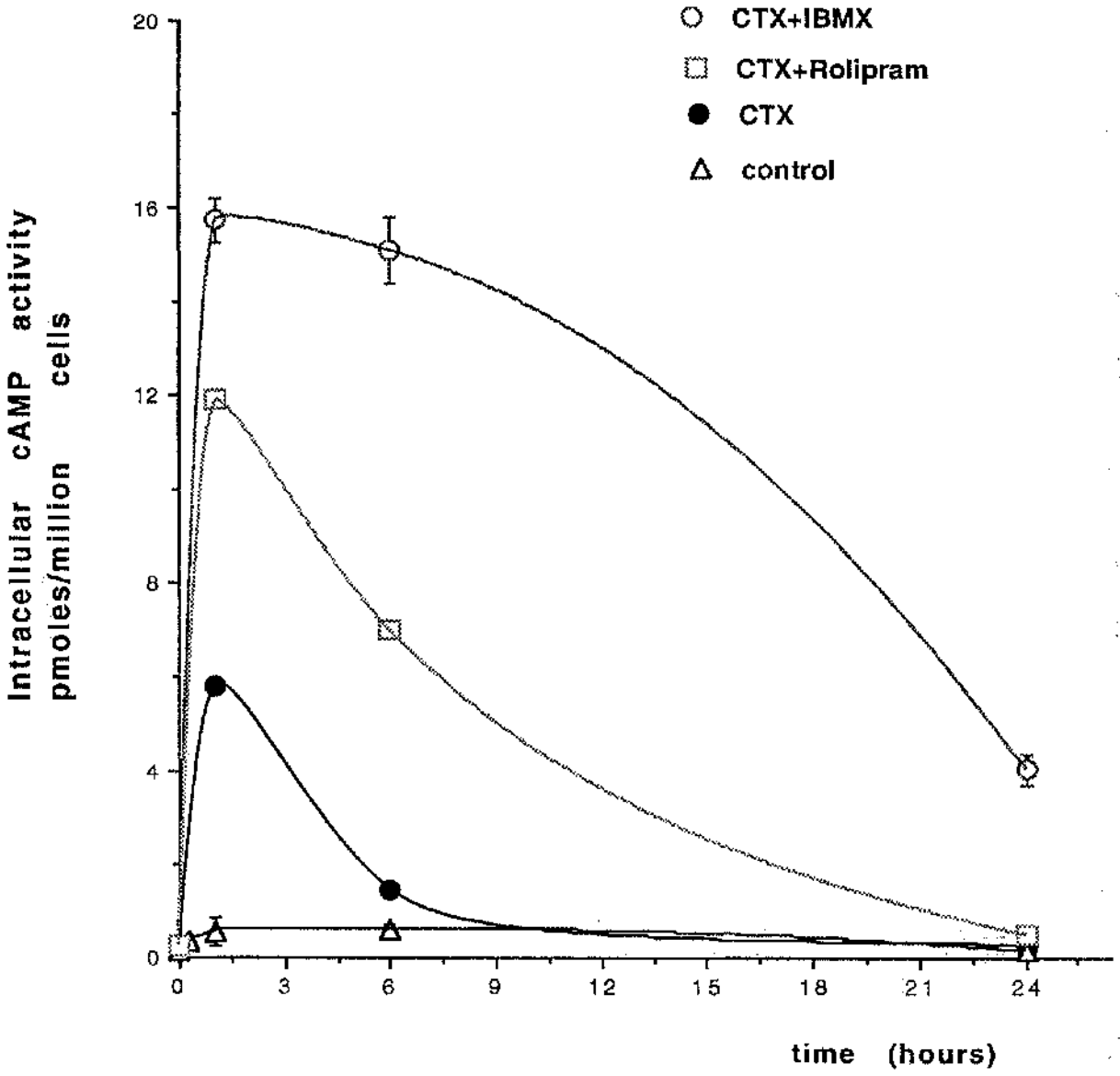
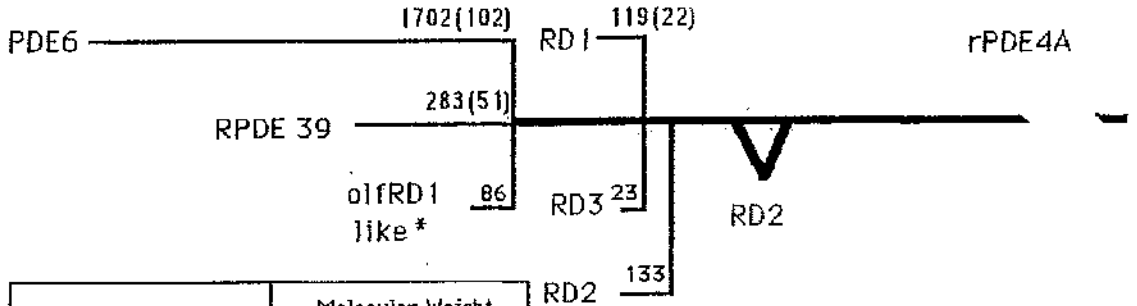

CTX+IBMX
CTX+Rolipram
CTX
control
Intracellular cAMP activity
pmoles/million cells
20
16
12
8
4
0
0
3
6
9
12
15
18
21
24
time (hours)

Figure 4.5 (a) Design of primers for RT-PCR detection of PDE4A

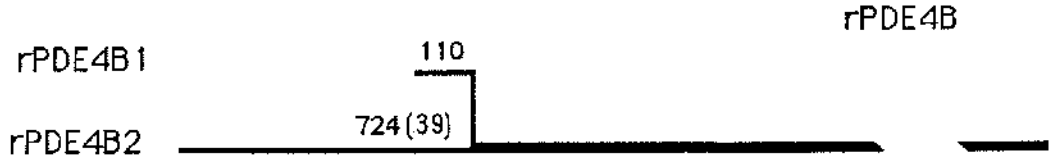
Alignment of rat PDE4A cDNAs with the consensus. Thick line is sequence found in more than one cDNA in a given species. Thin line is sequence unique to a given cDNA in a given species. Numbers above the sequences are the amount of unique sequence in nucleotides (without brackets) and amino acids (brackets). Molecular weights of conceptual translates of each sequence are given along with real molecular weights where available.



	Molecular Weight Calculated	Actua
PDE6	93 kD	
RPDE39	87 kD	
olf RD1 like*	77 kD	
RD1	68 kD	73kD
RD2	55 kD	
RD3	65 kD	

Figure 4.5 (b) Design of primers for RT-PCR detection of PDE4B

Alignment of rat (r) PDE4B cDNAs with the consensus. Thick line is sequence found in more than one cDNA in a given species. Thin line is sequence unique to a given cDNA in a given species. Numbers above the sequences are the amount of unique sequence in nucleotides (without brackets) and amino acids (brackets). Molecular weights of conceptual translates of each sequence are given along with real molecular weights where available.



	Molecular Weights	
	Calculated	Actual
rPDE4B1	56kD	64-68kD
rPDE4B2	59kD†	61kD

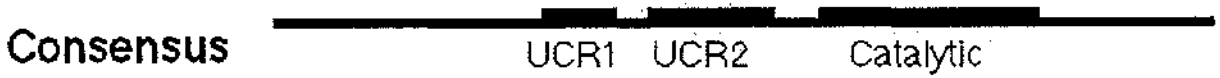


Figure 4.5 (c) Design of primers for RT-PCR detection of PDE4C

Alignment of rat PDE4C cDNA with the consensus. Thick line is sequence found in more than one cDNA. Thin line is sequence unique to this cDNA. Molecular weight of conceptual translate of this sequence is shown to be 60kDa.

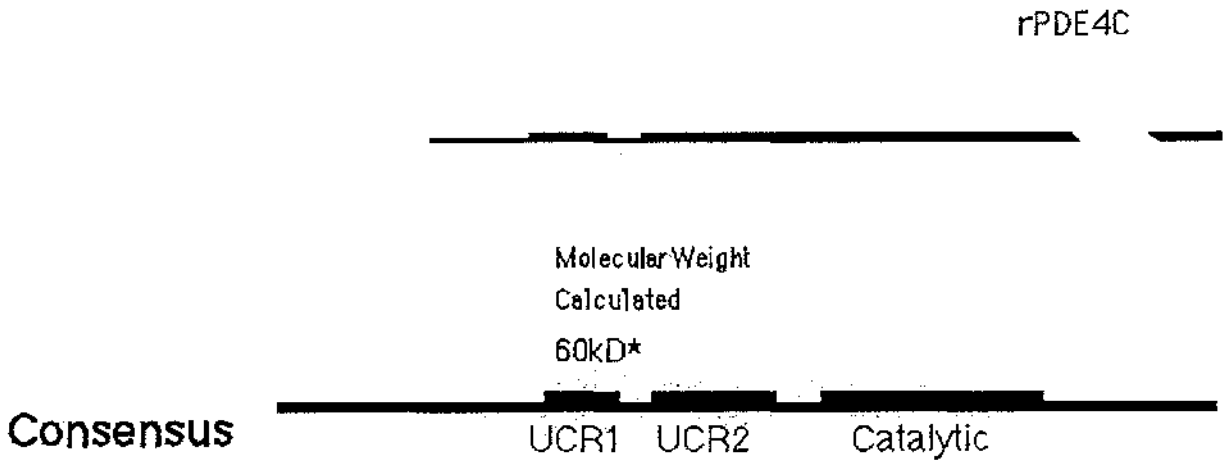
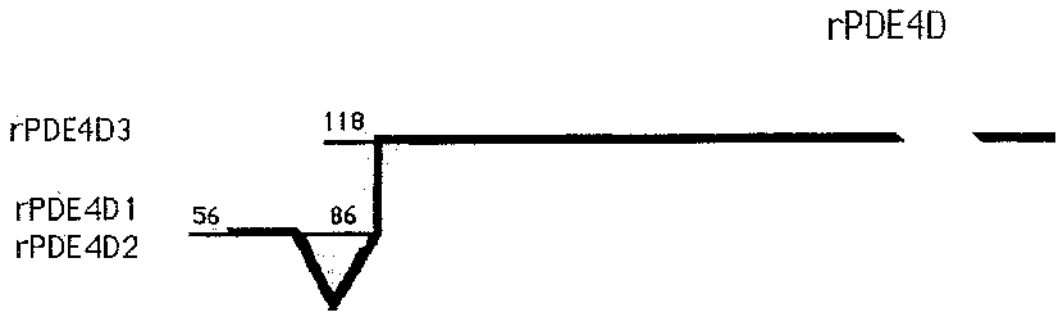

rPDE4C
Molecular Weight
Calculated
60kD*
Consensus
UCR1
UCR2
Catalytic

Figure 4.5 (d) Design of primers for RT-PCR detection of PDE4D

Alignment of rat (r) PDE4D cDNAs with the consensus. Thick line is sequence found in more than one cDNA in a given species. Thin line is sequence unique to a given cDNA in a given species. Numbers above the sequences are the amount of unique sequence in nucleotides (without brackets) and amino acids (brackets). Molecular weights of conceptual translates of each sequence are given along with actual molecular weights where available.

N.B. Due to leaky scanning (or removal of the 86nt intron) rPDE4D1 produces two translation products, one identical to the rPDE4D2 translation product (Monaco *et al.*, 1994)



	Molecular Weight	
	Calculated	Actual
rPDE4D1	66kD	72kD
rPDE4D2	58kD	66-67kD
rPDE4D3	76kD	93kD

Figure 4.6 RT-PCR analyses of hormone effects on PDE4A 'generic' transcripts in P9 cells

This was done as described in methods using the degenerate primers designed to detect transcripts selectively for PDE4A forms in a species independent fashion. (Sense; GCGGGACCTRCTGAAGAAATTCC. Antisense; CAGGGTGRTCCACATCGTGG). PCR reaction condition employed here were denaturation; 94°C for 1min, annealing; 57°C for 2min and extension; 72°C for 3min. 40 cycles of each condition were utilized. This reaction condition allowed the detection of a 232bp fragment.

Analyses were performed on Marker (M), Brain control (B), P9 control (C) and forskolin (F) treated (24h) sample.

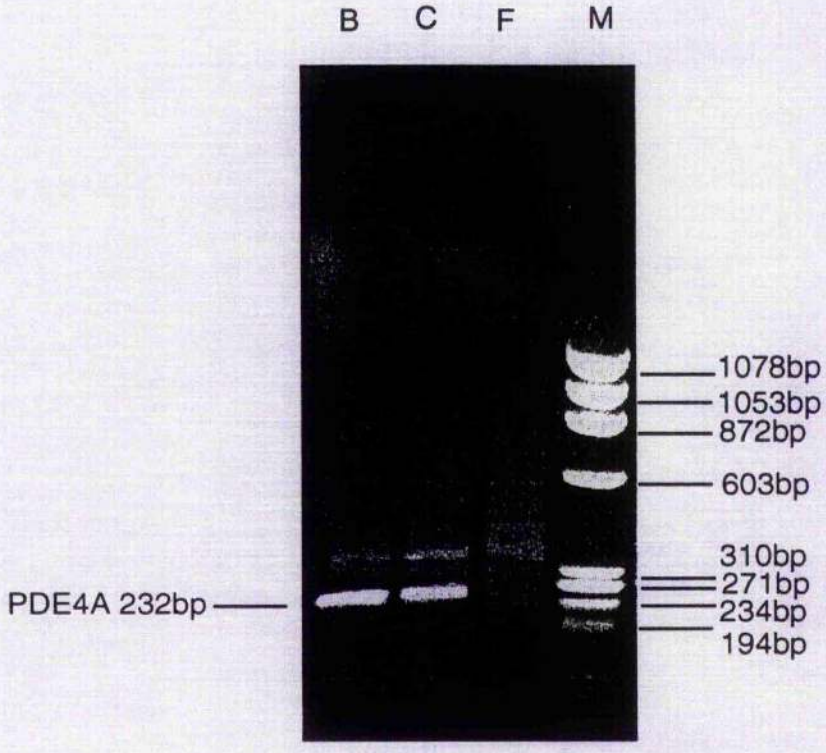

B
C
F
M
1078bp
1053bp
872bp
603bp
310bp
271bp
234bp
194bp
PDE4A 232bp

Figure 4.7 RT-PCR analyses of hormone effects on PDE4D 'generic' transcripts in P9 cells

This was done as described in methods using the degenerate primers designed to detect transcripts selectively for PDE4D forms in a species independent fashion. (Sense; CCYYTGACTGTTATCATGCACACC. Antisense; GATCYACATCATGTATTGCACTGGC). PCR reaction condition employed here were denaturation; 94°C for 1min, annealing; 55°C for 2min and extension; 72°C for 3min. 40 cycles of each condition were utilized. This reaction condition allowed the detection of a 262bp fragment.

Analyses were performed on Marker (M), P9 control (C), 100μM forskolin (F) and 1mg/ml cholera toxin (CTX) treated (24h) samples.

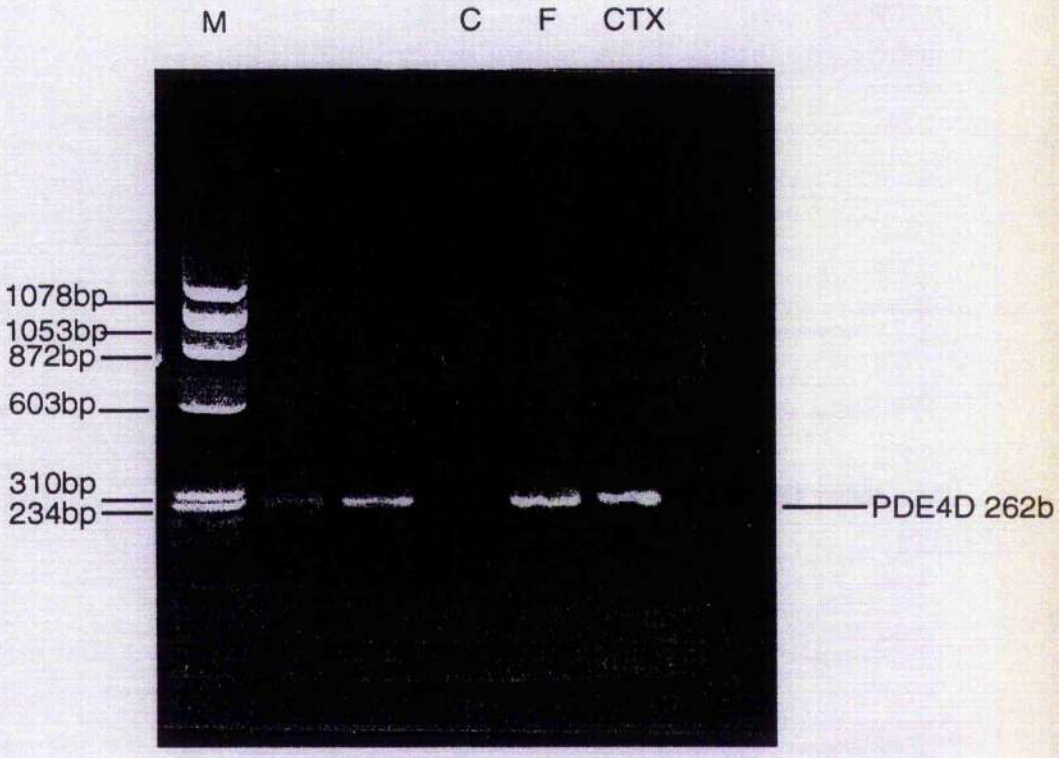

M
C
F
CTX
1078bp
1053bp
872bp
603bp
310bp
234bp
PDE4D 262b

Figure 4.8 RT-PCR analyses of hormone effects on PDE4B 'generic' transcripts in P9 cells

This was done as described in methods using the degenerate primers designed to detect transcripts selectively for PDE4B forms in a species independent fashion. (Sense; CAGCTCATGACCCAGATAAGTGG. Antisense; GTCTGCACARTGTACCATGTTGCG). PCR reaction condition employed here were denaturation; 94°C for 1min, annealing; 55°C for 2min and extension; 72°C for 3min. 40 cycles of each condition were utilized. This reaction condition allowed the detection of a 786bp fragment.

Analyses were performed on Marker (M), P9 control (C), 100μM forskolin (F) and 1mg/ml cholera toxin (CTX) treated (24h) samples.

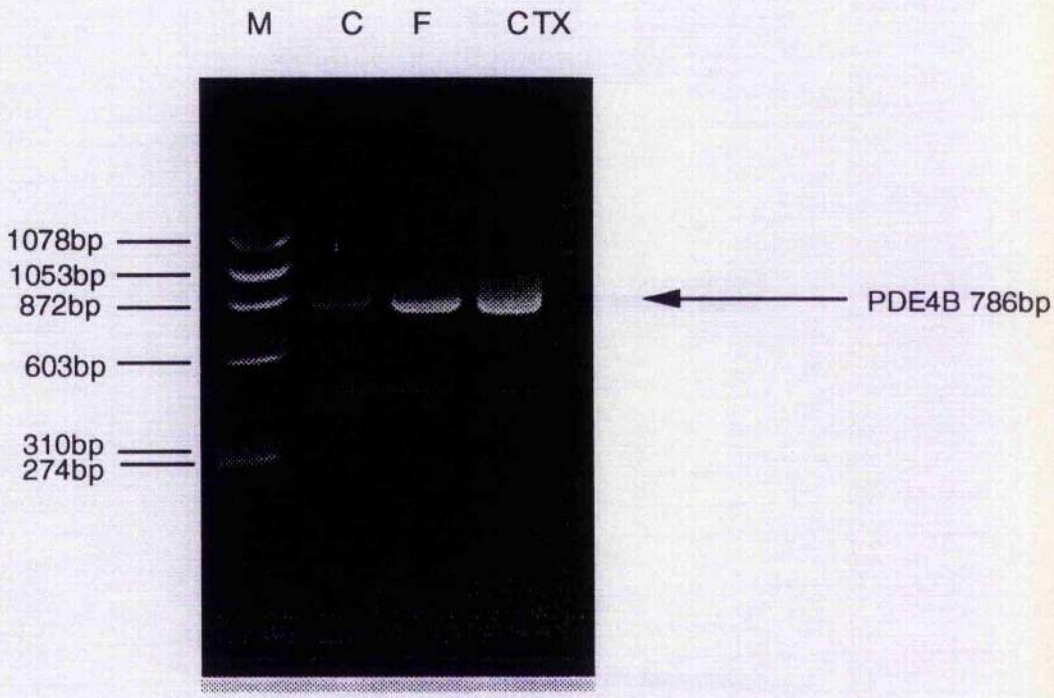

M
C
F
CTX
1078bp
1053bp
872bp
603bp
310bp
274bp
PDE4B 786bp

Figure 4.9 RT-PCR analyses of hormone effects on PDE4C 'generic' transcripts in P9 cells

This was done as described in methods using the degenerate primers designed to detect transcripts selectively for PDE4C forms in a species independent fashion. (Sense; ACTGAGTCTGCGCAGGATGG. Antisense; CMTCCTCTTCCTCTGYCTCCTC). PCR reaction condition employed here were denaturation; 94°C for 1min, annealing; 58°C for 1 min 20sec and extension; 72°C for 1min 10sec. 40 cycles of each condition were utilized. Thess reaction conditions were optimised for the detection of a 388bp fragment (see Fig 5.5), although no transcript could be detected here.

Analyses were performed on Marker (M), P9 control (C), 100μM forskolin (F) and 1mg/ml cholera toxin (CTX) treated (24h) samples.

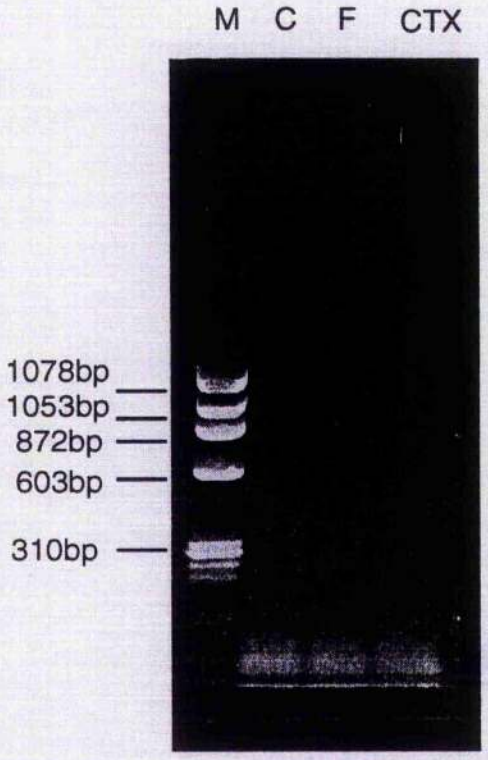

M C F CTX
1078bp
1053bp
872bp
603bp
310bp

Figure 4.10 Immunoblot analysis of rPDE39 in P9 cells

Immunoblot analyses were done with PDE4A antisera as described in methods section 2.3.23. In these samples, P9 cell homogenates containing 200μg of protein were analysed by SDS-PAGE, transfered to nitrocellulose and Western blotting was performed with antisera specific for the indicated forms of PDE4A. In experiments using testis as a positive control (C) for rPDE39 (Bolger *et al.*, 1995), the strong band of 97kDa reflects the specific identification of rPDE39. This species was also found in P9 cells as the arrow points out.

P9

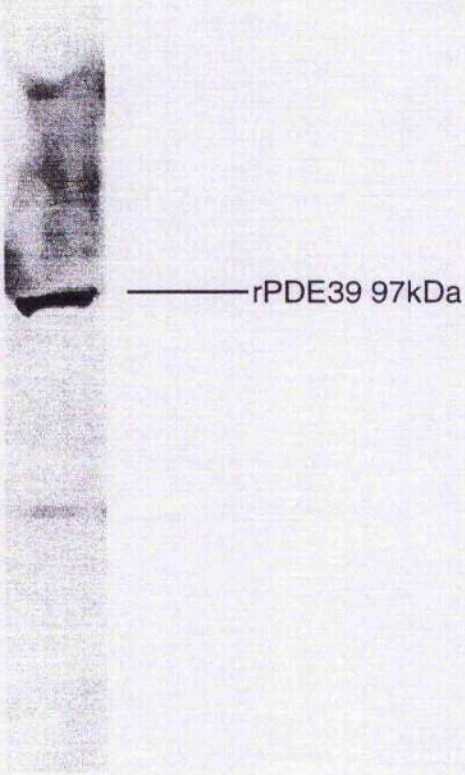


Figure 4.11 RT-PCR analyses of hormone effects on PDE4D3 transcripts in P9 cells

This was done as described in methods using the degenerate primers designed to detect transcripts selectively for PDE4D3 forms in a species independent fashion. (Sense; CTAATTTGCAAGATCGCGCACCCAGC. Antisense; CCTGGTTGCCAGACCGACTCATTTCA). PCR reaction condition employed here were denaturation; 94°C for 1min, annealing; 58°C for 1min 20sec and extension; 72°C for 1min 10sec. 40 cycles of each condition were utilized. This reaction condition allowed the detection of a 255bp fragment. Analyses were performed on Marker (M), P9 control (C), 100μM forskolin (F) and 1mg/ml cholera toxin (CTX) treated (24h) samples.

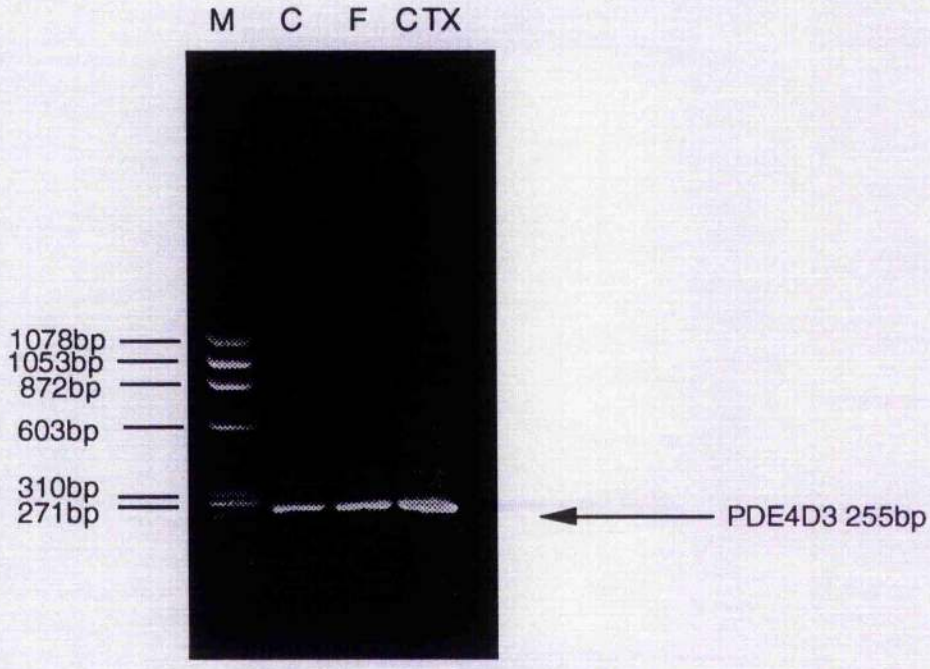

M
C
F
CTX
1078bp
1053bp
872bp
603bp
310bp
271bp
PDE4D3 255bp

Figure 4.12 RT-PCR analyses of hormone effects on PDE4D1 transcripts in P9 cells

This was done as described in methods using the degenerate primers designed to detect transcripts selectively for PDE4D1 forms in a species independent fashion. (Sense; TCCGGTGAAGCGCTTAAGAACTGAGTCC. Antisense; CCTGGTTGCCAGACCTACTCATTTCA). PCR reaction condition employed here were denaturation; 94°C for 1min, annealing; 62°C for 1min 20sec and extension; 72°C for 1min. 40 cycles of each condition were utilized. These reaction conditions were optimised for the detection of a 226bp fragment (see Figure 5.11), although no transcripts were detected here.

Analyses were performed on Marker (M), P9 control (C), 100μM forskolin (F) and 1mg/ml cholera toxin (CTX) treated (24h) samples.

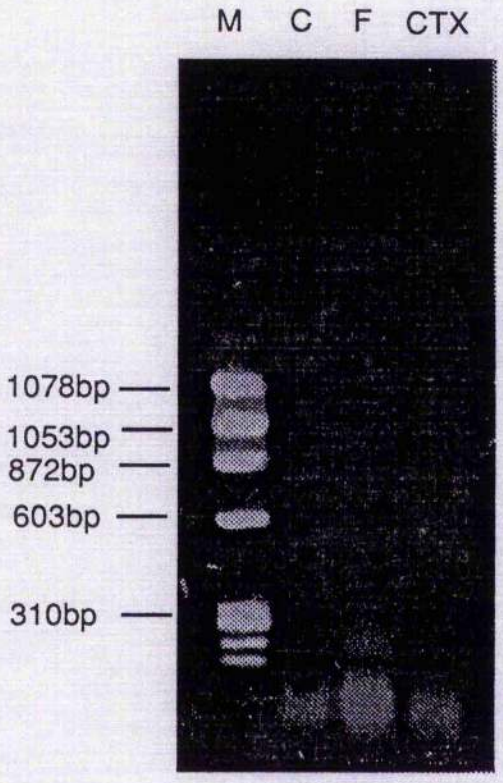

M
C
F
CTX
1078bp
1053bp
872bp
603bp
310bp

Figure 4.13 RT-PCR analyses of hormone effects on PDE4B1 transcripts in P9 cells

This was done as described in methods using the degenerate primers designed to detect transcripts selectively for PDE4B1 forms in a species independent fashion. (Sense; AAACCTTCACGGAGCACCGAACAAGAGG. Antisense; GCCACGTTGAAGATGTTAAGGCCCCATT). PCR reaction condition employed here were denaturation; 94°C for 1min, annealing; 58°C for 1min 20sec and extension; 72°C for 1min 10SEC. 40 cycles of each condition were utilized. These reaction conditions were optimised for the detection of a 507bp fragment (see Figure 5.9), although no transcripts were detected here.
Analyses were performed on Marker (M), P9 control (C), 100μM forskolin (F) and 1mg/ml cholera toxin (CTX) treated (24h) samples.

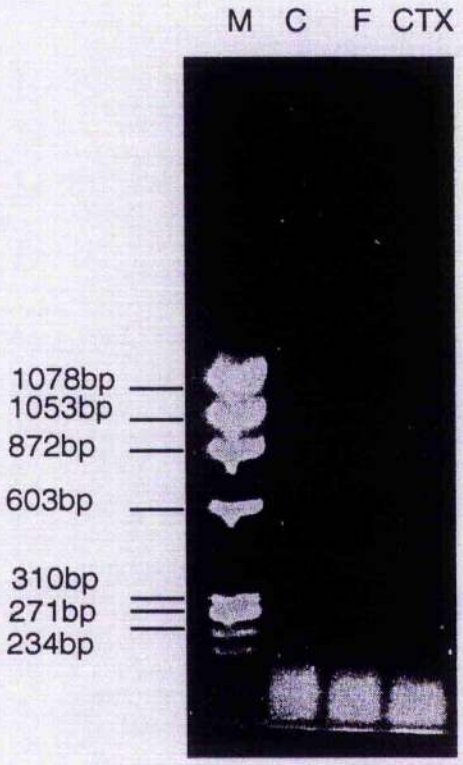

M C F CTX
1078bp
1053bp
872bp
603bp
310bp
271bp
234bp

Figure 4.14 RT-PCR analyses of hormone effects on PDE4B2 transcripts in P9 cells

This was done as described in methods using the degenerate primers designed to detect transcripts selectively for PDE4B2 forms in a species independent fashion. (Sense; TTGGTAGATCACTGACACCTCATCCCG. Antisense; GCCACGTTGAAGATGTTAAGGCCCCATT). PCR reaction condition employed here were denaturation; 94°C for 1min, annealing; 58°C for 1min 20sec and extension; 72°C for 1min 10SEC. 40 cycles of each condition were utilized. These reaction conditions were optimised for the detection of a 667bp as shown.

Analyses were performed on Marker (M), P9 control (C), 100μM forskolin (F) and 1mg/ml cholera toxin (CTX) treated (24h) samples.

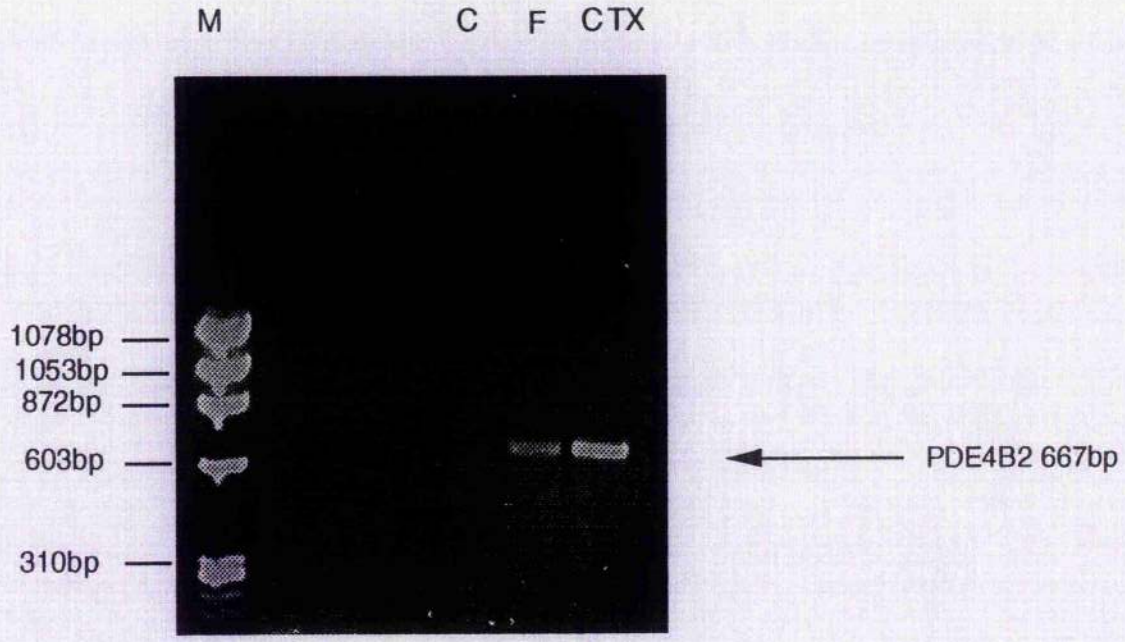

M
C
F
CTX
1078bp
1053bp
872bp
603bp
310bp
PDE4B2 667bp

Figure 4.15 Immunoblot analysis of hormone effects on PDE4D3 in P9 cells

In the samples of P9 cell homogenates containing 200µg of protein were analysed by SDS-PAGE, transfered to nitrocellulose and Western blotting was done with antisera specific for the indicated forms of PDE4D (see method section 2.3.23). Experiments used P9 control (C), 100µM forskolin (F) and 1mg/ml cholera toxin (CTX) treatment for 24h. The band of 93kDa reflects the specific identification of PDE4D3. Result shows typical experiments of ones done at least three times using different cell preparations.

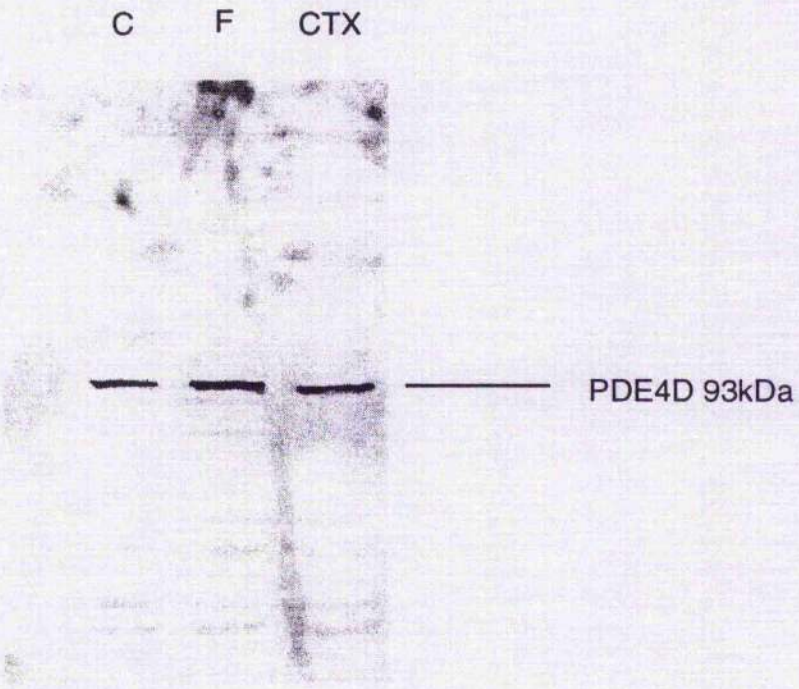

C
F
CTX
PDE4D 93kDa

Table 4.1 Assessment of cAMP phosphodiesterase activities in P9 cells and hepatocytes.

PDE assays of hepatocyte and P9 cell homogenates were performed in the absence (regarded as total PDE activity) or presence of either 10μM rolipram (regarded as PDE4 activity) or 10μM cilostimide (regarded as PDE3 activity) or 100μM IBMX (IBMX insensitive PDE activity). EHNA (10μM) was added in either the presence or absence of cGMP (10μM) to determine PDE2 levels. Data is given as with mean±S.E. of at least 3 separate experiments employing different cell preparations. In each experiment triplicate PDE assays were performed and an average value taken. PDE assays were done in the presence of 1μM cAMP as substrate (see method section 2.3.11).

PDE Activity Assayed	P9 PDE Activity. (pmoles/min/mg protein)	Hepatocyte PDE Activity (pmolesl/min/mg protein)
Total	8.7±0.4	23.6±1.8
PDE2	1.2±0.3	13.9±2.1
PDE3	3.6±0.3	9.5±2.0
PDE4	4.5±0.4	4.0±0.4
IBMX insensitive	1.1±0.9	3.64±0.82

Table 4.2 Sub-cellular distribution of PDE4 in P9 cells

Preparation of P9 cells homogenate, cytosol and membrane fractions were carried out as described by method section 2.3.7.1 PDE4 activity assays were performed in the presence of a final concentration of 10μM rolipram using 1μM cAMP as substrate (for details see method section 2.3.11) in the assay. Each point represents the mean±S.E. of at least three different experiments, each carried out in triplicate.

Fraction studied	% Total distribution	Rolipram inhibited PDE activity (pmol/min/mg)	% PDE4 distribution
Homogenate	-	3.5±1.8	-
Cytosol	34	7.3±1.8	88
Membrane	66	3.3±1.5	20

Table 4.3 Attenuation by actinomycin D of forskolin and cholera toxin stimulated induction of PDE4 activity in P9 cells

P9 cells were treated with 100μM forskolin or 1mg/ml cholera toxin in either the presence or absence of 1μg/ml actinomycin D (see method section 2.3.7.2) for 9hrs. After this, cells were harvested, homogenised and PDE4 activity was assayed in the presence of a final concentration of 10μM rolipram using 1μM cAMP as substrate (for details see method section 2.3.11). Each point represents the mean±S.E. of at least three different experiments, each carried out in triplicate.

Condition (9h treatment)	PDE4 activity. (pmol/min/mg protein)
Control	3.2±0.3
Forskolin	12.1±1.1
Forskolin+Actinomycin D	1.6±0.5
Cholera toxin	18.1±1.8
Cholera toxin +Actinomycin D	1.3±0.3

Table 4.4 Summary of hormone mediated induction of PDE4 splice variants.

This table summaries the occurence of PDE4 splicing transcripts after treating P9 cells for 24h with 100μM forskolin and/or 1mg/ml cholera toxin.

	Untreated	Forskolin treated	Cholera toxin treated
4A generic	+	<	n.d.
RD1	-	=	=
rPDE6	-	=	=
rPDE39	+	=	n.d.
4B generic	-	>	>
DPD	-	=	=
pde4	-	>	>
4C generic	-	=	=
4D generic	+	>	>
3.1	-	=	=
3.2	-	>	n.d.
3.3	+	>	>

N.B. - stands for no signal expressed, = stands for equal signal expressed, + stands for normal signal expressed , > or < stands for stronger signal or weaker signal than untreated respectively, n.d. stands for not determined.

Discussion

Four different genes coding for the low K_m cAMP-specific PDE4 have now been identified in the rat (Swinnen *et al.*, 1989a and 1989b) where their expression is cell and tissue-specific (Swinnen *et al.*, 1989b). Hormonal regulation of the mRNA level and of the protein expression of some of these isoforms has been reported (Swinne *et al.*, 1989a; Swinnen *et al.*, 1991; Takahashi *et al.*, 1991).

Considerable attention has been focused on two general mechanisms by which the activity of PDE4 is regulated by hormones, particularly those that stimulate adenylyl cyclase activity. One regulatory mechanism, designated "short term activation", involves a protein kinase A-mediated phosphorylation of a specific splice variant PDE4D3 (Sette *et al.*, 1994a). This phosphorylation results in an increase in catalytic activity, perhaps by allosteric modification of the catalytic domain (Sette *et al.*, 1994b). The short-term activation of a PDE and the consequent increase in cAMP degradaton contributes to the termination of the hormone stimulation. A second regulatory mechanism, designated "long term activation", has been shown to occur with two splice variants of PDE4D1 and PDE4D2 (Swinnen *et al.*, 1989b and 1991). Activation of protein kinase A in intact cells increases the expression of these later forms by enhancing mRNA synthesis or increasing mRNA stability.

Despite the growing body of evidence suggesting that the activity of PDE4 can be up-regulated by hormonal stimulation, little is known about the biological importance of this regulation. We have begun to address these deficiencies by examining the nature and functional consequences of PDE4 up-regulation in P9 cells; an SV40 transformed immortalized hepatocyte cell line (Livingston *et al.*, 1994).

In these studies, mRNA transcripts encoding PDE4 subtypes were identified through RT-PCR methodology using subtype-specific oligonucleotide

primers (**Table 4.4**). In untreated P9 cells, the only PCR products detected were those corresponding to PDE4A (**Figure 4.6**) and PDE4D (**Figure 4.7**), neither PDE4B (**Figure 4.8**) nor PDE4C (**Figure 4.9**) were expressed in P9 cells. A distinct pattern of splice variant expression is then seen, with rPDE39 (**Figure 5.8**), an enzyme of highly restricted distribution previously only reported in testis (Bolger *et al.,* 1995), also appearing to provide the PDE4A enzyme in P9 cells; whereas the PDE4D3 species provides the sole PDE4D component in P9 cells (**Figure 4.11**). Thus, P9 cells appear to express a distinctive pattern of PDE4 splice variants.

Intriguingly, elevation of intracellular cyclic AMP levels achieved using either forskolin or cholera toxin appeared to have a profound effect in inducing PDE4 activity by around 4-8 fold (**Figure 4.1** and **Figure 4.2**). This appeared to be due to the increased levels of mRNA and new protein synthesis of PDE4 splice variants. Specifically, PCR products for PDE4B transcripts was barely detectable in untreated cells, but a PCR product of the appropriate length was observed clearly and consistently in stimulated cells (**Figure 4.8**). In addition, the amount of PCR product corresponding to PDE4D appeared to increase (**Figure 4.7**), whereas that corresponding to PDE4A appeared to decrease (**Figure 4.6**). The functional consequences of these apparent changes in steady-state transcripts levels as they relate to PDE4A and PDE4B protein expression remains to be determined. Furthermore, we have detected profound changes in levels of specific PDE4 splice variants, namely PDE4B2 (**Figure 4.14**) and PDE4D3 (**Figure 4.11**), which are generated from the PDE4B and PDE4D genes repectively. These findings suggested that prolonged treatment with hormones was accompanied by an increase in total cAMP hydrolysing capability that appears to include up-regulation of PDE4. This increase in the activity of PDE4 appears to be due to at least two distinct forms namely PDE4B and PDE4D which are also differentially regulated, thereby adding further flexibility.

The increased activity of PDE4 by hormone treatment is prevented by actinomycin D (**Table 4.3**), an inhibitor of RNA synthesis. Such data indicate that the up-regulation of PDE4 activity depends upon the synthesis of mRNA. Western blot analysis confirmed the presence of PDE4D in P9 cells and showed protein levels to be increased after treatment with forskolin and cholera toxin (**Figure 4.15**). The results of PCR analysis are consistent with those obtained from immunoblot experiments and indicate, in light of earlier actinomycin D experiments, that the mechanism of protein induction probably involves increases in the rates of PDE4D gene transcription. However it is possible that transcription of some other genes (PDE4B) results in stabilization of PDE4 message. Our ability to detect decreased PDE4A mRNA in forskolin-treated P9 cells leads to speculation that this subtype could be down-regulated by cAMP. Indeed, recently Edorgan and Houslay (1996 unpublished data) have shown that human Jurkat T cells, challenged with forskolin for 9hrs demonstrated a decreased PDE4A mRNA level which was also accompanied by decreased protein expression.

PDE4D primers can be used to amplify a portion of cDNA refered to as rat PDE4D3 which codes for a 93kDa enzyme that is rapidly activated by cAMP-dependent protein kinase mediation (Sette *et al.*, 1994a). We have found that in P9 cells long time challenge with hormone leads to PDE4D protein levels being increased (**Figure 4.15**). This is due to the activation of PDE4D3, as Western blotting showed enhanced levels of a 93kDa protein following such hormone treatment. This change in PDE4D3 protein synthesis by hormones had also been confirmed by their expression at mRNA levels (**Figure 4.11**). This might contrast with the notion by Nemoz *et al.*, (1996) that the activation and expression of the PDE4D1 and PDE4D2 variants are involved in the long-term adaptation and desensitization of target cells to hormones. However, our hypothesis is in agreement with a recent report describing the induction of proteins very similar to PDE4D1 and PDE4D2 by cAMP in a human

promonocytic cell line (Verghese *et al.*, 1995). It may be that there are cell-specific controls in the regulation of the PDE4D gene. The newly identified human PDE4D1 and PDE4D2 lack the so-called UCR1, a region of high homology with the drosophila dunce PDE sequence, which is present at the N-terminus in human and rat PDE4D3 and other PDE4 variants (Conti *et al.*, 1995b). This region may not be essential for catalysis, instead, it may play a role in the regulation of enzyme activity. Indeed, the consensus site for PKA-mediated phosphorylation/activation in rat PDE4D3 resides within this region (Sette and Conti, 1995). Taken together, these findings suggest the possibility that expression of the longer, rapidly activated splice variant is down-regulated in favour of the smaller forms under conditions of prolonged hormone stimulation. Further investigation seems warranted at both protein and mRNA levels in various cell types.

The evidence that the PDE4 subtypes found in P9 cells differ in their susceptibility to long-term up-regulation by cAMP offers only a partial demonstration of the complexity of their regulation. The present studies are in agreement with other work indicating that certain subtypes may function as "house keeping" enzymes under basal conditons and play lesser roles in the presence of hormone, whereas other subtypes are more strongly induced when high levels of cAMP are present. As an example, rat PDE4B mRNA, which is readily detectable in unstimulated Sertoli cells (Sette *et al.*, 1994b), increases no more that 5-fold after exposure to follicle-stimulating hormone, forskolin or dibutyryl cAMP. Under the same conditions, rat PDE3/4D mRNA increased as much as 100-fold, though it is barely detectable in unstimulated Sertoli cells (Sette *et al.*, 1994a). Furthermore, Conti *et al.*, (1995a) reported the partial purification and characterization of a cAMP-PDE protein from the rat Sertoli cell and the association of the catalytic activity of this form with a 67kDa polypeptide derived from the PDE4D gene. These studies suggest that a single subtype may be differently regulated in disparate cell types. Although it has

been shown that human PDE4A is up-regulated by cAMP in both monocytes and U937 cells (Torphy *et al.*, 1995), other investigators have found that rodent homologues of this PDE are insensitive to cAMP in FRTL-5 cells and in the mouse MA-10 Leydig tumour cell line (Swinnen *et al.*, 1991a and1991b). These differences, although they are quite possibly species-related, might be indicative of different regulatory processes among different cell types. Additionally, various N-terminal domains are spliced to the PDE4A unit which determine subcellular localisation and activity (Bolger, 1994) and these are also differently expressed within different cell types. For example, we have observed **(Table 4.2)** that most of the immunoreactive PDE4A is in the membrane fraction in P9 cells. This protein is also detectable only in the membrane fraction of human brain homogenates (Torphy unpublished data, 1996), unlike U937 cells where most of the monocyte PDE4A is found in the soluble fraction (Manning *et al.*, 1996). Our studies using P9 cells **(Figure 4.3** and **Figure 4.4)** have also shown that an increase in total PDE activity is associated with significant suppression of both the cells' ability to elevate cAMP in response to a hormone stimulus and the ability of the prototypical PDE4 inhibitor rolipram to elevate cAMP at submicromolar concentratons in the presence of hormone. These results are consistent with other findings which demonstrated that up-regulation of cAMP-PDE activity plays a major role in cellular refractoriness after hormone stimulation (Dwinnen *et al.*, 1989b).

In conclusion, long-term treatment of immortalized hepatocytes with agents that activate the cAMP/protein kinase A cascade results in PDE activity being up-regulated. The elevation in total cellular PDE4 catalytic activity is an increase in steady-state levels of the PDE4B and PDE4D isoforms.

Chapter 5

Cyclic AMP-Phosphodiesterase expression in normal and diabetic hepatocytes

Introduction

Diabetes is a metabolic disease in which in the absence of insulin, unbridled catabolism and engergy waste is rampant. Insulin, a powerful anabolic hormone, reverses this and restores energy conservation. Diabetes is commonly broken down into two types: insulin-dependent diabetes mellitus (IDDM) or Type I diabetes and non-insulin-dependent mellitus (NIDDM) or Type II diabetes. Type I diabetes occurs in 10% to 15% of diabetic cases in the Western world and appears heterogeneous in terms of genetic, autoimmune, and environmental factors that bring about the disease (Rimoin and Rotter, 1981; Rotter and Rimoin, 1981). Most important, Type I diabetes is characterized by an eventual absence of endogenous insulin production, as measured by circulation *C-peptide* levels. Insulin resistance (when normal insulin levels fail to produce a normal response) is also present, but its pathogenesis is not well understood (DeFronzo *et al.*, 1982). Of individuals with diabetes, 90% have Type II diabetes where environmental factors play a role in the development of NIDDM because the prevalence rate increases with obesity and aging (Fournier *et al.*, 1986). NIDDM is characterized by diminished insulin secretion relative to serum glucose levels and peripheral insulin resistance, individuals with the disease have abnormalities in both hepatic glucose production and glucose uptake in the peripheral tissues (DeFronzo *et al.*, 1982; Kolterman *et al.*, 1881). Three major metabolic abnormalities in NIDDM are impairment in pancreatic beta cell insulin secretion in response to a glucose stimulus (DdFronzo *et al.*, 1983; Halter *et al.*, 1979); a reduced sensitivity to the action of insulin in major organ systems such as muscle, liver and adipose tissue (DeFronzo *et al.*, 1979; DeFronzo *et al.*, 1983; Olefsky *et al.*, 1982) and excessive hepatic glucose production in the basal state (Gerich, 1984; Revers *et al.*, 1984).

Besides the insulin secretion abnormalities which can be demonstrated in subjects with NIDDM, another disorder associated with NIDDM is hepatic overproduction of glucose resulting in progressive hyperglycemia and continued reduced insulin sensitivity (DeFronzo *et al.*, 1982); also lipid and lipoprotein abnormalities are present in both Type I and Type II diabetes (Stanby, 1968; Swislocki *et al.*, 1989). Assessment of nucleotide signalling in animal models of diabetes and insulin-resistance have identified lesions in the control and expression of adenylyl cyclase and specific G-proteins (Griffiths *et al.*, 1990; Bushfield *et al.*, 1990). This may contribute to diabetes-induced alterations in the metabolic functions regulated by adenylyl cyclase in certain cells (Herberg and Coleman, 1977; Levilliers *et al.*, 1978). Thus analysis of streptozotocin-induced diabetes indicated that tissue-specific alterations occurred in G-protein expression and mRNA levels with hepatocytes (Griffiths *et al.*, 1990; Gawler, *et al.*, 1987, Bushfield *et al.*, 1990a;) and adipocytes (Griffiths *et al.*, 1990; Strassheim *et al.*, 1990) being cell types that are particularly affected. In hepatocytes of streptozotocin-induced diabetic rats, levels of the G-proteins G_s, G_{i-2} and G_{i-3} were decreased (Bushfield *et al.*, 1990b). Whilst in adipocytes, levels of G_{i-3} were actually increased (Strassheim *et al.*, 1990). In studies done on platelets from type II, non-insulin dependent diabetic male subjects markedly lower levels of G_{i-2} and somewhat reduced levels of G_{i-3} were found compared to normal subjects (Livingstone *et al.*, 1991). Such analyses indicate an abnormality of G_i function in diabetes, which can be expected and indeed has been shown, to perturb cAMP metabolism in these central metabolically active sites. As we and others have noted that changes in cAMP levels can affect PDE expression then it is possible to envisage that an altered PDE enzyme complement ensues in diabetes.

Uncontrolled diabetes is believed to be associated with increased levels of cAMP in both plasma and tissues (hepatocytes and adipocytes) of the affected animals. Intriguingly, adding to this problem has been the observation

that low K_m cAMP PDE activity is apparently decreased in streptozotocin-induced diabetic rats (Solomon *et al.*, 1994). This may also be compounded by reduced levels of calmodulin in the streptozotocin-induced diabetic rat which might attenuate any PDE1 activity (Solomon *et al.*, 1994). Such actions, however, appear to be fully reversible as when insulin is administered to streptozotocin-induced diabetic rats, then cAMP levels fall to normal (Solomon *et al.*, 1987; Solomon, 1975) **(Table 5.1).** The underlying mechanisms accounting for this are unknown. However, there is evidence that G_i expression in hepatocytes may be insulin regulated (Livingston *et al.*, 1991), and hence corrected by insulin therapy. This may be true for other components or may ensue as a consequence of altered G-protein expression.

Results

Table 5.2 shows that the majority of PDE activity, whether in normal or diabetic hepatocytes, is IBMX sensitive. For in the the presence of 100μM IBMX, a considerable decrease in PDE activity was detected. However, IBMX is a reversible inhibitor which, under the conditions used, might be expected to inhibit some 95% of the PDE activity of PDE1/2/3/4 isoforms. That inhibition was not as great as this suggests the presence of an IBMX-insensitive PDE7 component. Indeed, in this regard, such a species has been noted in hepatocytes (Lavan *et al.*, 1989). Employing selective PDE inhibitors, it can be seen that PDE2 and PDE3 are prominent in hepatocytes for both normal and diabetic animals and that levels of these isoforms exceed that of PDE4 **(Table 5.2)**. Such analyses were made by treating PDE assays of hepatocyte homogenates with cilostimide for PDE3, with rolipram for PDE4 and assessing the EHNA-inhibited component in the presence of low cGMP concentration for PDE2. In **Table 5.2**, we also see the profound decrease in activity of PDE2 and PDE3 in hepatocytes from diabetic animals compared to those from normal animals. In contrast to this, there was little significant change in PDE4 activity induced upon diabetes **(Table 5.2)**. Also, estimating PDE7 activity, then that also appeared unaffected by diabetes.

In this study, I found it hard to detect any Ca^{2+}/CaM-dependent PDE (PDE1) activity in normal hepatocytes. This was done by the addition of Ca^{2+}/CaM (50μM; 10unit=10ng/ml) **(Table 5.3)** to assays. This indicates the absence of any PDE1 activity in these cells. There is a possibility that sufficient endogenous Ca^{2+} might have been carried over into the assays, from the cell homogenates, to stimulate, in full, any PDE1 enzyme (Spence *et al.*, 1995). To obviate this, I added 1mM (or 2mM, 5mM)-EGTA to the assays and then determined PDE activity in both the presence and absence of 5mM (or 5mM, 8mM) Ca^{2+}/CaM respectively. However, in such assays no activation was elicited by the addition of Ca^{2+}/CaM **(Table 5.3)**. Using the primers GR18

and GR19, which were designed to be specific for PDE1 transcripts (**Figure 5.1a**), RT-PCR was done using RNA extracted from rat brain, which is known to provide a good source of PDE1 (Repaske *et al.,* 1992; Bentley *et al.,* 1992, Sonnenburg *et al.,* 1993), as control. This allowed for the amplification, from brain DNA, of a species of ~600bp (**Figure 5.1b**). This indicates that such primers are able to allow the amplification of a species of the appropriate size. When these primers were used in RT-PCR with RNA extracted from normal and diabetic hepatocytes then no such species was amplified (**Figure 5.1b**), which confirmed the absence of PDE1 activity as suggested by the assay (**Table 5.3**).

PDE4 has recently drawn particular attention as selective inhibitors play an important role in pharmacological areas such as the study of asthma, where the control of expression of members of the PDE4 gene families involved and their multiple splicing, is ill-understood. Because of this, PDE4 enzymes were studied in more detail. The PDE4 enzyme distribution between cytosol and membrane was assessed for hepatocytes from both normal and diabetic states. Cell homogenates were fractionated into a low speed pellet (P_1), a high speed pellet (P_2; includes membranes) and a high speed supernatant (cytosol). **Table 5.4** demonstrated that, for normal hepatocytes, PDE4 activity predominated (72%) in cytosol compared with membrane. This changed dramatically for hepatocytes from diabetic animals where the ratio of cytosol and membrane distribution was almost equal (43% cytosol). To characterize further the PDE4 subtypes, cell extracts from normal hepatocytes were incubated with antisera for PDE4A, PDE4B or PDE4D (see section 2.3.25) which had been pre-adsorbed to pansorbin. These specific antisera were generated to rat PDE4A and PDE4B species using dodecapeptides representing the extreme C-terminal of these enzymes (Shakur *et al.,* 1995, Lobban *et al.,* 1994). The activity of both the adsorbed and the supernatant fractions was then determined in the presence or absence of 10μM rolipram to determine PDE4 activity. This

immunoprecipitation utilised the specificity of the antibody-PDE4 interaction to determine which PDE4 subtypes were contributing to the rolipram-sensitive activity known to exist here. Results reported in **Table 5.5** indicate that PDE4 activity was quantitatively immunoprecipitated by the antibody specific for PDE4A, 4B and 4D. Neither pansorbin alone nor the antibody preadsorbed to the synthetic peptide nor the preimmune serum precipitated significant PDE activity (data not shown). The particular fraction which has been immunoprecipitated by the specific antisera reflected that PDE4A are mostly membrane associated, PDE4B are equally distributed between membrane and cytosol, whereas, PDE4D are mostly cytosol associated (**Table 5.5**).

Thus, in order to determine which PDE4 splice variant mRNAs are expressed in normal and diabetic hepatocytes, we used generic PCR primer pairs specific for PDE4A, B, C and D family members. Using such generic primers together with appropriate positive controls, we were able to detect the expression of PDE4A (**Figure 5.2**) in both normal and disease states. Also the presence of PDE4B (**Figure 5.3**) in both normal and diabetic hepatocytes, although a weak signal for PDE4B was seen in the latter state. It was a similar situation for PDE4D (**Figure 5.4**) with lower transcript levels being detected in the diabetic state. There was no detectable expression of PDE4C in either normal or diabetic hepatocytes (**Figure 5.5**), although the primers did amplify specific products from testis which provided the positive control.

Whilst, PDE4A mRNA was observed in both diabetic and normal hepatocytes, primers designed (**Figure 4.5a**) for the RD1 and rPDE6 splice variants of PDE4A, failed to identify transcripts in both cell types (**Figure 5.6 and Figure 5.7** respectively). However, a weak signal for rPDE39 expression was detected in normal hepatocytes but not expressed in the diabetic state (**Figure 5.8**).

Splice variants PDE4B1(DPD) and PDE4B2 ("pde 4") of the rat PDE4B gene which differ at the 5'-end were studied here and results indicated that

PDE4B1 was expressed in both normal and diabetic hepatocytes (**Figure 5.9**), whereas PDE4B2 was only detected in the normal but not in the diabetic state (**Figure 5.10**).

A similar situation exists with PDE4D where complex multiple splice variants are known. Using specific primer pairs we were able to detect the presence of the PDE4D variants PDE4D1 (**Figure 5.11**) in diabetic hepatocytes and PDE4D3 (**Figure 5.12**) in both states but with a strong signal expressed in normal hepatocytes.

From the results detailed above, it is apparent that induction of diabetes causes complex changes in the expression of hepatocyte PDEs and that the PDE4 family is no exception to this. We were able to assess changes in the PDE4 forms by not only assaying specific activity of the various subfamilies, but by detection of the level of changes in the level of their mRNA transcripts (**Table 5.6** for summary).

Figure 5.1a Design of degererate primers for RT-PCR detection of PDE1 transcripts

Analysis of the complete and partial sequences of PDE1 enzymes in GenBank (Bilofsky and Burks, 1988) from mouse, rat, human and bovine sources revealed substantial homology across a 1252 bp stretch of sequence (labelled 439-1691 in mouse) which is known to include coding sequence for the catalytic unit of this enzyme (Jin *et al.*, 1992). The majority of this sequence is also highly homologous to other PDE types (thin horizontal line). Within this region three stretches of sequence (hatched boxes) were found to be unique to PDE1 forms. Degenerate primers were designed to anneal within two of these stretches such that they would be predicted to allow for the amplification of a 601 bp fragment from all known PDE1 forms.

Adapted from Spence *et al.*, 1995

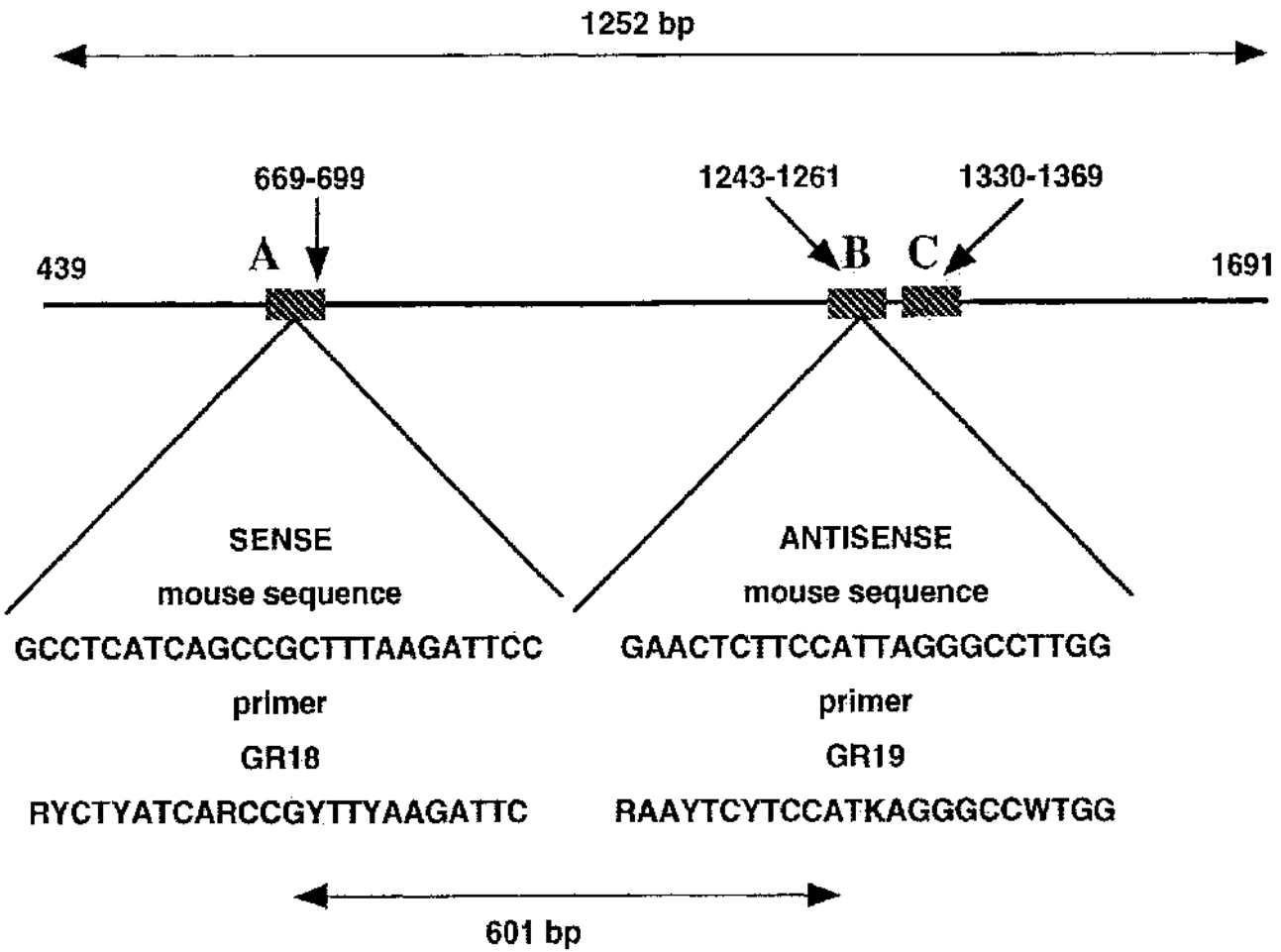

1252 bp
669-699
1243-1261
1330-1369
439
A
B
C
1691
SENSE
mouse sequence
GCCTCATCAGCCGCTTTAAGATTCC
primer
GR18
RYCTYATCARCCGYTTYAAGATTC
ANTISENSE
mouse sequence
GAACTCTTCCATTAGGGCCTTGG
primer
GR19
RAAYTCYTCCATKAGGGCCWTGG
601 bp

Figure 5.1b RT-PCR analyses of PDE1 transcripts in normal and diabetic hepatocytes and P9 cells

This analysis was done as described in methods using degenerate primers designed to detect transcripts selectively for PDE1 forms in a species independent fashion. Nucleotide sequences of sense and antisense primers ar shown in Figure 5.1(a). RT-PCR reaction conditions were as follows: Denaturation: 1min at 94°C, Annealing; 1min 20sec at 50°C and Extension, 1min 10sec at 72°C.

The predicted position, from a marker ladder, of the 607bp fragment is shown with an arrow.

Analyses were performed on Marker (M), Brain control (B), P9 cells (P9), normal (H) and diabetic hepatocytes (D).

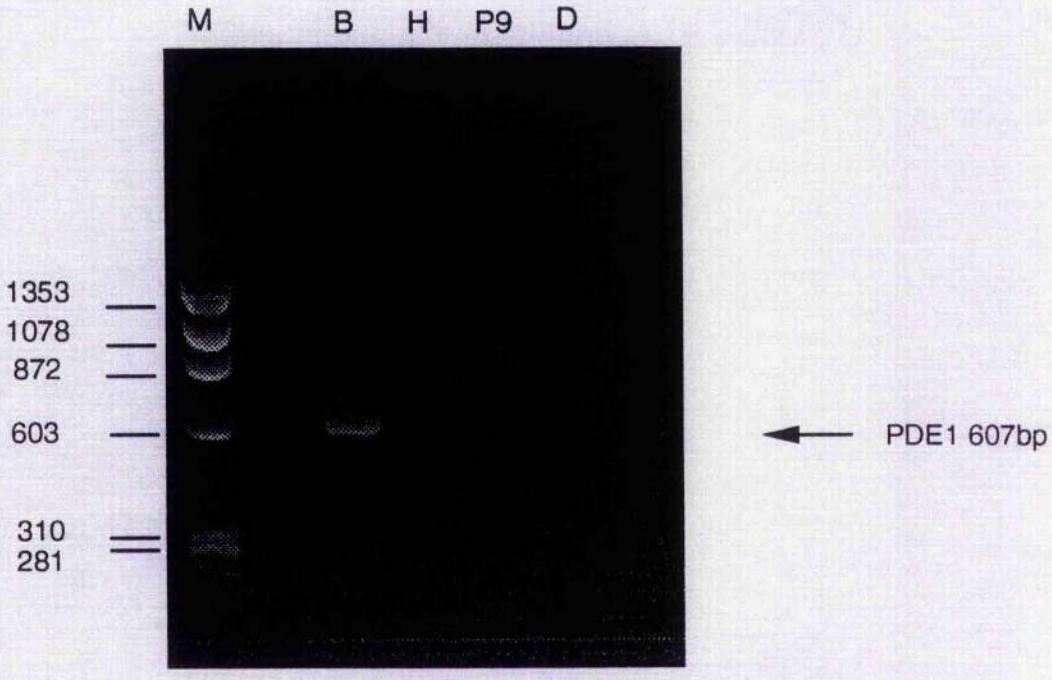

M
B
H
P9
D
1353
1078
872
603
310
281
PDE1 607bp

Figure 5.2 RT-PCR analyses of PDE4A 'generic' transcripts in normal and diabetic hepatocytes.

This analysis was done as described in methods using degenerate primers designed to detect transcripts selectively for PDE4A forms in a species independent fashion. (Sense; GCGGGACCTRCTGAAGAAATTCC. Antisense; CAGGGTGRTCCACATCGTGG). PCR reaction conditions employed here were denaturation; 94°C for 1min, annealing; 57°C for 2min and extension; 72°C for 3min. 40 cycles of each condition were utilized. The predicted position, from a marker ladder, of the 232bp fragment is shown with an arrow.

Analyses were performed on Marker (M), Brain control (B), P9 cells (P9), normal (H) and diabetic hepatocytes (D).

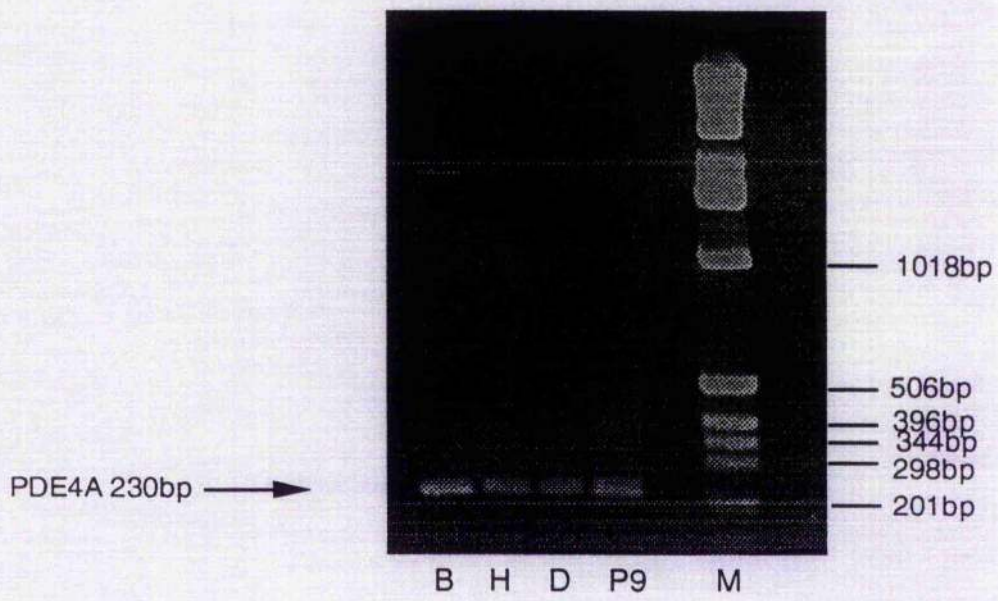

PDE4A 230bp
1018bp
506bp
396bp
344bp
298bp
201bp
B
H
D
P9
M

Figure 5.3 RT-PCR analyses of PDE4B 'generic' transcripts in normal and diabetic hepatocytes.

This was done as described in methods using degenerate primers designed to detect transcripts selectively for PDE4B forms in a species independent fashion. (Sense; CAGCTCATGACCCAGATAAGTGG. Antisense; GTCTGCACARTGTACCATGTTGCG). PCR reaction conditions employed here were denaturation; 94°C for 1min, annealing; 55°C for 2min and extension; 72°C for 3min. 40 cycles of each condition were utilized. This reaction condition allowed the detection of a 786bp fragment.

Analyses were performed on Marker (M), normal (H) and diabetic hepatocytes (D).

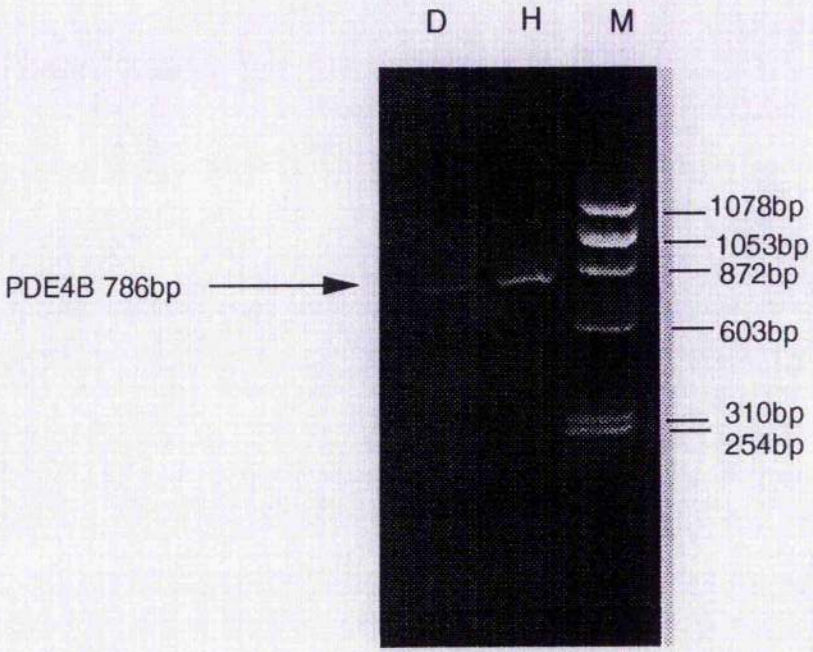

D
H
M
PDE4B 786bp
1078bp
1053bp
872bp
603bp
310bp
254bp

Figure 5.4 RT-PCR analyses of PDE4D 'generic' transcripts in normal and diabetic hepatocytes

This was done as described in methods using degenerate primers designed to detect transcripts selectively for PDE4D forms in a species independent fashion. (Sense; CCYYTGACTGTTATCATGCACACC. Antisense; GATCYACATCATGTATTGCACTGGC). PCR reaction conditions employed here were denaturation; 94°C for 1min, annealing; 55°C for 2min and extension; 72°C for 3min. 40 cycles of each condition were utilized. This reaction condition allowed the detection of a 262bp fragment.

Analyses were performed on Marker (M), normal (H) and diabetic hepatocytes (D).

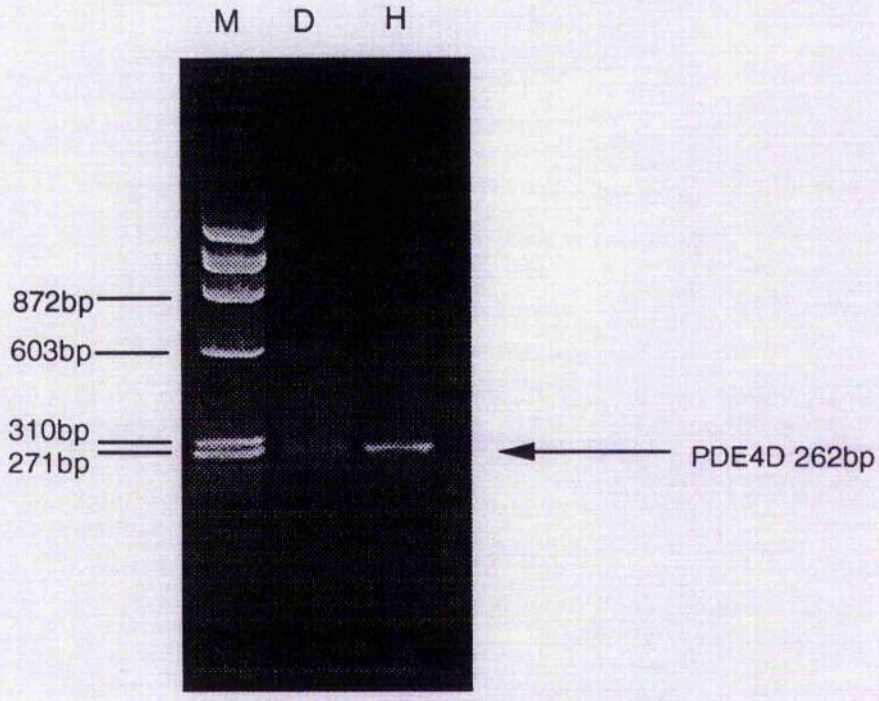

M
D
H
872bp
603bp
310bp
271bp
PDE4D 262bp

Figure 5.5 RT-PCR analyses of PDE4C 'generic' transcripts in normal and diabetic hepatocytes

This was done as described in methods using degenerate primers designed to detect transcripts selectively for PDE4C forms in a species independent fashion. (Sense; ACTGAGTCTGCGCAGGATGG. Antisense; CMTCCTCTTCCTCTGYCTCCTC). PCR reaction conditions employed here were denaturation; 94°C for 1min, annealing; 58°C for 1 min 20sec and extension; 72°C for 1min 10sec. 40 cycles of each condition were utilized. Thess reaction conditions were optimised for the detection of a 388bp fragment as shown with an arrow.

Analyses were performed on Marker (M), testis control (T), normal (H) and diabetic hepatocytes (D).

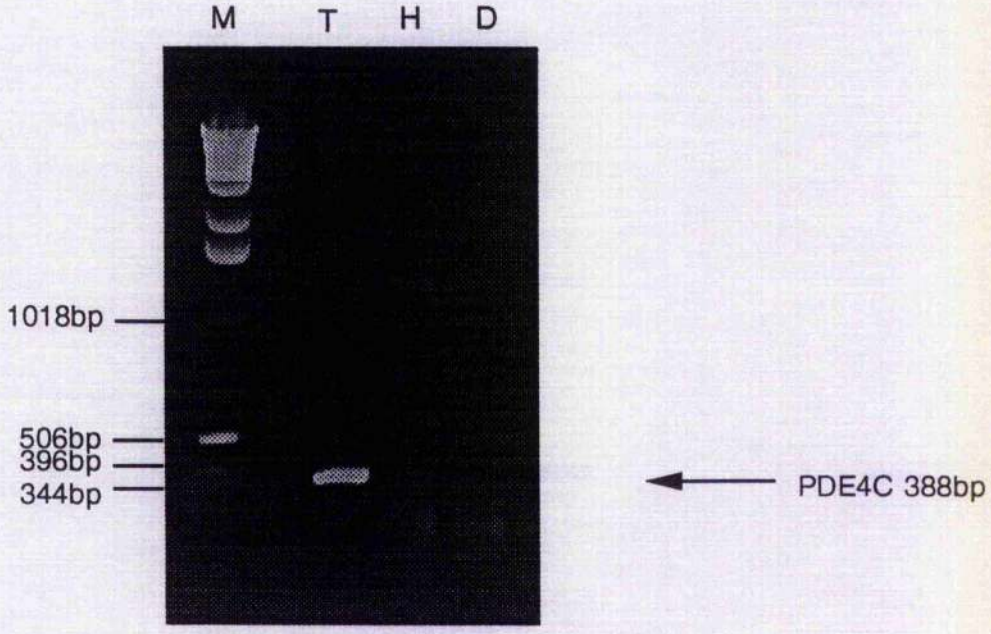

M
T
H
D
1018bp
506bp
396bp
344bp
PDE4C 388bp

Figure 5.6 RT-PCR analyses of RD1 transcripts in normal hepatocytes

This was done as described in methods using degenerate primers designed to detect transcripts selectively for RD1 forms in a species independent fashion. (Sense; TTCTTCTGCGAGACCTGCTCCAAGC. Antisense; CAGGCCCCATTTGCTCAAGTTCTCC). PCR reaction conditions employed here were denaturation; 94°C for 1min, annealing; 57°C for 2 min and extension; 72°C for 3min. 40 cycles of each condition were utilized. Thess reaction conditions were optimised for the detection of a 372bp fragment as shown with an arrow.

Analyses were performed on Marker (M), brain control (B), normal hepatocytes (H) and P9 cells (P9).

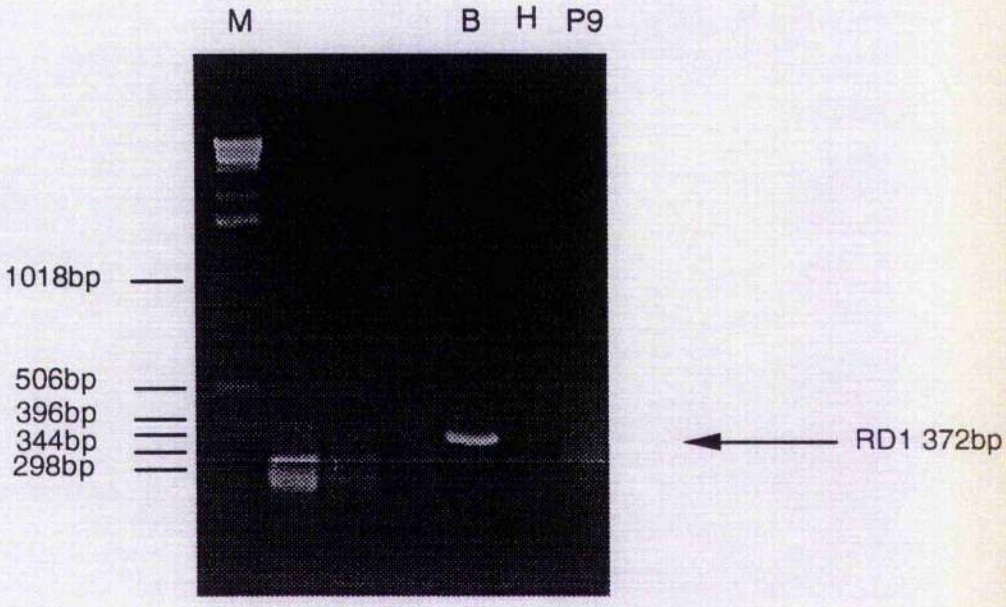

M
B
H
P9
1018bp
506bp
396bp
344bp
298bp
RD1 372bp

Figure 5.7 RT-PCR analyses of rPDE6 transcripts in normal and diabetic hepatocytes

This was done as described in methods using degenerate primers designed to detect transcripts selectively for rPDE6 forms in a species independent fashion. (Sense; AAGGAGCCTGTCTCTCTCTCTTCCG. Antisense; GGTACCGGTGCCGTGGAAGGA). PCR reaction conditions employed here were denaturation; 94°C for 1min, annealing; 62°C for 1 min 20sec and extension; 72°C for 1min. 40 cycles of each condition were utilized. Thess reaction conditions were optimised for the detection of a 825bp fragment as shown with an arrow.

Analyses were performed on Marker (M), brain control (B), normal hepatocytes (H1 and H2), diabetic hepatocytes (D) and P9 cells (P9).

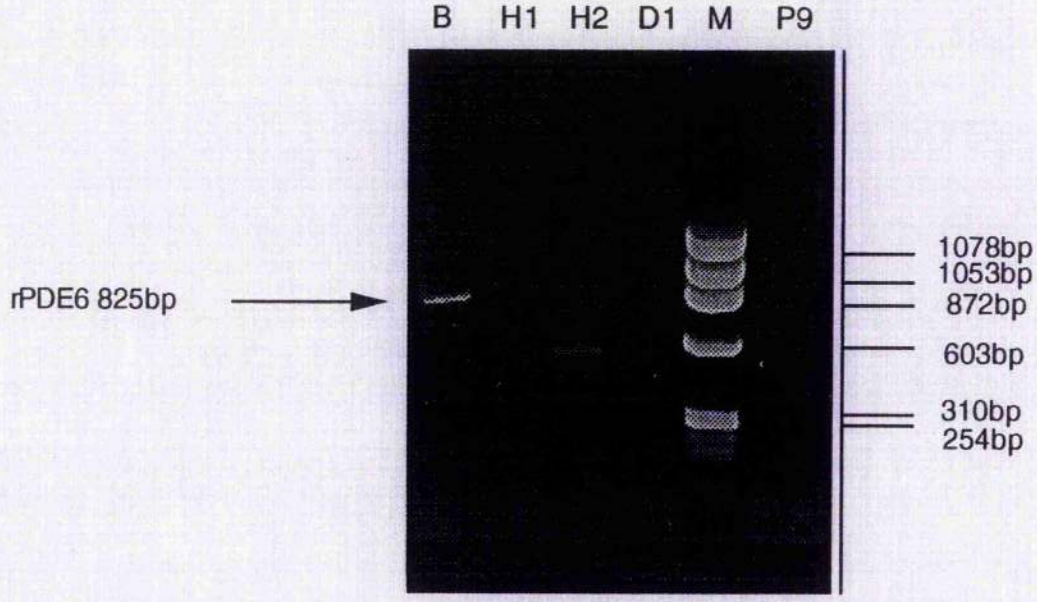

B
H1
H2
D1
M
P9
rPDE6 825bp
1078bp
1053bp
872bp
603bp
310bp
254bp

Figure 5.8 RT-PCR analyses of rPDE39 transcripts in normal and diabetic hepatocytes

This was done as described in methods using degenerate primers designed to detect transcripts selectively for rPDE39 forms in a species independent fashion. (Sense; GCCCAGAGAGGCTTGGTGATTTATCC. Antisense; ATATTCGAGGCAGTGTCAGCCTCTTGC). PCR reaction conditions employed here were denaturation; 94°C for 1min, annealing; 62°C for 1 min 20sec and extension; 72°C for 1min. 40 cycles of each condition were utilized. Thess reaction conditions were optimised for the detection of a 215bp fragment as shown with an arrow.

Analyses were performed on Marker (M), testis control (T), normal hepatocytes (H), diabetic hepatocytes (D) and P9 cells (P9).

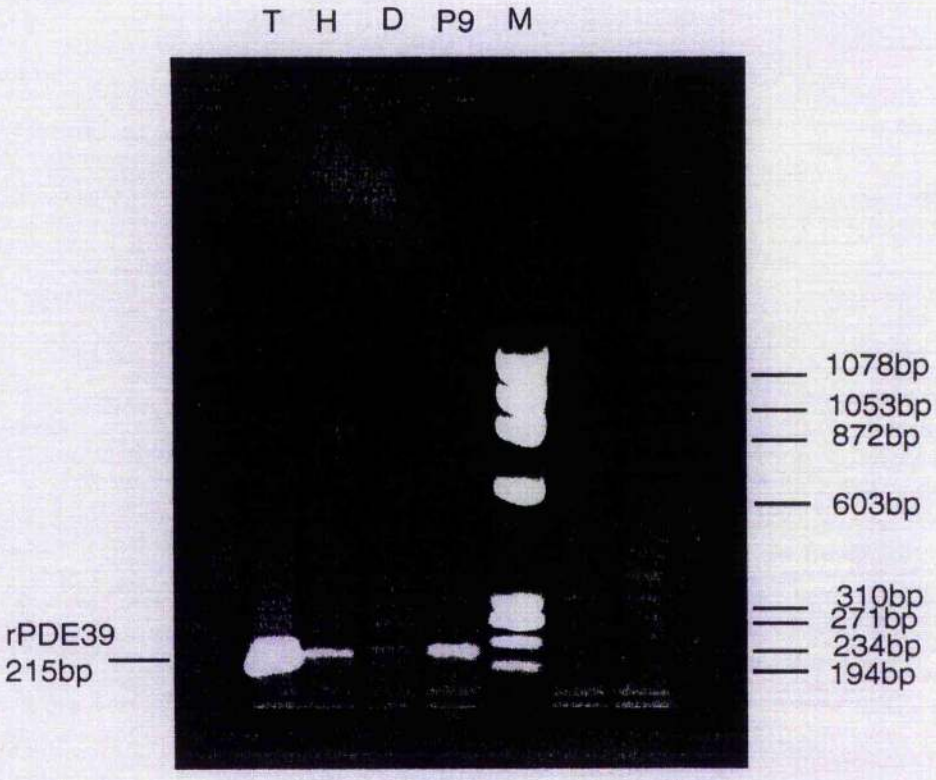

T H D P9 M
1078bp
1053bp
872bp
603bp
310bp
271bp
234bp
194bp
rPDE39
215bp

Figure 5.9 RT-PCR analyses of PDE4B1 transcripts in normal and diabetic hepatocytes.

This was done as described in methods using the degenerate primers designed to detect transcripts selectively for PDE4B1 forms in a species independent fashion. (Sense; AAACCTTCACGGAGCACCGAACAAGAGG. Antisense; GCCACGTTGAAGATGTTAAGGCCCCATT). PCR reaction condition employed here were denaturation; 94°C for 1min, annealing; 58°C for 1min 20sec and extension; 72°C for 1min 10sec. 40 cycles of each condition were utilized. These reaction conditions were optimised for the detection of a 507bp fragment.

Analyses were performed on Marker (M), P9 cells (P9), normal hepatocytes (H) and diabetic hepatocytes (D1 and D2).

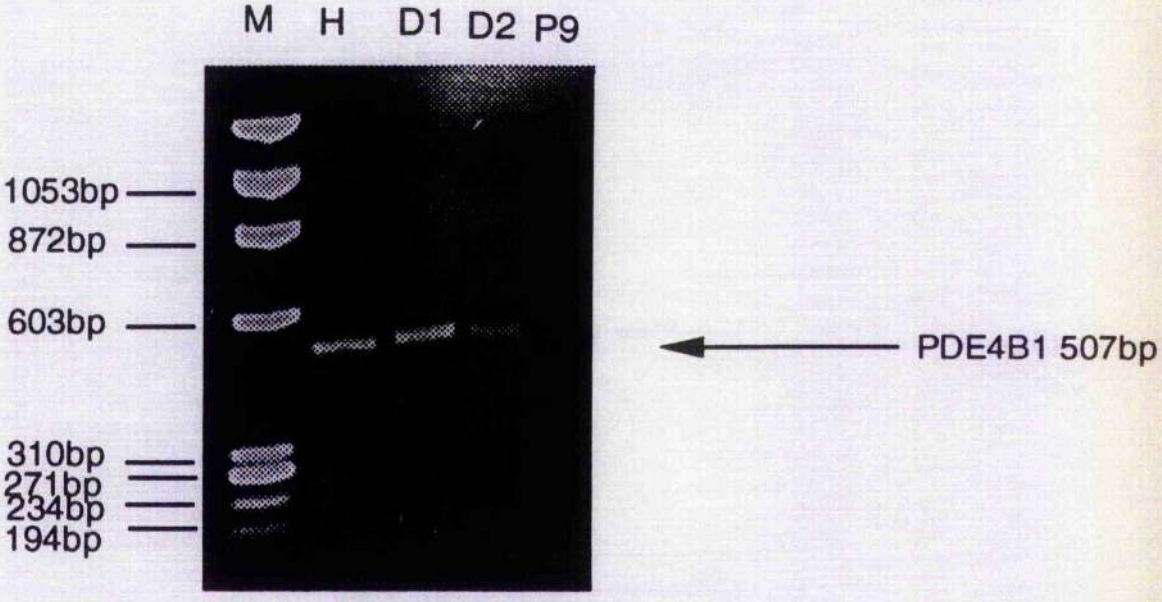

M
H
D1
D2
P9
1053bp
872bp
603bp
310bp
271bp
234bp
194bp
PDE4B1 507bp

Figure 5.10 RT-PCR analyses of PDE4B2 transcripts in normal and diabetic hepatocytes

This was done as described in methods using degenerate primers designed to detect transcripts selectively for PDE4B2 forms in a species independent fashion. (Sense; TTGGTAGATCACTGACACCTCATCCCG. Antisense; GCCACGTTGAAGATGTTAAGGCCCCATT). PCR reactions condition employed here were denaturation; 94°C for 1min, annealing; 58°C for 1min 20sec and extension; 72°C for 1min 10sec. 40 cycles of each condition were utilized. These reaction conditions were optimised for the detection of a 667bp as shown.

Analyses were performed on Marker (M), P9 cells (P9), normal hepatocytes (H) and diabetic hepatocytes (D).

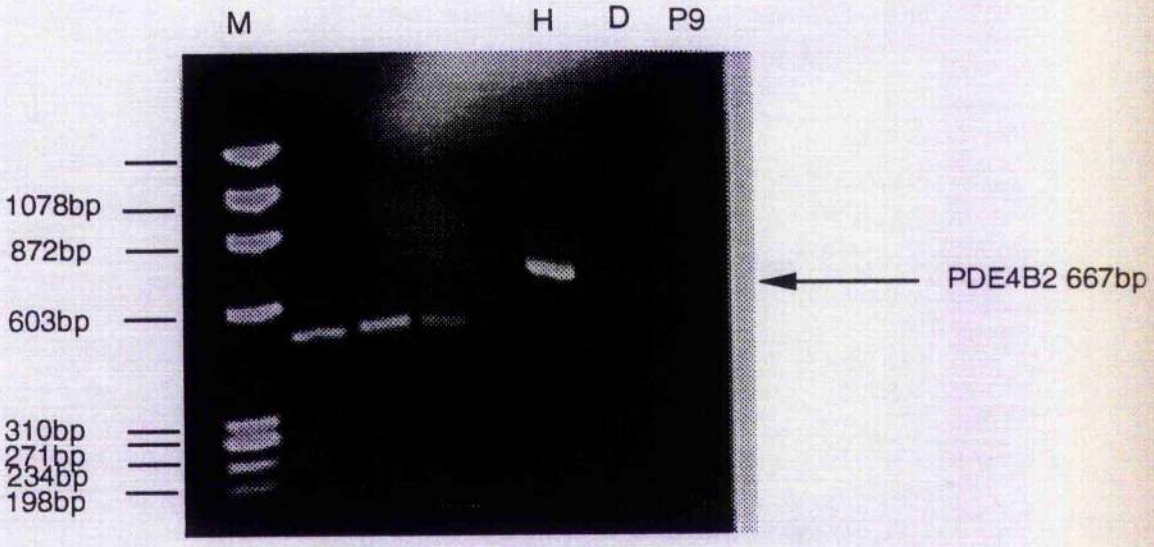

M
H
D
P9
1078bp
872bp
603bp
310bp
271bp
234bp
198bp
PDE4B2 667bp

Figure 5.11 RT-PCR analyses of PDE4D1 transcripts in normal and diabetic hepatocytes

This was done as described in methods using degenerate primers designed to detect transcripts selectively for PDE4D1 forms in a species independent fashion. (Sense; TCCGGTGAAGCGCTTAAGAACTGAGTCC. Antisense; CCTGGTTGCCAGACCTACTCATTTCA). PCR reaction condition employed here were denaturation; 94°C for 1min, annealing; 62°C for 1min 20sec and extension; 72°C for 1min. 40 cycles of each condition were utilized. These reaction conditions were optimised for the detection of a 227bp fragment.
Analyses were performed on Marker (M), testis control (T), P9 cells (P9), normal (H) and diabetic hepatocytes (D).

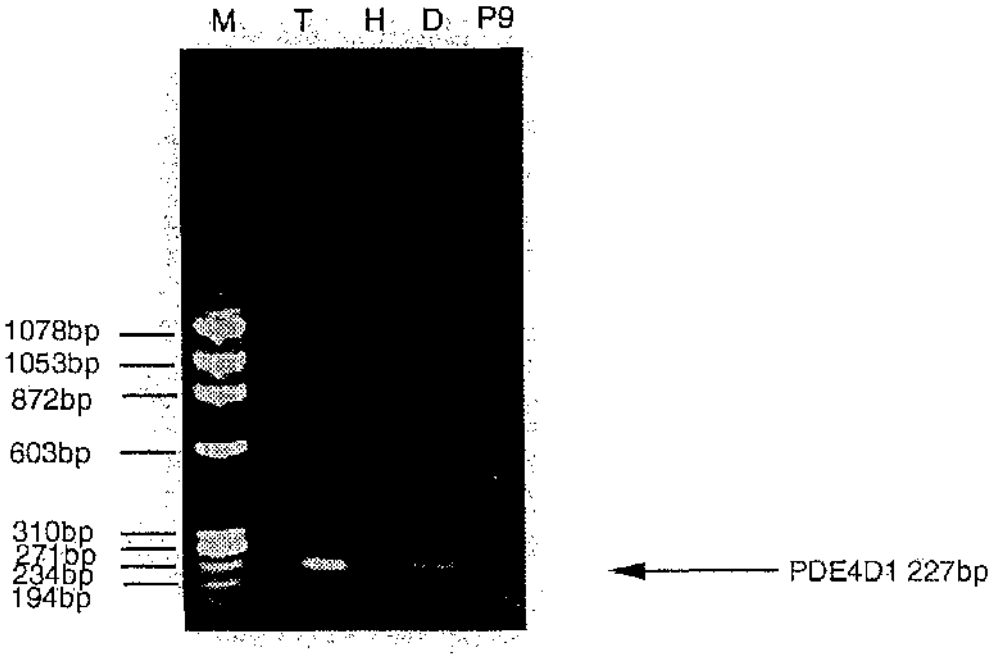

M
T
H
D
P9
1078bp
1053bp
872bp
603bp
310bp
271bp
234bp
194bp
PDE4D1 227bp

Figure 5.12 RT-PCR analyses of PDE4D3 transcripts in normal and diabetic hepatocytes

This was done as described in methods using degenerate primers designed to detect transcripts selectively for PDE4D3 forms in a species independent fashion. (Sense; CTAATTTGCAAGATCGCGCACCCAGC. Antisense; CCTGGTTGCCAGACCGACTCATTTCA). PCR reaction condition employed here were denaturation; 94°C for 1min, annealing; 58°C for 1min 20sec and extension; 72°C for 1min 10sec. 40 cycles of each condition were utilized. This reaction condition allowed the detection of a 255bp fragment. Analyses were performed on Marker (M), P9 cells (P9), normal (H) and diabetic hepatocytes (D1 and D2).

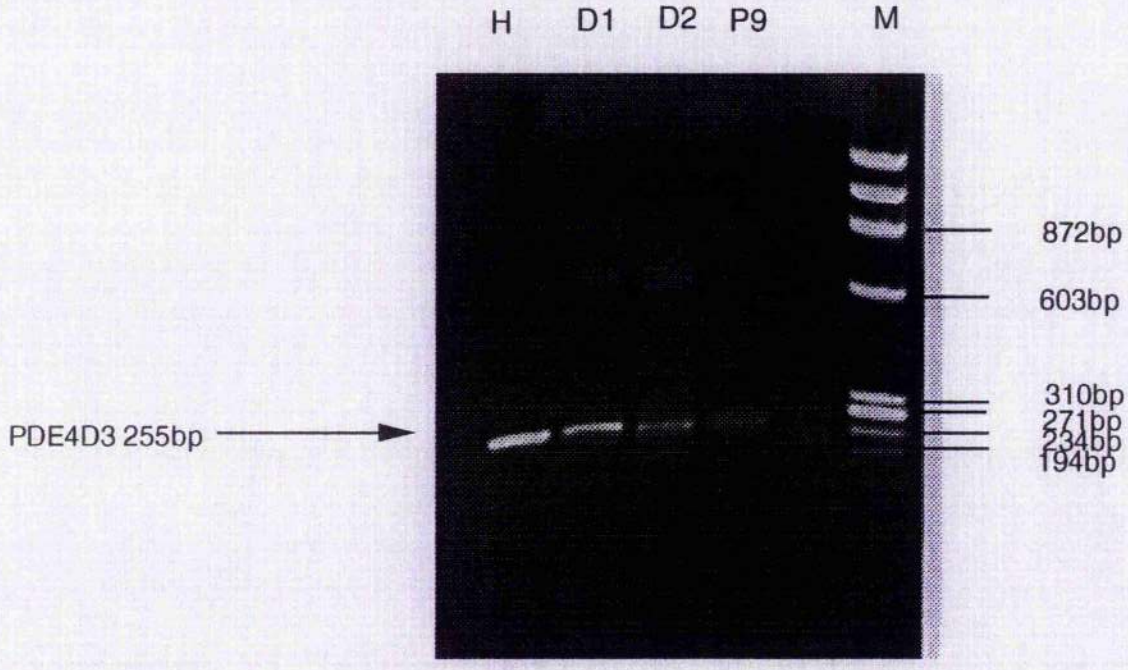

H
D1
D2
P9
M
872bp
603bp
310bp
271bp
234bp
194bp
PDE4D3 255bp

Table 5.1 Summary of the effects of insulin on components of the cAMP signalling pathway in normal and diabetic states.

References:

1. Coleman, 1977
2. Heyworth *et al.*, 1983
3. Soloman *et al.*, 1987
4. Soloman *et al.*, 1986
5. Heyworth *et al.*, 1983 and 1984
6. Solomon *et al.*, 1979
7. Soloman *et al.*, 1995
8. Bushfield *et al.*, 1990a and 1990b
9. Gawler *et al.*, 1987
10. Reaven *et al.*, 1995
11. Livingstone *et al.*, 1994

cAMP signalling	Changes in diabetic state	Effect of insulin treatment on	
		Normal	Diabetic
Intracellular cAMP activity in rat liver	increase [1]	decrease [2]	reverse to normal [3]
Low Km-PDE expression in rat liver, hepatocytes	decrease [4]	increase [5]	reverse to normal [3,6]
CaM (Ca^{2+} binding protein) expression in rat liver, adipocytes	decrease [3,4]	increase [4,7]	reverse to normal [3,6]
G_i level in rat liver adipocytes, platelets	decrease [8,9,10]	increase [11]	reverse to normal [9]
G_s level expression in rat hepatocytes	increase [8,9]	decrease [11]	not known
Adenylyl cyclase activity in rat liver hepatocytes, adipocytes	increase [8]	decrease [8,9]	not known

Table 5.2 Assessment of cAMP phosphodiesterase activity in hepatocytes from normal and diabetic animals.

Homogenate PDE activity of hepatocytes from normal and diabetic animals was determined in the absence (regarded as total PDE activity) or presence of either 10μM rolipram (regarded as PDE4 activity) or 10μM cilostimide (regarded as PDE3 activity), or 100μM IBMX (regarded as IBMX insensitive component). In both normal and diabetic hepatocytes the presence of EHNA (10μM), led to a decreased activity of cGMP (10μM) to stimulate activity which was taken as an index of PDE2 activity. Data is given with means±S.E. of at least 4 separate experiments employing different cell preparations. In each experiment triplicate PDE assays were performed and an average value taken. Assays were done in the presence of 1μM cAMP as substrate (see method section 2.3.11).

PDE activity	Normal hepatocytes (pmols/min/mg)	Diabetic hepatocytes (pmols/min/mg)	diabetic x100 (%) / normal
Total	23.6±1.8	13.8±2.0	58.5
PDE2	13.9±2.1	7.9±1.2	56.8
PDE3	9.6±2.0	5.4±0.3	56.2
PDE4	4.0±0.4	3.1±0.3	77.5
IBMX insensitive	3.7±0.8	3.2±0.3	86.5

Table 5.3 Assessment of PDE1 activity in hepatocytes and P9 cells.

PDE1 activity was assessed by determining the effect of adding Ca^{2+} (50μM) and CaM (10units) to the assays. This did not cause any change in PDE activity (none; <5%). Also, in order to detect whether endogenous Ca^{2+} might be carried over in this assay, EGTA (at the indicated concentrations) was added in the assay. Additional Ca^{2+} was then added to ensure an excess of free Ca^{2+} was present in the PDE assay. In all cases, CaM was present at 10units/tube. PDE activity assays were done in the presence of 1μM cAMP as substrate (see method section 2.3.11). Data is given as mean±S.E. of at least three separate experiments employing different cell preparations. In each experiment triplicate PDE assays were performed and an average value taken.

	Normal hepatocytes	P9 cells
	(pmols/min/mg)	(pmols/min/mg)
Total	17.8±2.1	10.7±3.1
50μM Ca^{2+}/CaM	17.7±3.2	8.6±4.2
1mM EGTA	18.3±2.4	7.4±3.9
1mM EGTA/2mM Ca^{2+}/CaM	18.9±2.0	5.3±3.6
1mM EGTA/5mM Ca^{2+}/CaM	18.9±3.0	6.4±4.0
2mM EGTA	18.5±0.9	8.2±4.8
2mM EGTA/5mM Ca^{2+}/CaM	18.9±1.6	4.5±2.4
2mM EGTA/8mM Ca^{2+}/CaM	18.6±2.0	5.7±2.5
5mM EGTA	17.2±2.1	7.7±3.1
5mMEGTA/8mM Ca^{2+}/CaM	6.1±1.0	2.5±1.2
5mM EGTA/12mM Ca^{2+}/CaM	5.8±0.6	4.1±2.0

Table 5.4 Sub-cellular distribution of PDE4 activity in normal and diabetic hepatocytes.

Preparation of cytosol and membranes from cell homogenates was carried out as described by method section 2.3.1. PDE4 activity was determined as that component of homogenate PDE activity which was inhibited by the presence of final concentrations of 10μM rolipram using 1μM cAMP as substrate (for details see method section 2.3.11) in the assay. Each point represents the mean±S.E. of at least four different experiments, each carried out in triplicate.

Fractions	hepatocytes (pmols/min/mg of protein)	Diabetic hepatocytes (pmole/min/mg of protein)
Cytosol	11.16±0.8	3.9±1.6
Membrane	4.4±0.7	5.2±0.88

Table 5.5 Immunoprecipitation of PDE4 activity in hepatocytes.

Cytosol and membrane fractions were prepared from cell homogenates as described by methods section 2.3.1. Immunoprecipitation with PDE4A, 4B and 4D antisera was also carried out as detailed in methods section 2.3.23. PDE activity assays were done in the presence of 1μM cAMP as substrate (see method section 2.3.11). Data is given as mean±S.E. of at least three separate experiments employing different cell preparations. In each experiment triplicate PDE assays were performed and an average value taken. Results are shown as pmoles/min/mg

Fractions	PDE4A	PDE4B	PDE4D
Cytosol	0.5±0.2	1.1±0.2	0.7±0.2
Membrane	2.45±0.5	0.8±0.1	0.3±0.1

Table 5.6. Summary of PDE4 splice variant expression in normal and diabetic hepatocytes.

Transcripts analyses were performed in the tissues indicated using primers and RT-PCR conditions as detailed previously. See Figures 5.2-5.12 inclusive.

	Normal hepatocytes	Diabetic hepaotcytes	Brain	Testis
4A generic	=	=	+	n.d.
RD1	-		+	n.d.
rPDE6	-	-	+	n.d.
rPDE39	<	-	n.d.	+
4B generic	=	<	+	n.d.
4B1	=	=	+	n.d.
4B2	=	-	+	n.d.
4C generic	-	-	n.d.	+
4D generic	=	<	+	n.d.
4D1	<	=	n.d.	+
4D3	=	<	+	n.d.

N.B. - stands for no signal expressed, = stands for equal signal expressed, + stands for normal signal expressed , < stands for weaker signal than control, n.d. stands for signal not determined.

Discussion

Type 2 diabetes is associated with insulin resistance in peripheral tissues with the result that these subjects have supraphysiological levels of glucose despite normal or elevated insulin levels. There is extensive evidence that E series prostaglandins (PGE1) which, when coupled to its specific receptor active intracellular cAMP, elicit a reduced responsiveness in platelets from type 2 diabetics (Livingstone *et al.*, 1991), implying an effect of diabetes on adenylyl cyclase signalling. Indeed, in adipose tissue where the prostanoid receptor couples to G_i, a reduction in PGE1 responsiveness would tend to enhance intracellular cAMP accumulation in response to stimulatory agonists and therefore promote lipolysis. A decreased antilipolytic response to PGE1 in adipocytes from diabetic rats has been proposed to result from a decrease in G_i function (Green and Johnson, 1991). Whilst it is becoming increasing clear that diabetic states alter the generation of the cAMP signal relatively less is known about the effects of this disease state on the termination of this cyclic nucleotide message.

Phosphodiesterases (PDEs) act as a terminator of the cAMP signal and therefore play an important role in pharmacology. It is thus interesting to establish any connection between elevated cAMP levels in the diabetic state and their effect on PDE expression and activity. Reports by other groups (eg Solomon, 1994) have shown the decreased activity of low-K_m cyclic AMP in the streptozotocin-induced diabetic rat. Here, our data support this notion and demonstrate decreased cAMP-utilizing PDE activity in streptozotocin-induced diabetic rat hepatocytes (**Table 5.2**) is associated specifically with a reduction in PDE2 and 3 and changes in PDE4 activities.

We focused here on an analysis of the PDE4 subtypes, attemping to determine the subcellular distribution of these enzymes in normal and diabetic hepatocytes and isoform expression. It was shown that total homogenate expressed a slightly lower level of PDE4 activity in diabetic hepatocytes

compared with normal hepatocytes (**Table 5.4**). However, profound differences were noted upon analysis of subcellular distribution. Thus, levels of cytosolic PDE4 activity fell profoundly from 72% to 43% in diabetic hepatocytes, whilst those for membrane PDE4 were unchanged (**Table 5.4**). As splice variants of PDE4 families can show membrane targetting (Shakur *et al.*, 1995), it is possible that changes in isoform expression underlie these differences. To examine this, we used antibodies and PCR. Thus, mRNA transcripts encoding PDE4 subtypes were identified through RT-PCR methodology using subtype-specific oligonucleotide primers (**Table 4.4**). These results showed that there was a clear preferential decrease in the steady-state levels of specific PDE4 splice variant mRNAs, namely PDE4B2 (**Figure 5.10**) and rPDE39 (**Figure 5.8**), arising from the PDE4B and PDE4A genes respectively, in diabetic hepatocytes. Meanwhile, even though a reduced signal for generic PDE4D (Fig 5.4) was detected in the diabetic hepatocyte, this was found not to be due to the reduced expression of PDE4D1 (**Figure 5.11**) but was due, possibly, to changes in the PDE4D3 (**Figure 5.12**) splice variant. However, it could be that changes were due to additional splice variants of PDE4D which we were not able to detect. For example, PDE4D4 might be expressed in these cells but their sequence for the rat are not known so that no specific primer can be designed according to its unique sequence. Thus, RT-PCR could not be employed to detect their expression at the mRNA level. Also, the primer we designed specifically for PDE4D2 failed to detect transcript in these samples, although some reports indicated the existance of PDE4D2 in sertoli cells (Swinnen *et al.*, 1989; Sette *et al.*, 1994b). Due to lack of any other "positive" controls, we were therefore unable to detect its expression in the other samples.

Antisera raised against the C-terminal region of PDE4B failed to detect any change in the normal and diabetic hepatocytes. This contrasts with the RT-PCR results where lower 4B and 4D levels occurred in the disease state. This

discrepancy might be due to the proteolysis of these samples so that the reduced protein synthesis was not able to be detected in diabetic hepatocytes. It is also not certain if the reduction in the steady state levels of these transcripts seen in the diabetic tissues was caused by impaired transcription or enhanced degradation of the target mRNAs. Is the system protecting itself to handle the stress of the diabetes, a catabolic state, by shutting down its anabolic mechanisms? Examples of such situations are common in nature and are part of the explanation of the specialized role of ubiquitin and other heat shock proteins in response to severe stress (Lindquist and Craig, 1988).

Soloman with co-workers (1994) have shown levels of CaM and its mRNA to be reduced in diabetes and stimulated by insulin. This ability of insulin to inhibit the degradation of mRNA suggests a major post-transcriptional effect which has been demonstrated in a number of insulin-regulated systems. For example, Tewari *et al.*, (1991) proposed that post-transcriptional regulation accounts for major differences in insulin receptor mRNA in different tissues.

The pathophysiologic processes involved in diabetic ketoacidosis are accompanied by increased levels of cAMP in plasma and tissue (Solomon *et al.*, 1987). This results from excess counter-regulatory hormones and the unavailability of insulin with resultant inability to increase the activity of low-K_m cAMP PDE. PDE hydrolysis of cAMP is not only stimulated by insulin, through specific isoforms (Pyne *et al.*, 1986, 1988, 1989) but is also modified by other regulatory components which are insulin sensitive, eg, CaM (Cheung, 1971; Solomon *et al.*, 1987, Solomon *et al.*, 1986, Smoake *et al.*, 1981). Soloman and co-workers have previously demonstrated that PDE activity is reduced in diabetes and restored to normal levels with insulin. Insulin-sensitive low-K_m cAMP PDE appears to be a key regulatory enzyme in insulin's actions in tissue, presumably through its ability to regulate levels of the second messenger cAMP.

These studies illustrate regulation of cAMP-specific PDE enzymes in the insulin-independent diabetic state. The biological role of particular PDE variants remains to be elucidated. However, the complex changes in cAMP signalling incurred in diabetes at the level of synthesis and degradation is likely to have profound effects on cellular functioning.

Chapter 6
General Discussion

General discussion

The cAMP signalling system provides adenylyl cyclase and PDE isoforms which can couple to signals emanating from every known signalling pathway. Thus the cAMP signalling system may actually provide the 'core' machinery for integrating signalling with the cell. Far from there being a single source of production, degradation and recognition there are now over 8 isoforms of adenylyl cyclase (Cooper *et al.*, 1994; Jacobowitz *et al.*, 1993, Krupinski *et al.*, 1992), over 25 isoforms of cAMP phosphodiesterase (Beavo *et al.*, 1994; Conti *et al.*, 1991) and multiple forms of PKA-I (Corbin *et al.*, 1977) and PKA-II families (Nigg *et al.*, 1985; Salavatori *et al.*, 1990). This vast stock of components allows the cAMP signalling system to be tailored in a highly cell-specific fashion.

The complex family of PDEs lends itself to a role in the interactions between signalling pathways, due to its ability to be regulated by a number of different effectors (Beavo, 1990; Bolger, 1994; Conti *et al.*, 1995b; Houslay and Kilgour, 1990; Manganiello *et al.*, 1990a)). This, coupled with the enormous capacity for differential regulation of adenylyl cyclase (Chen and Iyengar, 1993; Krupinski *et al.*, 1992), demonstrates the immense opportunity for dynamic control of a vast network of intracellular signals.

The action of PKC has been shown to regulate a number of elements of the cyclic AMP signalling pathway (Houslay, 1991b), leading to an inhibition (Beckner and Farrar, 1986; Kelleher *et al.*, 1984) or an enhancement (Cronin *et al.*, 1986; Yoshimasa *et al.*, 1987) of agonist-induced increases in cyclic AMP, depending on the cell type. The regulation of cyclic AMP signalling by PKC can occur at various different stages of the signalling pathway, such as the modification of stimulatory and inhibitory receptors coupled to adenylyl cyclase such as the β2-adrenoceptor (Bouvier *et al.*, 1987), the α2-adrenoceptor (Convents *et al.*, 1989) and the inhibitory G_i protein (Bell and

Brunton, 1986; Morris *et al.,* 1994; O'Brien *et al.,* 1987) . Adenylyl cyclase has been shown to be modulated by PKC phosphorylation in intact cells (Jacobowitz and Iyengar, 1994; Kawabe *et al.,* 1994; Williams *et al.*, 1987) , as well as in purified cell membranes (Nagshineh *et al.,* 1986; Pyne *et al.,* 1994). Moreover, differing sensitivities of the cells may occur due to the different complement of PKC and adenylyl cyclase/PDE isoforms which are expressed within the cell types. In accord with this, Chapter 3 illustrated the differences in PMA-mediated inhibition of cholera toxin-stimulated adenylyl cyclase in hepatocytes, P9 cells, CHO cells and NIH3T3 cells, These results indicated that a particular isoform of adenylyl cyclase (type V) may be phosphorylated by PKC; which is expressed in hepatocytes and P9 cells only. It has been shown that PKC-δ and PKC-α selectively activated type-V adenylyl cyclase (Kawabe *et al.,* 1994) and we note here that PKC-α is expressed in hepatocytes (Tang *et al.,* 1993).

PDE isoform expression is tissue specific and can be differentially regulated by hormones. That individual PDE isoenzymes are regulated in both positive and negative manners has highlighted their pivotal role in mediating cross-talk between second messenger pathways. It has also provided a conceptual basis for understanding many of the distinctive, cell type-specific differences in the amplitude and duration of cAMP and cGMP signals produced in response to stimulation of adenylyl and guanylyl cyclase. In our studies the up-regulation of PDE4 by chronic stimulation of adenylyl cyclase, led to an additional mRNA message of PDE4B2, accumulation of PDE4D3 transcripts and increased protein expression. These studies provide a novel tissue specific model for analysing the functional regulation of PDE4 genes by cAMP. It has been concluded that increases in PDE-4 activity may arise via two mechanisms: one being activation by phosphorylation (rapid) and the other probably reflecting constitutive activation (delayed) (Conti *et al.,* 1995a; Sette *et al.,* 1994b). It seems likely that both of these mechanisms

are imortant contributors to the tachyphylaxis many tissues exhibit with continued stimulation of adenylyl cyclase. The situation described here differs from that described by Sette *et al.* (1994b) where PKA has been shown to mediate the expression of certain splice variants of PDE4D (PDE4D1 and PDE4D2), whilst increasing activity of PDE4D3 by phosphorylation in response to thyroid-stimulating hormone. This investigation demonstrated that splice variants from the same locus could be differentially regulated on stimulation of the cell by a particular hormone. An elevation of cyclic AMP has also been shown to cause an increase in ratPDE3 (PDE4D) expression in immature Sertoli cells on stimulation with FSH (Monaco *et al.*, 1994; Sette *et al.*, 1994b; Swinnen *et al.*, 1991b) , in cardiac myoblasts (Kovala *et al.*, 1994) and in Jurkat cells (Engels *et al.*, 1994). It would be of interest to see whether different PDE4 activities are regulated by different signalling networks.

Although an additional transcript of PDE4B2 has been detected upon elevation of cAMP, we have been unable to detect a significant increase in enzyme activity under such conditions, which might suggest that this PDE4B protein is highly unstable (Alvarez *et al.*, 1995; Conti *et al.*, 1995a) or that transcript levels are not sufficiently high enough to allow for significant expression.

The concept of differentially expressed and regulated PDE isozymes also implies that individual PDEs are likely to be good targets for therapeutic intervention in diseases caused or regulated by cyclic nucleotide-modulated transduction mechanisms. Pharmaceutical interest in the area has been further sparked by the promise that different PDE isozymes having distinct sequences at regulatory and catalytic sites should allow the development of selective therapeutic agents that can target a specific cyclic nucleotide pool in a very limited number of cell types. As such, an appreciation of the molecular details of the enzymes involved and aberrations occurring in disease states may be of profound significance. On this basis, the altered PDE4 expression shown in

this study may contribute to our understanding of changes occuring in diabetes.

References

Abdelatif, M.M., Neubauer, C.F., Lederer, W.J. and Rogers, T.B. (1991) **Circ. Res.** 69, 800-809.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) **J. Mol. Biol.** 215, 403-410.

Alvarez, R., Sette, C., Yang, D., Eglen, R.M., Wilhelm, R., Shelton, E.R. and Conti, M. (1995) **Mol. Pharmacol.** 48, 616-622.

Anderson, N. G., Kilgour, E. and Houslay, M.D. (1989) **Biochem. J.** 262, 867-872.

Ansanay, H., Sebben, M., Bockaert, J. and Dumuis, A. (1992) **Mol. Pharmacol.** 42, 808-816.

Asaoka, Y., Nakamura, S., Yoshida, K. and Nishizuka, Y. (1992) **Trends Biochem. Sci.** 17, 414-417.

Ase, K., Berry, N., Kikkawa, U., Kishimoto, A. and Hishizuka, Y. (1988) **FEBS Lett.** 236, 396-400.

Atkinson, B.N. and Minneman, K.P. (1992) **Mol. Pharmacol.** 41, 688-694.

Attali, B. and Vogel, Z. (1989) **J. Neurochem.** 53, 1636-1639.

Bacher, N., Zisman, Y., Berent, E. and Livneh, E. (1992) **Mol. Cell. Biol.** 12, 1404.

Bakalyar, H.A., and Reed, R.R. (1990) **Science** 250, 1403-1406.

Ball, E.H., Seth, P.K. and Sanwal, B.D. (1980) **J. Biol. Chem.** 255, 2962-2968.

Balmfort, A.J., Warburton, P. and Ball, S.G. (1990) **J. Neurochem.** 55, 2111-2116.

Barnette, M.S., Grous, M., Cieslinski, L.B., Burman, M., Christensen, S.B., and Torphy, T.J. (1995) **J. Pharmacol. Exp. Ther.** 273, 1396-1402.

Barovsky, K., K., Pedone, C. and Brooker, G. (1984) **Mol. Pharmacol.** 25, 256-260.

Barton, A.C., Black, L.E. and Sibley, D.R. (1990) **Mol. Pharmacol.** 38, 531-541.

Beavo, J.A. (1988) **Adv. Second Messenger Phosphoprotein Res.** 22, 1-30.

Beavo, J.A. (1990) **Molecular Pharmacology of Cell Regulation** Vol. 2, 3-15.

Beavo, J.A. and Reifsnyder, D.H. (1990) **Trends Pharm. Sci.** 11, 150-155.

Beavo, J.A., Conti, M., and Heaslip, R.J. (1994) **Mol. Pharmacol.** 46, 399-405.

Beckner, S.K. and Farrar, W.L. (1986) **J. Biol Chem.** 261, 3043-3047.

Bell, J.D. and Brunton, L.L. (1986) **J. Biol. Chem.** 261, 12936-12941.

Bell, R.M. and Burns, D.J. (1991) **J. Biol. Chem.** 266, 4661-4664.

Beltman, J., Sonnenberg, W.K., Beavo, J.A. (1993) **Mol. Cell Biol.** 127, 239-253.

Benovic, J.L., Shorr, R.G.L., Caron, M.G. and Lefkowitz, R.J. (1984) **Biochemistry** 23, 4519-4525.

Benovic, J.L., Pike, L.J., Cerione, R.A., Staniszewski, C., Yoshimasa, T., Codina, J., Caron, M.G., and Lefkowitz, R.J. (1985) **J. Biol. Chem.** 260, 7094-7101.

Benovic, J.L., Strasser, R.H., Caron, M.G. and Lefkowitz, R.J. (1986) **Proc. Natl. Acad. Sci. USA** 83, 2797-2801.

Benovic, J.L., Kuhn, H., Weyand, I., Codina, J., Caron, M.G., and Lefkowitz, R.J. (1987) **Proc. Natl. Acad. Sci. U.S.A.** 84, 8879-8882.

Benovic, J.L., DeBlasi, A., Stone, W.C., Caron, M.G. and Lefkowitz, R.J. (1989) **Science** 246, 235-240.

Benovic, J.L., Onorato, J.J., Arriza, J.L., Stone, C.W., Lohse, M.J., Jenkins, J., Gilbert, N.G., Caron, M.G. and Lefkowitz, R.J. (1991) **J.Biol. Chem.** 266, 14939-14946.

Bentley, J.K., Kadlecek, A., Sherbert, C.H., Seger, D., Sonnenburg, W.K., Charbonneau, H., Novack, J.P. and Beavo, J.A. (1992) **J. Biol. Chem.** 267, 18676-18682.

Berridge, M.J.and Irvine, R.F. (1984) **Nature** 312, 315-321.

Berry, M.N. & Friend, D.S. (1969) **J. Cell. Biol.** 43, 506-520.

Bhat, S.V., Bajwa, B.S., Dornauer, H. and de Souza, N.J. (1977) **Tetrahedron Lett.** 19, 1669-1672.

Bialojan, C. and Takai, A. (1988) **Biochem. J.** 256, 283-290.

Bilofsdy, H.S. and Burks, C. (1988) **Nucleic Acids Res.** 16, 1861-1864.

Birnbaumer, L. (1973) **Biochim. Biophys. Acta** 300, 129-158.

Birnbaumer, L., Codina, J., Mattera, R., Cerione, R.A., Hildebrandt, J.D., Sunya, T., Rojas, F.J., Caron, M.J., Lefkowitz, R.J. and Iyengar, R. (1985) **Mol. Aspects Cell Regul.** 4, 131-182.

Birnbaumer, L., Abramowitz, J. & Brown, A.M. (1990) **Biochim. Biophys. Acta** 1031, 163-224.

Birnbaumer, L., Antaramian, A., Themmen, A.P. and Gilbert, S. (1992) **J. Biol. Chem.** 267, 11783-11788.

Bitensky, M.W., Wheeler, G.L., Yamazaki, A., Rasenick, M.M. and Stein, P.J. (1981) **Curr. Top. Membr. Transp.** 15, 237-271.

Blackmore, P & Exton, J.H. (1986) **J. Biol. Chem.** 261, 11056-11063

Boeckino, S.B., Blackmore, P.F. and Exton, J.H. (1985) **J. Biol. Chem.** 260, 14201-14207.

Boekhoff, I. and Breer, H. (1992) **Proc. Natl. Acad. Sci. USA** 89, 471-474.

Boekhoff, I., Schleicher, S., Strotmann, J. and Breer, H. (1992) **Proc. Natl. Acad. Sci. USA** 89, 11983-11987.

Bokoch, G.M., Katada, T., Northup, J.K., Hewlett, E.L. and Gilman, A.G. (1983) **J. Biol. Chem.** 258, 2071-2075.

Bolger, G., Michaeli, T., Martins, T., St-John, T., Steiner, B., Rodgers, L., Riggs, M., Wigler, M. and Ferguson, K. (1993) **Mol. Cell. Biol.** 13, 6558-6571.

Bolger, G. (1994) **Cell Signalling** 6, 851-859.

Bolger, G.B., Rodgers, L. and Riggs, M. (1994) **Gene**, 149, 237-244.

Borrelli, E., Montmayeur, J.P., Foulkes, N.S., and Sassone-Corsi, P. (1992) **Crit. Rev. Oncog.** 3, 321-338.

Bourne, H.R., Lichtenstein, L.M., Melmon, K.L., Henney, C.S., Weinstein, Y. and Shearer, B.M. (1974) **Science** 184, 19-28.

Bourne, H.R., Sanders, D.A. and McCormick, F. (1990) **Nature** 348, 125-132.

Bouvier, M. Leeb-Lundberg, L.M.F., Benovic, J.L., Caron, M.G. and Lefkowitz, R.J. (1987) **J. Biol. Chem.** 262, 3106-3113.

Bouvier, M., Guilbault, N. and Bonin, H. (1991) **FEBS Lett.** 279, 243-248.

Bradford, M.M. (1976) **Analytical Biochemistry** 72, 248-254.

Bray, P., Carter, A., Simons, C., Gou, V. and Rickett, C. (1986) **Proc. Natl. Acad. Sci. U.S.A.** 83, 8893-8897.

Brooker, G., Pedone, C. and Barovsky, K. (1983) **Science** 220, 1169-1170.

Brown, B.L., Ekins, R.P. and Albano, J.D.M. (1972) **Advances in Cyclic Nucleotide Research** 2, 25-40.

Bruns, R.F., Miller, F.D., Merriman, R.L., Howbert, J.J., Heath, W.F., Kobayashi, E., Takahashi, I., Tamaoki, T. and Nakano, H. (1991) **Biochem. Biophys. Res. Commun.** 176, 288-293.

Burns, D.J., Bloomenthal, J., Lee, M.H. and Bell, R.M. (1990) **J. Biol. Chem.** 265, 12044-12051.

Burns, D.J. and Bell, R.M. (1991) **J. Biol. Chem.** 266, 18330-18338.

Bushfield, M., Murphy, G.J., Lavan, B.E., Parker, P.J., Hruby, V.J., Milligan, G. and Houslay, M.D. (1990a) **Biochem. J.** 268, 449-457.

Bushfield, M., Griffiths, S.L., Murphy, G.J., Pyne, N., Knowler, J.T., Milligan, G., Parker, P.J., Mollner, S. and Houslay, M.D. (1990b) **Biochem. J.** 271, 365-372.

Bushfield, M., Griffiths, .S.L., Strassheim, D., Tang, E., Shakur, Y., Lavan, B. and Houslay, M.D. (1990c) **Biochem. Soc. Symp.** 56, 137-154.

Bushfield, M., Lavan, B.E. & Houslay, M.D. (1991) **Biochem. J.** 274, 317-321.

Butcher, R.W and Sutherland, E.W. (1962) **J Biol. Chem.** 237, 1244-1250.

Bygrave, F.L. and Benedetti, A. (1993) **Biochem. J.** 296, 1-14.

Caldwell, K.K., Boyajian, C.L., and Cooper, D.M.F. (1992) **Cell Calcium** 13, 107-121.

Cali, J.J., Zwaagstra, J.C., Mons, N., Copper, D.M.F., and Krupinski, J. (1994) **J. Biol. Chem.** 269, 12190-12195.

Camps, M., Hou, C., Sidiropoulos, D., Stock, J.B., Jakobs, K.H.and Gierschik, P. (1992) **Eur. J. Biochem.** 206, 821-831.

Cantiello, H.F., Patenaude, C.R., Codina, J., Birnbaumer, L., Ausiello, D.A. (1990) **J. Biol. Chem.** 265, 21624-21628.

Carlson, K.E., Brass, L.F., and Manning, D.R. (1989) **J. Biol. Chem.** 264, 13298-13305.

Caro, J.F., Raju, M.S., Exton, J.H., Thakkar, J.K., Roy, L., Hale, J.C. and Pories, W.J. (1991) **J. Biol. Chem.** **(Check 15B, 13.)**

Carr, D.W., Hausken, Z.E., Fraser, E.D.C., Stofko-Hahn, R.E., Scott, J.D. (1992) **J. Biol. Chem**. 267, 13376-13382.

Carr, D.W., DeMamo, D.A., Atwood, A., Hunzicker-Dunn, M., Scott, D.J. (1993) **J. Biol. Chem.** 268, 20729-20732.

Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishezuka, Y. (1982) **J. Biol. Chem**. 257, 7847-7851.

Cerione, R.A., Codina, J., Benovic, J.L., Lefkowitz, R.J., Birnbaumer, L. and Caron, M.G. (1984) **Biochemistry** 23, 4519-4525.

Chabre, M. and Deterre, P. (1989) **Eur. J. Biochem.** 179, 255-266.

Chamras, H., Foucherau-Peron, M. and Rosselin, G. (1980) **Diabetologia** 19, 74-80.

Charbonneau, H. Beier, N., Walsh, K.A., Beavo, J.A. (1986) **Proc. Natl. Acad. Sci. U.S.A.** 63, 9308-9312.

Charbonneau, H., Prusti, R.K., LeTrong, H., Sonnenburg, W.K., Stith-Coleman, I.E., Vaughan, M., Mullaney, P.J., Walsh, K.A.and Beavo, J.A. (1989) **Proc. Natl. Acad. Sci. U.S.A.** 87, 288-292.

Charbonneau, H. (1990) **Mol. Pharmacol. Cell Regulation** 2, 267-296.

Charbonneau, H., Kumar, S., Novack, J.P., Blumenthal, D.K., Griffin, P., Shabanowitz, J., Hunt D.F., Beavo, J.A. and Walsh, K.A .(1991) **Biochemistry** 30, 7931-7940.

Chauhan, A., Chauhan, V.P.S. and Brockerhoff, H. (1991) **Biochem. Biophys. Res. Commun.** 175, 852-857.

Chen J. & Iyengar, R. (1993) **J. Biol. Chem.** 268, 12253-12256.

Chen, K.H., Widen, S.G., Wilson, S.H. and Huang, K.P. (1990) **J. Biol. Chem.** 265, 19961-19965.

Cheung, W.Y. (1967) **Biochem. Biophys. Res. Commun.** 29, 478-482.

Cheung, W.Y. (1970) **Biochem. Biophys. Res. Commun.** 38, 533-538.

Cheung, W.Y. (1971) **J.Biol. Chem.** 246, 2859-2869.

Chin, J.E., Dickens, M., Tavare, J.M. and Roth, R.A. (1993) **J. Biol. Chem.** 268, 6338-6347.

Choi, E., Xia, Z., and Storm, D.R. (1992) **Biochemistry** 31, 6492-6498.

Chou, J.Y. (1989) **Mol. Endocrinology.** 3, 1511-1514.

Clark, R.B., Friedman, J., Dixon, R.A.F., and Strader, C.D. (1989) **Mol. Pharmacol.** 36, 343-348.

Codina, J., Hildebrandt, J.D., Iyengar, R., Birnbaumer, L., Sekura, R.D. and Manclark, C.R. (1983) **Proc. Natl. Acad. Sci. U.S.A.** 80, 4276-4280.

Codina, J., Hildebrandt, J.D., Sekura, R.D., Birnbaumer, M., Bryan, J., Manclark, C.R., Iyengar, R. and Birnbaumer, L. (1984) **J. Biol. Chem.** 259, 5871-5886.

Codina, J., Olate, J., Abramowitz, J., Mattera, R., Cook, R.G. and Birnbaumer, L. (1988) **J. Biol. Chem.** 263, 6746-6750.

Coghlan, V.M., Jangeberg, L.K., Fernandez, A., Lamb, N.J.C., Scott, J.D. (1994) **J. Biol. Chem.** 269, 7658-7665.

Coghlan, V.M., Perrino, B.A., Howard, M., Langeberg, L.K., Hicks, J.B., Ballatin, W.M., Scott, J.D. (1995) **Science** 267, 108-111.

Cohen, P. (1985) **Eur. J. Biochem.** 151, 439-448.

Cohen, P., Holmes, C.F.B. and Tsukitani, Y. (1990) **Trends Biochem. Sci.** 15, 98-102.

Collins, S., Caron, M.G., and Lefkowitz, R.J. (1992) **Trends Biochem. Sci.** 17, 37-39.

Comb, M., Birnberg, M.C., Seasholtz, A., Herbert, E., and Goodman, H.M. (1986) **Nature** 323, 353-356.

Conti, M., Geremia, R., Adamo, S., and Stefanini, M. (1981) **Biochem. Biophys. Res. Commun.** 98, 1044-1050.

Conti, M., Toscano, M.V., Petrelli, L., Geremia, R., and Stefanini, M. (1982) **Endocrinology** 110, 1189-1196.

Conti, M. and Swinnen, J.V. (1990) **Molecular Pharmacology of Cell Regulation** vol. 2, 243-266.

Conti, M. Jin, S.-L.C., Monaco, L., Repaske, D.R., and Swinnen, J.V. (1991a) **Endocrine. Rev.** 12, 218-234.

Conti, M., Swinnen, J.V., Tsikalas, K.E. and Jin, S.L.C. (1991b) **Adv. in Second Messenger and Phosphoprotein Res.** 25, 87-99.

Conti, M., Nemoz, G., Sette, C. and Vicini, E. (1995b) **Endocrine Rev.** 16, 370-389.

Conti, M., Iona, S., Cuomo, M., Swinnen, J.V., Odeh, J. and Svoboda.M.E. (1995a) **Biochemistry** 34, 7979-7987.

Cooper, D.M.F. (1991) **Biochem. J.** 278, 903-904.

Cooper, D.M., Mons, N & Fagan, K (1994) **Cell. Signalling**. 6, 823-840.

Convents, A., De Backer, J.P., Andre, C. and Vauquelin, G. (1989) **Biochem. J.** 262, 245-251.

Corbin, J.D., Sugden, P.H., Lincolin, T.M., Keely, S.L. (1977) **J. Biol. Chem.** 252, 3854-3861.

Corbin, J.D., Beebe, S.J., and Blackmore, P.E. (1985) **J. Biol. Chem**. 260, 8731-8735.

Creba, J.A., Downes, C.P., Hawkins, P.T., Brewster, G., Michell, R.H. and Kirk, C.J. (1983) **Biochem. J.** 212, 733-747.

Cronin, M.J. Summers, S.T., Sortino, M.A. and Hewlett, E.L. (1986) **J. Biol. Chem**. 261, 13932-13935.

Crouch, M.F. and Lapetina, E.G. (1988) **J. Biol. Chem.** 263, 3363-3371.

Cryer, P.E. (1980) **New England Journal of Medicine** 303, 436-444.

Danidl-Issakani, S., Spiegel, A.M., and Strulovice, B. (1989) **J. Biol. Chem**. 264, 20240-20247.

Darfler, F.J., Mahon, L.C., Koachman, A.M. and Insel, P.A. (1982) **J. Biol. Chem.** 257, 11901-11907.

Davis, R.L., Takayasu, H., Eberwine, M.and Myres, J. (1989) **Proc. Natl. Acad. Sci. U.S.A.** 86, 3604-3608.

Dawson, T.M., Arriza, J.L., Jaworsky, D.E., Borisy, F.F., Attramadal, H., Lefkowitz, R.J. and Ronnett, G.V. (1993) **Science** 259, 825-829.

de Croot, R.P., den Hertog, J., Wandenheede, J.R., Goris, J., Sassone-Corsi, P. (1993) **EMBO J.** 12, 3903-3911.

DeFronzo, R., Deibert, D., Hendler, R., Flig, P. and Soman, M. (1979) **J. Clin. Invest.** 63, 939-946.

DeFronzo, R., Ferrannini, E.and Koivisto, V. (1983) **American J. of Medicine** 74, 52-81.

DeFronzo, R.A., Hendler, R. and Simonson, D. (1982) **Diabetes** 31, 795-801.

DeFronzo, R.A., Simonson, D. and Ferrannini, E. (1982) **Diabetologia** 23, 313-319.

Degerman, E., Smith, C.J., Tornqvist, H., Vasta, V., Belfrage, P. Manganiello, V.C. (1990) **Proc. Natl. Acad. Sci. U.S.A.** 87, 533-537.

Dekker, L.V., Parker, P.J. and McIntyre, P. (1992) **FEBS Lett.** 312, 195-199.

Dekker, L.V. and Parker, P.J. (1994) **Trends Biochem Sci.** 19, 73-77.

Diaz-Laviada, I., Larrodera, P., Nieto, J.L., Cornet, M.E., Diaz-Meco, M.T., Sanchez M.J., Guddal, P.H., Johansen, T., Haro, A. and Moscat, J. (1991) **J. Biol. Chem.** 266, 1170-1176.

DiSanto, M.E. and Heaslip, R.J. (1993) **Biochem. Biophys. Res. Commun.** 197, 1126-1131.

DiSanto, M.E. and Heaslip, R.J. (1994) **FASEB J.** 8, A368.

DiSanto, M.E., Glaser, K.B. and Heaslip, R.J. (1995) **Cell Signalling** 7, 827-835.

DiSanto, M.E. and Heaslip, R.J. (1995) **Eur. J. Pharmacol.** 290, 169-172.

Dixon, R.A.F., Kobilka, B.K., Strader, D.J., Benovic, J.L., Dohlman H.G., Frielle, T., Bolanowski, M.A., Bennett, C.D., Rands, E., Dichl, R.E., Mumford R.A., Slater, E.E., Sigal, E.S., Caron, M.G., Lefkowitz, R.J. and Strader, C.C (1986), **Nature** 321, 75-79.

Dohlman, H.G., Bouvier, M., Benovic, J.L., Caron, M.G. and Lefkowitz, R.J. (1987) **J. Biol. Chem.** 262, 14282-14288.

Dohlman, H.G., Thorner, J., Caron, M.G., and Lefkowitz, R.J. (1991) **Ann. Rev. Biochem.** 60, 653-688.

Duronio, V., Nip, L. and Pelech, S.L. (1989) **Biochem. Biophys. Res. Commun.** 164, 804-808.

Efrat, S. and Hanahan, D. (1989) **Current Topics in Microbiology and Immunology** 144, 89-95.

Eicholtz, T., Ablas, J., van Overveld, M., Moolenaar, W. and Poegh, H. (1990) **FEBS Lett**. 261, 147-150.

Elliott, L.H., Wilkinson, S.E., Sedgwick, A.D., Hill, C.H., Lawton, G., Davis, P.D. and Nixon, J.S. (1990) **Biochem. Biophys. Res. Commun.** 171, 148-154.

Emorine, L.J., Marullo, S., Briend-Sutren, M.M., Patey, G., Tate, K.M., Delavier-Klutchko, C. and Strosberg, A.K. (1989) **Science** 245, 1118-1121.

Engels, P., Fichtel, K. and Lubbert, H. (1994) **FEBS Letts.** 350, 291-295.

Engels, P., Abdel'Al. S., Hulley, P. and Lubbert, H. (1995) **J. Neurosci. Res.** 41, 169-178.

Erneux, C., Moit, F., Boeynaems, J. and Dumont, J.E.(1982) **FEBS Lett.** 142, 251-254.

Erneux, C., Moit, F., Van Haastert, P.J.M. and Jastorff, B.(1985) **J. Cyclic Nucleotide Prot. Phosphor. Res**. 10, 463-470.

Farese, R.V., Standaert, M.L., Arnold, T., Yu, B., Ishizuka, T., Hoffma, J., Vila, M. and Cooper, D.R. (1992) **Cell. Signalling**. 4, 133-144.

Federman, A.D., Conklin, B.R., Schrader, K.A., Reed, R.R., and Bourne, H.R. (1992) **Nature** 356, 159-161.

Feinsten, P.G., Schrader, K.A., Bakalyar, H.A., Tang, W.J., Krupinski, J., Gilman, A.G., and Reed, R.R. (1991) **Proc. Natl. Acad. Sci. U.S.A.** 88, 10173-10177.

Finkelstein, R.A. (1973) **Crit. Rev. Microbiol.** 2, 553-623.

Fishman, P.H. (1990) in text book **"ADP-ribosylation Toxins and G proteins"** pp.127-140, American Society for Microbiology, Washington, D.C.

Florio, V.A. and Sternweis, P.C. (1989) **J. Biol. Chem.** 264, 3909-3915.

Foulkes, N.S., Sassone-Corsi, P. (1992) **Cell** 68, 411-414.

Francis, S.H., Colbran, J.L., McAllister-Lucas, M.l. and Corbin, J.D. (1994) **J. Biol. Chem.** 269, 77-80.

Freissmuth, M., Casey, P.J. and Gilman, A.G. (1989) **FASEB J.** 3, 2125-2131.

Frielle, T., Collins, S., Daniel, K.W, Caron, M.G., Lefdowitz, R.J. and Kobilka, B.K. (1987) **Proc. Natl. Acad. Sci. USA** 84, 7920-7924.

Furuyama, T., Inagaki, S., and Tagaki, H. (1993). **Brain Res.** 19, 165-170.

Fournier, A.M., Gadia, M.T., Kubrusly, D.B., Skyler, J.S. and Sosenki, J.M. (1986) **Am. J. Medicine** 80 (5), 861-864.

Fournier, A.and Murray, A.W.(1987) **Nature** 330, 767-769.

Gao, B., and Gilman, A.G. (1991) **Proc. Natl. Acad. Sci. U.S.A**. 88, 10178-10182.

Gawler, D., Milligan, G., Spiegel, A.M., Unson, C.G. and Houslay, M.D. (1987) **Nature** 327, 229-232.

Gawler, D., Milligan, G., Spiegel, A,M., Unson, C.G. and Houslay, M.D. (1990) **Mol. Pharmacol. Cell Regul**. 1, 197-230.

Genaro, A.M., Hortelano, S. Alverez, A. and Martinez, A.C. (1995) **J. Clin. Invest.** 95, 1884-1890.

Gerich, J.E. (1984) **J. Lab Clin. Medicine** 103 (4), 497-505.

Gerich, J.E. (1990) Adv. in **Second Messenger and protein Phosphoprotein Research** 24, 507-510.

Gettys, T.W., Blackmore, P.F., Redmon, J.B., Beebe, S.J., and Corbin, J.D. (1987) **J. Biol. Chem.** 262, 10353-10358.

Gierschik, P & Jakobs, H.K (1990) Wiley Series. **Mol. Pharmacol. Cell. Regul.** vol 1, pp 67-82.

Gill, D.M. (1976) **J. Infectous. Diseases.** 133, S55-S63.

Gillespie, P.G. and Beavo, J.A.(1989) **Mol. Pharmacol.** 36, 773-781.

Gilman, A.G. (1984) **Cell** 36, 577-579.

Gilman, A.G (1987) **Annu. Rev. Biochem.** 56, 615-649.

Glantz, S.B., Li, Y., Rubin, C.S. (1993) **J. Biol. Chem**. 268, 12796-12804.

Glynn , B.P., Colliton, J.W., McDermoot, J. M. and Witters, L.A. (1986) **Biochem. Biophys. Res.**135, 1119-1125.

Goldsmith, P., Backlund, P.S., Jr., Rossiter, K., Carker, A., Milligan, G., Unson, C.G. and Spiegel, A. (1988) **Biochemistry** 227, 7085-7090.

Gordge, P.C & Ryves, W.J. (1994) **Cell. Signalling**. 6, 871-882.

Graziano, M.P., Casey, P.J. and Gilman, A.G. (1987) **J. Biol. Chem.** 262, 11375-11381.

Graves, D.B. and McDonald, J.M. (1985) **J. Biol. Chem**. 260, 11286-11292.

Greene, D.A., Lattimer, S.A., Sima, A.A.F. (1987) **New England Journal of Medicine** 316, 599-606.

Green, A. and Johnson, J.L.(1989) **J. Biol. Chem.** 258, 607-610.

Green, A. and Johnson, J.L. (1991) **Diabetes** 40, 88-94.

Griffiths, S.L., Knowler, J.T. and Houslay, M.D. (1990) **Eur. J. Biochem**. 193, 367-374.

Gschwendt, M., Kittstein, W. and Marks, F. (1991) **Trends Biochem. Sci.** 16, 167-169

Haga, K. and Haga, T. (1990) **FEBS Lett.** 268, 43-47.

Haga, K. and Haga, T. (1992) **J. Biol. Chem.** 267, 2222-2227.

Hagawara, M., Alberts, A., Brindle, P., Meindoth, J., Feramisco., J. Deng, T., Karin, M., Shenolikar, S., Montminy, M.R. (1992) **Cell** 20, 105-113.

Hai, T. and Curran, T. (1991) **Pro. Natl. Acad. Sci. U.S.A.** (1991) 88, 3720-3724.

Helenda, S.P., Violpi, M., Zavoica, G.B., Sha'afi, RI. and Feinstein, M.B. (1986) **FEBS Lett** 204, 341-346.

Hall, I.P. and Hill, S.J. (1992) **Biochem. Pharmacol.** 43, 15-17.

Halter. J.B., Graf, R.J. and Porte, D.Jr. (1979) **J. Clin. Endocrin. Metabolism** 48 (6), 946-954.

Hannun, Y.A. and Bell, R.M. (1987) **Science** 235, 670-674.

Hardie, T. (1988) **Nature** 335, 592-593.

Haystead, T.J., Sim, A.T.R., Carling, D., Honnor, R.C., Tsukitani, Y., Cohen, P. and Hardie, D.G. (1989) **Nature** 337, 78-81.

Hausdorff, W.P., Bouvier, M., O'Dowd, B.F., Irons, G.P., Caron, M.G., and Lefkowitz, R.J. (1989) **J. Biol. Chem.** 264, 12657-12663.

Hausken, Z.E., Coghlan, V.M., Schafer-Hastings, C.A., Reimann, E.M., Scott, J.D. (1994) **J. Biol. Chem.** 269, 24245-24251.

Helluvo, K., Yoshimura, M., Kan, M., Hoffman, P.L., Cooper, D.M.F., and Tabakoff, B. (1993) **Biochem. Biophys. Res. Comm.** 192, 311-318.

Henkel-Tigges, J. and Davis, R.L. (1989) **Mol. Pharmacol.** 37, 7-10.

Henkel-Taiggs, J. and Davis, R.L. (1990) **Mol. Pharmacol.** 37, 7-10.

Hepler, J.R. and Gilman, A.G. (1992) **Trends Biochem. Sci.** 17, 383-387.

Herberg, L. and Coleman, D.L. (1977) **Metabolism** 26, 59-99.

Herbert, J.M., Augereau, J.M., Gleye, J. and Maffrand, J.P. (1990) **Biochem. Biophys. Res. Commun.** 172, 993-999.

Heyworth, C.M. & Houslay, M.D. (1983) **Biochem. J.** 214, 93-98.

Heyworth, C.M., Wallace, A.V. and Houslay, M.D. (1983a) **Biochem. J.** 214, 99-110.

Heyworth, C.M., Rawal, S., and Houslay, M.D. (1983b) **FEBS Lett.** 154, 87-91.

Heyworth, C.M., Hanski E. and Houslay, M.D. (1984) **Biochem. J.** 222, 189-194

Heyworth, C.M., Whetton, A.D., Kinsella, A.R. & Houslay, M.D. (1984) **FEBS Lett.** 170, 38-42.

Hidaka, H., Inagaki, M., Kawamoto, S. and Sasaki, Y. (1984) **Biochemistry** 23, 5036-5041.

Hildebrandt, F.D., Sekura, R.D., Codina, J., Iyengar, R., Manclark, C.R. and Birnbaumer, L. (1983) **Nature** 302, 706-709.

Hod, Y., Cook, J.S., Weldon, S.L., Short, T.M., Wynshaw-Boris, A. and Hanson, R.W. (1986) **Annals New York Academy of Science** 478, 31-45.

Horton Y., Sulivan, M. and Houslay, M.D. (1995) **Biochem. J.** 308, 683-691.

House, C. and Kemp, B.E. (1987) **Science** 238, 1726-1728.

Houslay, M.D., Metcalfe, J.C., Warren, G.B., Hesketh, T.R. and Smith, G.A. (1976) **Biochim. Biophys. Acta** 436, 489-494.

Houslay, M.D. & Elliott, K.R.F. (1979) **FEBS Lett.** 104, 359-363.

Houslay, M.D. & Elliott, K.R.F. (1981) **FEBS Lett.** 128, 289-292.

Houslay, M.D. (1989) **Trends Endocrinol. Metab**. 1, 83-89.

Houslay, M.D. (1990) **Cell. Signalling.** 2, 85-98.

Houslay, M.D. and Kilgour, E. (1990) **Molecular Pharmacology of Cell Regulation** 2, 185-224

Houslay, M.D., Gawler, D.J., Milligan, G. and Wilson, A. (1989) **Cell Signalling** 1, 9-22.

Houslay, M.D. (1991a) **Cell Signalling** 3, 1-9.

Houslay, M.D. (1991b) **Eur. J. Biochem.** 195, 9-27.

Houslay, M.D. (1994) Wiley Seres. **Mol. Pharmacol. Cell Regul.** vol. 3, 129-168.

Houslay, M.D., Scotland, G., Pooley, L., Spence, S., Wilkinson, I., McCallum, F., Julien, P., Rena, N.G., Michie, A.M., Erdogan, S., Zeng, L., O'Connell, J.C., Tobias, E.S. and MacPhee, I. (1995) **Biochem. Soc. Trans.** 23, 393-398.

Huang, K.P., Nakabayashi, H. and Huang, F.L. (1986A) **Proc. Natl. Acad. Sci. U.S.A.** 83, 8535-8539.

Huang, K.P., Chan, K.F.J., Singh, T.J., Nakabayashi, H. and Huang, F.L. (1986b) **J. Biol. Chem**. 261, 12134-12140.

Hug, H. and Sarre, T.F. (1993) **Biochem. J**. 291, 329-343.

Illiano, G. and Cuatrecasas, P. (1972) **Science** 175, 906-908.

Inoue, M, Kishimoto, A, Takai, Y. and Nishizuka, Y. (1977) **J. Biol. Chem.** 252, 7610-7616.

Irvine, F.J. & Houslay, M.D. (1988) **Biochem. J.** 251, 447-452.

Ishikawa, I., Katsudhika, S., Chen, L., Halnon, N.J., Kawabe, J.I., and Homoy, C.J. (1992) **J. Biol. Chem**. 267, 13553-13557.

Ivins, K.J. and Molinoff, P.B. (1991) **J. Pharmacol. Exp. Ther.** 259, 423-429.

Iyengar, R. (1993) **FASEB J.**7, 768-775.

Jacobowitz, O., Chen, J., Premont, R.T & Iyengar, R (1993) **J. Biol. Chem.** 268, 3829-3832.

Jacobitz, S., McLaughlin, M.M., Livi, G.P., Ryan, M.D., and Torphy, T.J. (1994) **FASEB J.** 8, A371.

Jelinek, L.J., Lok,S., Rosenberh, G.B., Smith, R.A., Grant, F.J., Biggs, S., Bensch, P.A., Kuijper, F.L., Sheppard, P.O., Sprecher, C.A., O'Hara, P.J., Foster, D., Walker, K.M., Chen, L.H.J., McKernan, P.A. and Kindsvogel, W. (1993) **Science** 259 1614-1616.

Janicot, M., and Desbuquois, B. (1987) **Eur. J. Biochem.** 163, 433-442.

Janicot, M., Clot, J.P., and Desbuqouis, B. (1988) **Biochem. J.** 253, 735-743.

Janicot, M., Fouque, F. and Desbuquois, B. (1991) **J. Biol. Chem.** 266, 12858-12865.

Jin, S.-L.C., Swinnen, J.V. and Conti, M. (1992) **J. Biol. Chem.** 267, 18929-18939.

Johnson, M.E.M., Das, N.M., Butcher, F.R & Fain, J.N (1972) **J. Biol. Chem.** 247, 3229-3235.

Johnson, G.L., Kaslow, H.R. and Bourne, H.R.(1978) **Proc. Natl. Acad. Sci. U.S.A.** 75, 3113-3117.

Johnson, M.L. and Veneziale, C.M. (1980) **Biochemistry** 19, 2191-2195.

Kahn, R.A. and Gilman, A.G. (1984) **J. Biol. Chem.** 259, 6228-6234.

Kahn, R.A. and Gilman, A.G. (1986) **J. Biol. Chem.** 261, 7906-7911.

Kaibuchi, K., Miyajima, A., Arai, K. and Matsumoto, K. (1989) **Proc. Natl. Acad. Sci. U.S.A.** 83, 8172-8176.

Kassis, S., Hagmann, J., Fishman, P.H., Chang, P.P., and Moss, J. (1982) **J. Biol. Chem.** 257, 12148-12152.

Katada, T., Kusakabe, K., Oinuma, M., and Ui, M. (1987) **J. Biol. Chem.** 262, 11897-11900.

Katz, A., Wu, D., and Simon, M.I. (1992) **Nature** 360, 686-689.

Kawabe, J., Iwami, G., Ebina, T., Ohno, S., Katada, T., Ueda, Y., Homcy, C.J. & Ishikawa, Y. (1994) **J. Biol. Chem**. 269, 16554-16558.

Kaziro, Y (1990) in **Mol. Pharmacol. Cell. Regul.** vol 1, pp 47-66.

Kaziro, Y., Itoh, H., Kozasa, T., Nafafuku, M., Satoh, T. (1991) **Ann. Rev. Biochem.** 60, 349-400.

Kelleher, D.J., Pessin, J.E., Ruoho, R.E. and Johnson, G.L. (1984) **Proc. Natl. Acad. Sci. U.S.A.** 81, 4316-4320.

Kemp, B.E. and Pearson, R.B. (1991) **Biochim. Biophys. Acta** 1094, 67-76.

Kerger, G., Rios, R.M., Landmark, B.F., Skalbegg, B., Lohmann, S.M., Borneus, M (1993) **Expt. Cell Res**. 204, 230-240.

Kilgour, E, Anderson, N.G., and Houslay, M.D. (1989) **Biochem. J.** 260, 27-36.

Kincaid, R.L., Smith-Coleman, I.E., Vaughaan, M. (1985) **J. Biol. Chem.** 260, 9009-9015.

Kishihara, K., Penninger, J., Wallace. W.A., Kundig, T.M., Kawai, K., Wakeham, A., Timms, E., Pfeffer, K., Ohashi, P.S., Thomas, T.L., Furlonger, C., Paige, C.J., and Mak, T.W. (1993) **Cell** 74, 143-156.

Kishimoto, A., Mikawa, K., Hashimoto, K., Yasuda, I., Tanaka, S.I., Tominaga, M., Kuroda, T. and Nishizuka, Y. (1989) **J. Biol. Chem**. 264, 4088-4092.

Klip, A., Ramlal, T., Douen, A.G., Bilan, P.J. and Shorecki, K.L. (1988) **Biochem. J.** 255, 1023-1029.

Kochs, G., Hummel., R., Fiebich., B., Sarre, T.F., Marme, D. and Hug, H. (1993) **Biochem. J.** 291, 627-633.

Kolterman, O.G., Gray, R.S., Griffin, J., Burstein, P., Insel, J., Scarlett, J.A. and Olefsky, J.M. (1981) **J. Clin. Invest.** 68 (4), 957-969.

Koshio, O., Akanuma, Y. and Kasuga, M. (1989) **FEBS Lett.** 254, 22-24.

Kobayashi, E., Nakano, H., Morimoto, M. and Tamaoki, T. (1989) **Biochem. Biophys. Res. Commun.** 159, 548-553.

Kovala, T., Lorimer, I.A.J., Brickenden, A.M., Ball, E.H. and Sanwall, B.D. (1994) **J. Biol. Chem.** 269, 8680-8685.

Krebs, E.G., Beavo, J.A. (1979) **Ann. Rev. Biochem.** 48, 923-959.

Krupinski, J., Rajaram, R., Lakonishok, M., Benovic, J.L. and Cerione, R.A. (1988) **J. Biol. Chem.** 263, 12333-12341.

Krupinski, J., Coussen, F., Bakalyar, H.A., Tang, W.J., Feinstein, P.G., Orth, K., Slaughter, C., Reed, R.R. & Gilman, A.G (1989) **Science** 244, 1558-1564.

Krupinski, J., Lehman, T.C., Frankenfield, C.D., Zwaagstra, J.C. & Watson, P.A. (1992) **J. Biol. Chem.** 267, 24858-24862.

Kuhn, H. (1980) **Nature** 283, 587-589.

Kwatra, M.M., Benovic, J.L., Caron, M.G., Lefkowitz, R.J. and Hosey, M.M. (1989) **Biochemistry** 28, 4543-4547.

Laemmli, U.K. (1970) **Nature** 227, 680-685.

Lalli, E., and Sassone-Corsi, P. (1994) **J. Biol. Chem.** 269, 17359-17362.

Landschulz, W.H., Johnson, P.F., McKnight, S.L., (1988) **Science** 240, 1759-1764.

Lavan, B., Lakey, T. and Houslay, M.D. (1989) **Biochem. Pharmacol.** 38, 4123-4136.

Lee, M.H. and Bell, R.M. (1991) **Biochemistry** 30, 1041-1049.

Lee, T.S., Saltsman ,K.A., Ohashi, H. and King, G.L. (1989) **Proc. Natl. Acad. Sci. U.S.A.** 86, 5141-5145.

Lerner, R.A., Green, N. and Alexander, H. (1981) **Pro. Natl. Acad. Sci. U.S.A.** 78, 3403-3407

Levilliers, J., Pairult, J., Lecost, F., Tournenolle, A. and Levdat, M.H. (1978) **Eur. J. Biochem**. 88, 323-330.

Levin, L.R., Han, P.L., Hwang, P.M., Feinstein, P.G., Davis, R.L., and Reed, R.R. (1992) **Cell** 68, 479-489.

Lewis, R.E., Cao, L.K., Perregaux, D. and Czech, M.P. (1989) **Biochemistry** 29, 1807-1813.

Liggett, S.B., Ostrowski, J., Chesnut, L.C., Kurose, H., Raymond, J.R., Caron, M.G. and Lefkowitz, R.J. (1992) **J. Bol. Chem.** 267, 4740-4746.

Lindner, E., Dohadwalla, A.N. and Bhattacharya, B.K. (1978) **Arzneimittle-Forschung** 28, 284-289.

Lindquist, S. and Craig, E.A.(1988) **Ann. Rev. Genetics** 22, 631-677.

Livingstone, C., McLellan, A.R., McGregor, M.A., Wilson, A., Connell, M.F.C., Small, M., Milligan, G., Paterson, K.R. and Houslay, M.D. (1991) **Biochim. Biophy. Acta** 1096, 127-133.

Livingstone, C., MacDonald, C., Willett, B. & Houslay, M.D. (1994) **Biochem. J.** 300, 835–842.

Lobban, M., Shakur, Y., Beattie, J. and Houslay, M.D. (1994) **Biochem. J.** 304, 399-406.

Lohse, M.J., Benovic, J.L., Caron, M.G. and Lefkowitz, R.J. (1990) **J. Biol. Chem.** 265, 3203-3209.

Lohse, M.J, (1993) **Biochim. Biophys. Acta**, 1179, 171-188.

Luten, E.G. and Sneyd, J.G.T. (1970) **Biochem. J.** 120, 187-193.

Lustig, K.D., Conklin, B.R., Herzmark, P., Taussig, R., and Bourne, H.R. (1993) **J. Biol. Chem**. 268, 13900-13905.

Lynch, C.J., Blackmore, P.F., Johnson, E.H., Wange, R.L., Krone, P.K. and Exton, J.H. (1989) **J. Clin. Inves**. 83, 2050-2062.

Macaulay, S.L., Kiechle, F.L. and Jarett, L. (1983) **Arch. Biochem. Biophys**. 225, 130-136.

MacDonald, C., Watts, P., Stuart, B., Kreuzburg-Duffy, U., Scott, D.M. and Kinne, R.K.H. (1991). **Expt. Cell Res** 195, 458-461.

Madelian, V., and La Vigne, E. (1991) **Am. Soc. Neurochem.** p22.

Manaco, I. Vicini, E. and Conti, M. (1994) **J. Biol. Chem.** 269, 347-357.

Manganiello, V.C., Tanaka, T. and Murashima, S. (1990a) **Mol. Pharmacol. Cell Regulation** 2, 61-85.

Manganiello, V.C., Smith, C.J., Degerman, E. and Belfrage, P. (1990b) **Molecular Pharmacology of Cell Regulation** vol. 2, 87-116.

Manning.C.D., McLaughln, M.M., Livi, G.P., Cieslinski, L.B., Torphy, T.J., and Barnette, M.S. (1996) **J. Pharmacol. Experimental Therapeutics** 276, 810-818.

Marais, R.M. and Parker, P.J. (1989) **Eur. J. Biochem.** 182, 129-137.

Marchmont, R.J. and Houslay, M.D. (1980) **Biochem. J.** 187, 381-392.

Marchmont, R.J., Ayad, S.R., and Houslay, M.D. (1981) **Biochem. J.** 195, 645-652.

Marcoz, P., Nemoz, G., Prigent, A.F. and Lagarde, M. (1993) **Biochim. Biophys. Acta** 1176, 129-136.

Martins, T.J., Mumby, M.C., Beavo, J.A. (1982) **J. Biol. Chem.** 257, 1973-1979.

Mattera, R., Codina, J., Sekura, R.D. and Birnbaumer, L. (1986) **J. Biol. Chem**. 261, 11173-11179.

Mattera, R., Yatani, A., Kirsch, G.E., Graf, R., Okabe, K., Olate, J., Codina, J., Brown, A.M. and Birnbaumer, L. (1989) **J. Biol. Chem.** 262, 2131-2137.

Mauger, J.P., Poggioli, J. and Claret, M. (1985) **J. Biol. Chem.** 260, 11635-11642.

Mayer, J.B. and Baltimore, D. (1993) **Trends Cell Biol.** 3, 8-13.

McCartney, S., Little, B.M., Langeberg, L.E., Scott, J.D. (1995) **J. Biol. Chem.** 270, 1-7.

McClue, S.J., Selzer, E., Freissmuth, M. and Milligan, G. (1992) **Biochem. J.** 284, 565-568.

McFadzean, J., Mullaney, I., Brown, D.A. and Milligan, G. (1989) **Neuron** 3, 177-182.

McGlynn, E., Liebetanz, J., Reutener, S., Wood, J., Lydon, N.B., Hofstetter, H., Vanek, M., Meyer, T and Fabbro, K. (1992) **J. Cell. Biochem.** 49, 239-250.

McKenzie, F.R. and Milligan, G. (1990) **J. Biol. Chem.** 265, 17084-17093.

McLaughlin, M.M., Cieslinski, L.B., Burman, M., Torphy, T.J. and Livi, G.P. (1993), **J. Biol. Chem.** 268, 6470-6476.

McPhee, I., Pooley, L., Lobban, M., Bolger, G., and Houslay, M.D. (1995) **Biochem. J.** 310, 965-974.

Meek, D.W. and Street, A.J. (1992) **Biochem. J.** 287, 1-15.

Meldrum, E., Parker, P.J. and Carozzi, A. (1991) **Biochim. Biophys. Acta** 1092, 49-71.

Melloni, E., Pontremoli, S., Michetti, M., Sacco, O., Saratore, B. and Hrecker, B.L. (1986) **J. Biol. Chem**.261, 4101-4105.

Meyer, T.E., Habener, J.F. (1993) **Endocr. Rev.** 14, 269-290.

Michaeli, T., Bloom, T.J., Martins,T. *et al.*, (1993) **J. Biol. Chem.** 268, 12925-12932.

Michie, A., Harnett, M. and Houslay, M.D. (1996) **Cell Signalling** 8, 97-110.

Milatovich, A., Bolger, G., Michaeli, T. and Francke, U. (1994) **Somatic Cell and Molecular Genetics** 20, 75-86.

Miller, S.G. and Kennedy, M.B. (1986) **Cell** 44, 861-870.

Milligan, G (1990) **Cell Signalling** 1, 411-419.

Milligan, G. and Green, A. (1991) **Trends Pharmacol. Sci.** 12, 207-209.

Milligan, G. (1995) **Adv. Pharmacol.** 32, 1-29.

Milligan, G. (1996) **Cell Signalling** 8, 87-96.

Mine, T., Kojime, I. and Ogata, E. (1988) **Biochim. Biophys. Acta** 970, 166-171.

Molina, C.A., Foulkes, N.S., Lalli, E., Sassone-Corsi, P. (1993) **Cell** 75, 875-886.

Monaco, L., Vicini, E. and Conti, M. (1994) **J. Biol. Chem.** 269, 347-357.

Montminy, M.R., Sevarino, K.A., Wagner, J.A., Mandel, G., and Goodman, R.H. (1986) **Proc. Natl. Acad. Sci. U.S.A.** 83, 6682-6686.

Moore, J.B., Combs, D.W., Tobia, A.J. (1991) **Biochem. Pharmacol.** 42, 679-683.

Morgan, N. (1989) in **Cell Signalling** ch 3&4, 35-90. Open University Press, Milton Keynes.

Morris, N.J., Bushfield, M., Lavan, B.E. and Houslay, M.D. (1994) **Biochem. J.** 301, 693-702.

Moxham, C.M., Hod, Y. and Malbon, C.C. (1993) **Science** 260, 991-995.

Moxham, C.M., Hod, Y. and Malbon, C.C. (1993) **Devl. Gene.** 14, 266-273.

Moxham, C.M. and Malbon, C.C. (1996) **Nature** 379, 840-844.

Mumby, S.M., Kahn, R.A., Manning, D.R. and Gilman, A.G. (1986) **Proc. Natl. Acad. Sci. U.S.A.** 83, 265-269.

Munnich, A., Marie, J., Reach, G., Vaulont, S., Simon, M.P. and Kahn, A. (1984) **J. Biol. Chem.** 259, 10228-10231.

Muramatsu, M.A., Kaibuchi, K. and Arai, K.I. (1989) **Mol. Cell. Biol.** 9, 831-836.

Murphy, G.J., Hruby, V.J., Trivedi, D., Wakelam, M.J.O. and Houslay, M.D. (1987) **Biochem. J.** 243, 39-46.

Murphy, G. & Houslay, M.D. (1988) **Biochem. J.** 249, 543-547.

Murphy, G.J., Gawler, G.J., Milligan, G., Wakelam, M.J.O., Pyne, N.J. and Houslay, M.D. (1989) **Biochem. J.** 259, 191-197.

Musacchio, A., Gibson, T., Lehto, V.P. and Saraste, J. (1992) **FEBS Lett.** 307, 55-61.

Nagshineh, S., Nobuchi, M., Huang, K.P. and Londos, C (1986) **J. Biol. Chem.** 261, 14534-14538.

Nairn, A., Willett, B., Brant, M.H., Scott, A. and MacDonald, C. (1990) **Biochem. Soc. Trans.** 18, 1201-1202.

Narindrasorassak, S., Tan, L.U., Seth, P.K., and Sanwal, B.D. (1982) **J. Biol. Chem.** 257, 4618-4626.

Navon, S.E. and Fung, B.K.-K. (1987) **J. Biol. Chem.** 262, 15746-15751.

Némoz, G., Moueqqit, M., Pringent, A.F. and Pacheco, H. (1989) **Eur. J. Biochem.** 184, 511-520.

Némoz, G., Zhang, R.B., Sette, C. and Conti, M. (1996) **FEBS Lett.** 384, 97-102.

Newlands, C. and Houslay, M.D. (1991) **Biochem. J.** 277, 371-378.

Nicholson, C.D., Challis, R.A.J. and Shahid, M. (1991) **Trends Pharmacol. Sci.** 12, 19-27.

Nigg, E.A., Schafer, G., Hilz, G., Eppenberger, H. (1985) **Cell** 41, 1039-1051.

Nigg, E.A. (1990) **Adv. Cancer Res.** 55, 271-310.

Niino, Y.S., Ohno, S. and Suzuki, K. (1992) **J. Biol. Chem.** 267, 6158-6163.

Nishizuka, Y. (1986) **Science**, 233, 305-312.

Nishizuka, Y. (1988) **Nature** 334, 661-665.

Nishizuka, Y (1989) **Cancer** 63, 1892-1903.

Nishizuka, Y. (1992) **Science** 258, 607-614.

Nixon, J.S., Bishop, J., Bradshaw, D., Davis, P.D., Hill, C.H., Elliott, L.H., Kumar, H., Lawton, G., Lewis, E.J., Mulqueen, M., Westmacott, D., Wadsworth, J & Wilkinson, S.E (1992) **Biochem. Soc Trans.** 20, 419-434.

Northup, J.K., Sternweis, P.C., Smigel, M.D., Schleifer, L.S., Ross, E.M. and Gilman, A.G. (1980) **Proc. Natl. Acad. Sci. U.S.A.** 77, 6516-6520.

Northup, J.K. (1985) **Mol. Aspects Cell. Regul.** 4, 91-116.

Obernolte, R., Ratzliff, J., Baecker, P.A., Zuppan, P., Jarnagin, K and Shelton, E.R. (1996) (inpress)

O'Brien, R., Houslay, M.D., Milligan, G. and Siddle, K. (1987) **FEBS. Lett.** 212, 281-288.

O'Connell, J.C., McCallum, J.F., McPhee, I., Wakefield, J., Houslay, E.S., Wishart, W., Bolger, G., Frame, M. and Houslay, M.D. (1996) **Biochem. J.** (in press)

Ohno, S., Kawasaki, H., Imajoh, S., Suzuki, K., Inagaki, M., Yokokura, H., Sakoh, T. and Hidaka, H. (1987) Nature 325, 161-166.

Ohno, S., Konno, Y., Akita, Y., Yano, A. and Suzuki, K. (1990) J. Biol. Chem. 265, 6296-6300.

Olefsky, J.M., Koletrman, O.G.and Scarlett, J.A. (1982) Am. J. Physiol. 243, 1, E15-30.

Olivier, A.R. and Parker, P.J. (1991) Eur. J. Biochem. 200, 805-810.

Ono, Y., Kurokawa, T., Fujii, T., Kurokawa, K., Igarashi, K., Kikkawa, U., Ogita, K. and Nishizuka, Y. (1986) FEBS Lett. 206, 347-352.

Orlandi, P.A., Curran, P.K., Fishman, P.H. (1993) J. Biol. Chem. 268, 12010-12016.

Osada, H., Takahashi, H., Tsunoda, K., Kusakabe, H. and Isono, K. (1990a) J. Antibiotics 43, 163-167.

Osada, S., Mizuno, K., Saido, T.C., Akita, Y., Suzuki, K., Kuroki, T. and Ohno, S. (1990) J. Biol. Chem. 265, 22434-22440.

Osada, S., Mizuno, K., Saido, T.C., Akita, Y., Suzuki, K., Kuroki, T. and Ohno, S. (1992) Mol. Cell. Biol. 12, 3930-3938.

Pan, T. and Coleman, J.E. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 2077-2081.

Parker, P.J., Kour, G., Marais, R.M., Mitchell, F., Pears, C., Schaap, D., Stabel, S., and Webster, C. (1989) Mol. Cell. Endocrinol. 65, 1-11.

Parker, P.J., Cook, P.P., Olivier, A.R., Pears, C., Ways, D.K. & Webster, C. (1992) Biochem Soc Trans 20, 415-418.

Pawson, T and Gish, G.D. (1992) Cell 71, 359-362.

Peachell, P.T., Undem, B. J., Schleimer, R. P., MacGlashan Jr, D. W., Leichtenstein, L. M., Cieslinski, L. B. and Torphy, T. J. (1992) J. Immunol. 148, 2503 - 2510

Pears, C., Stabel, S., Cazaubon, S. and Parker, P.J. (1992) Biochem J. 283, 515-51.

Pilkis, S.J. (1970) Biochim. Biophys. Acta 461, 215-226.

Pilkis, S.J., Exton, J.H., Johnson, R.A. and Park, C.R. (1974) Biochim Biophys. Acta 343, 250-267.

Pinto, I.L., Buckle, D.R., Readshaw, S.A., and Smith, D.G. (1993) Bioorg. Medicinal. Chem. Lett. 8, 1743-1746.

Pitcher, J.A., Inglese, J., Higgins, J.B., Arriza, J.L., Casey, P.J., Kim, C., Benovic, J.L., Kwatra, M.M., Caron, M.G. and Lefkowitz, R.J. (1992) Science 257, 1264-1267.

Pitt, G.S., Milona, M., Borleis, J., Lin, K.C., Reed, R.R.,and Devreotes, P.N. (1992) Cell 69, 305-315.

Pittner, R.A. and Fain, J.N. (1991) Biochem. J. 277, 371-378.

Pobiner, B.F., Northup, J.K., Bauer, P.H., Fraser, E.D., Garrison, J.C. (1991) Mol. Pharmacol. 40, 156-167.

Podzuweit, T., Muller, A. and Nennstiel, P. (1992) J. Mol. Cardiol. 24, S102.

Premont, R.T., Jacobowitz, O., and Iyengar, R. (1992) Endocrinology 131, 2774-2783.

Pun, K.K., Ho, P.W., Nissenson, R.A. and Arnaud, C.D. (1990) J. Bone Min. Res. 5, 1193-1200.

Pyne, N.J., Cooper, M.E. and Houslay, M.D. (1986) Biochem. J. 234, 325-334.

Pyne, M.J., Murphy, G.J., Millgan, G. and Houslay, M.D. (1989a) FEBS Lett. 243, 77-82.

Pyne, N.J., Heyworth, C.M., Balfour, N.W. and Houslay, M.D. (1989b) Biochem. Biophys. Res. Commun. 165, 251-256.

Pyne, N.J., Cushley, W., Nimmo, H.G., Housley, M.D. (1989c) Biochem. J. 261, 897-904.

Pyne, N.J., Moughal, N., Stevens, P.A., Tolan, D. and Pyne, S. (1994) Biochem. J. 304, 611-616.

Reaven, G.M. (1995) Physiol. Rev. 75, 473-486.

Reeves, M.L. and England, P.J. (1990) Mol. Pharm. Cell Regul. 2, 299-316.

Refnes, M., Johansen, E.J. and Christoffersen, T. (1989) Pharmacol. Toxicol. 64, 397-403.

Repaske, D.R., Swinnen, J.V., Jin, S.-L.C., Van Wyck, J.J. and Conti, M. 1992) J. Biol. Chem. 267, 18683-18688.

Revers, R.R., Fink, R., Griffin, J., Olefsky, J.M. and Kolterman, O.G. (1984) J. Clin. Invest. 73, 664-672.

Richardson, R.M. and Hosey, M.M. (1992) J. Biol. Chem. 267, 22249-22255.

Rimoin, D.L. and Rotter, J.I. (1981) Hormone and Metabolic Res. 7, 63-72.

Roesler, W.J., Vandenbark, G.R. and Hanson, R.W. (1988) J. Biol. Chem 263, 9063-9066.

Rogue, P., Labourdette, G., Masmoudi, A., Yoshida, Y., Huang, F.L., Huang, K.P., Zwiller, J., Vincendon, G. and Malviya, A.N. (1990) J. Biol. Chem. 265, 4161-4165.

Ross, E.M. and Gilman, A.G. (1977) J. Biol. Chem. 252, 6966-6969.

Rothenberg, P.L. and Kahn, C.R. (1988) J. Biol. Chem. 263, 15546-15552.

Rotter, J.I. and Rimoin, D.L. (1981) Am. J. Medicine 70, 116-126.

Rubin, C.S., Erlichman, J., Rosen, O.R. (1972) J. Biol. Chem. 247, 6135-6139.

Ruegg, U.T. and Burgess G.M. (1989) Trends Pharmacol Sci. 10, 218-220.

Rutten, W.J., Shoot, B.M. and DePont, J.J.H.M. (1973) Biochim. Biophys. Acta 315 378-383.

Ryves, W.J., Evans, A.T., Olivier, A.R., Parker, P.J. and Evans, F.J. (1991) FEBS Lett. 288, 5-9.

Salvatori, S., Damiani, E., Barhanin, J., Furlan, S., Giovanni, S., Margerth, A. (1990) Biochem. J. 267, 679-687.

Savage, A., Zeng, L & Houslay, M.D (1995) Biochem J. 307, 281-285.

Schaap, D. and Parker, P.J. (1990) J. Biol. Chem. 265, 7301-7307.

Schaap, D., Parker, P.J., Bristol, A., Kriz, R. and Knopf, J. (1989) FEBS Lett. 243, 351-357.

Schaap, D., Hsuan, J., Totty, N. and Parker, P.J. (1990) Eur. J. Biochem. 191, 431-435.

Schneider, H.H., Schmiechen, R., Brezenski, M. and Seidler, J. (1986) Eur. J. Pharmacol. 127, 105-115.

Scotland, G. and Houslay, M.D. (1995) Biochem. J. 308, 673-681.

Scott, J.D. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 5192-5196.

Scott, J.D. (1991) Pharmacol. Ther. 14, 1-24.

Scott, J.D., Carr, D.W. (1992) News Physiol. Sci. 7, 143-148.

Seamon, K.B., Padgett, W & Daley, J.W (1981) Proc. Natl. Acad. Sci. USA 78, 3363-3367.

Seamon, K.B. and Daly, J.W. (1983) Trends in Pharmacological Sci. 4, 120-123.

Seamon, K.B. and Daly, J.W. (1986) Adv. Second Messenger and Protein Phosphorylation Res. 20, 1-150.

Sette, C., Iona, S and Conti, M. (1994a) J. Biol. Chem. 269, 9245-9252.

Sette, C., Vicini, E. and Conti, M. (1994b) J. Biol. Chem. 269, 18271-18274.

Sette, C., Vicini, E. and Conti, M. (1994c) Mol. Cell. Endocrinol. 100, 75-79.

Sette, C. and Conti, M. (1995) FASEB J. 33, A1262.

Shakur, Y., Pryde, J.G., and Houslay, M.D. (1993) Biochem. J. 292, 677-686.

Shakur, Y., Wilson, M.A., Pooley, L., Lobban, M., Griffiths, S.L., Campbell, A.M., Beattie, J., Daly, C., and Houslay, M.D. (1995) Biochem J. 306, 801-809.

Shen, S.S. and Buck, W.R. (1990) Dev. Biol. 140, 272-280.

Simon, M.I., Strathmann, M.P. and Gautam, N. (1991) Science 252, 802-808.

Sistare, F.D., Picking, R.C., Haynes, R.C. (1985) J. Biol. Chem. 260, 12744-12747.

Sixma, T.K., Pronk, S.E., Kalk, K.H., Wartna, E.S., van Zanten, B.A.M., Witholt, B., and Hol, W.G.J. (1991) Nature 351, 371-377.

Skalhegg, B.S., Tasken, K., Hausson, V., Huitfeldt, H.S., Jahnsen, T., Lea, T. (1994) Science 263, 84-87.

Smigel, M.D. (1986) J. Biol. Chem. 261, 1976-1982.

Smith, C. J. and Manganiello, V. C. (1988) Mol. Pharmacol. 35, 381-386.

Smith, M.K., Colbran, R.J. and Soderling, T.R. (1990) J. Biol. Chem. 265, 1837-1840.

Smoake, J.A., Johnson, L.S. and Peake, B.T. (1981) Arch. Biochem. Biophys. 206, 331-335.

Smoake, J.A. and Solomon, S.S. (1989) Life Science. 45 (23), 2255-2268.

Smoake, J.A., Moy, G.M., Fang, B.-H. and Solomon, S.S. (1995) Arch. Biochem. Biophys. 323, 223-232.

Solomon, S.S. (1975) Endocrinology 96, 1366-1373.

Solomon, S.S. Silberberg, M., and Palazzo, M. (1979) Biochim. Biophys. Acta 760, 293-299.

Solomon, S.S., Palazzolo, M., McPherson, J.M., and Smoake, J.A. (1981) Diabetes 30, 372-376.

Solomon, S.S. Steiner, M.S., Sanders, L. and Palazzolo, M.R. (1986) Endocrinology 119, 1839-1844.

Solomon, S.S., Steiner M.S., Little, W.L., Rao, R.H., Sanders, L.L.and Palazzolo, M.R. (1987) Diabetes 36, 210-215.

Solomon, S.S., Sibley, S.D. and Cunningham, T.M. (1990) Endocrinology 127, 1544-1546.

Solomon, S.S., Palazzolo, M.R., Elam, M.B., Green, S. and Raghow, R. (1994) J. Lab. Clin. Med. 124, 348-358.

Solomon, S.S., Palazzolo, M.R., Smoake, J.A., and Raghow, R.S. (1995) Biochem. Biophy. Res. Commun. 210, 921-930.

Sonnenburg, W.K., Seger, D. and Beavo, J.A. (1993) J. Biol. Chem. 268, 645-652.

Spence, J.T. (1983) J. Biol. Chem. 258, 9143-9146.

Spence, S., Rena, G, Sweeney, G. and Houslay, M.D. (1995) Biochem. J. 310, 975-982

Spiegel, A.M., Backlund, P.S., Butyrinski, J.E., Jones, T.L.Z. and Simonds, W.F. (1991) Trends Biochemistry Sci. 16, 338-341.

Srikant, C.B., Freeman, D., McCorkle, K. and Unger, R.H. (1977) J. Biol. Chem. 252, 7434-7436.

Stanby, N.H. (1968) Microbiologica Et Immun. Scandinavica. 194, 152-164.

Stow, J.L., Almeida, J.B., Narula, N., Holtzman, E.J., Ercolani, L. and Ausielo, D.A. (1991) J. Cell Biol. 114, 1113-1124.

Strålfros, P., Björgell, P. and Belfrage, P. (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 3317-3321.

Strassheim, D., Milligan, G. and Houslay, M.D. (1990) Biochem. J. 266, 521-526.

Stroop, S.D., Charbonneau, H. and Beavo, J.A. (1989) J. Biol. Chem. 260, 9009-9015.

Stroop, S.D. Beavo, J.A. (1991) J. Biol. Chem. 266, 23802-23809.

Stroop, S.D. and Beavo, J.A. (1992) Adv. Second Messenger and Phosphoprotein Res. 25, 55-71.

Stryer, L., Hurley, J.B. and Fung, B.K. (1981) Curr. Top. Membr. Transp. 15, 93-108.

Stryer, L. (1986) Ann. Rev. Neurosci. 9, 87-119.

Sullivan, M., Egerton, M., Shakur, Y., Marquardsen, A. and Houslay, M.D. (1994) Cell Signalling 6, 793-812.

Swinnen, J.V., Joseph, D.R. and Conti, M. (1989a) Proc. Natl. Acad. Sci. U.S.A. 86, 5325-5329.

Swinnen, J.V., Joseph, D.R. and Conti, M. (1989b) Proc. Natl. Acad. Sci. U.S.A. 86, 8197-8201.

Swinnen, J.V., Tsikalas, K.E. and Conti, M. (1991) J. Biol. Chem. 266, 18370-18377.

Swislocki, A.L.M., Hoffman, B.B. and Reaven, G.M. (1989) Am. J. Hypertension 2, 419-423.

Tachibana, K., Scheur, P.J., Tsukitani, Y., Kikuchi, H., Van Enden, D., Clardy, J., Gopichand, Y. and Schmitz, F.J. (1981) J. Am Chem. Soc. 103, 2469-2471.

Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M. and Tomita, F. (1986) Biochem. Biophys. Res. Commun. 135, 397-402.

Takayama, S., White, M.F. and Kahn, C.R. (1988) J. Biol. Chem. 263, 3440-3447.

Takahashi, S., Swinnen, J.V., Van Wyk, J.J., and Conti, M. (1991) Endocrine Soc. 1045.

Tamborlane, W.V., Sherwin, R.S., Koivisto, B., Hendler, R., Genel, M. and Felig, P. (1979) Diabetes 28, 785-788.

Tang, E.Y. & Houslay, M.D. (1992) Biochem. J. 283, 341–346.

Tang, E.Y., Parker, P.J., Beattie, J., and Houslay, M.D. (1993) FEBS Lett. 326, 117-123.

Tang, W.J., and Gilman, A.G. (1991) Science 254, 1500-1503.

Tang, W.J., Krupinski, J., and Gilman, A.G. (1991) J. Biol. Chem. 266, 8595-8603.

Tang, W.G. and Gilman, A.G. (1992) Cell 70, 869-872.

Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T. and Nishizuka, Y. (1979) J. Biol. Chem. 254, 3692-3695.

Taussig, R., Quarmby, L.M., and Gilman, A.G. (1993) J. Biol. Chem. 268, 9-12.

Taussig, R., Iniguez-Lluhi, J.A., and Gilman, A.G. (1993) Science, 261, 218-221.

Taussig, R and Gilman, A.G. (1995) J. Biol. Chem. 270, 1-4.

Taylor, S.S. (1989) J. Biol. Chem. 264, 8443-8446.

Taylor, S.S., Buechler, J.A., Yonemoto, W. (1009) Ann. Rev. Biochem. 59, 971-1005.

Terasawa, H., Kohda, D., Hatanaka, H., Tsuchiya, S., Ogura, K., Nagata, K., Ishii, S., Mandiyan, V., Ullrich, A., Schlessinger, J. and Inagaki, F. (1994) Nature Structural Biology 1, 891-897.

Tewari, M, Tewari, D.S., Taub, R. (1991) Mol. Endocrinol. 5, 653-660.

Thissen, J.A., Casey, P.J. (1994) Hardb. Expt. Pharmacol. 108/II, 131-146.

Thomason, P.A., James, S.R., Casey, P.J. and Downes, C.P. (1994) J. Biol. Chem. 269, 16525-16528.

Thompson, W.J. and Appleman, M.M. (1971) Biochemistry 10, 31-316.

Tomasi, M., and Montecucco, C. (1981) J. Biol. Chem. 256, 11177-11181.

Torphy, T.J., Stadel, J.M., Burman, M., Cieslinski, .L. B., McLaughlin, M.M., White, J.R. and Livi, G.P. (1992) J. Biol. Chem. 267, 1798-1804.

Torphy, T.J., Zhou, H.L., Foley, J.J., Sarau, H.M., Manning, C.D. and Barnette, M.S. (1995) J. Biol. Chem. 270, 23598-23604.

Tsai, S. C., Noda, M., Adamik, R., Chang, P.P., Chen, H. C., Moss, J. and Vaughan, M. (1988) J. Biol. Chem. 263, 1768-1772.

Ui, M. (1984) Trends Pharmacol. Sci. 5, 277-279.

Unger, R.H. and Foster, D. (1992) Textbook of Endocrinology 8, 1255-1333.

Unson, C.G., Gurzenda, E.M. and Merrifield, R.B. (1989) Peptides 10, 1171-1177.

Vaartjes, W.J., deHaas, C.G.M. and Van denBergh, S.C. (1986) Biochem. Biophys. Res. Commun. 138, 1328-1333

Valette, L., Prigent, A.F., Nemoz, G., Anker, G., Macvschi, O., and Lagarde, M. (1990) Biochem. Biophys. Res. Commun. 169, 864-872.

van Heyningen, S. (1977) Biol. Rev. Cambridge Philos. Soc. 52, 509-549.

van Heyningen, S (1983) Curr. Top. Membr. Trans. 18, 445-471.

Vasta, V., Smith, C.J., Calvo, J., Belfrage, P. and Manganiello, V. C. (1992) Biochem. Biophys. Res. Commun. 183, 1070-1075.

Vaughan, M. and Moss, J. (1978) J. Supramol. Struct. 8, 473-488.

Verma, I.M., Sassone-Corsi, P. (1987) Cell 51, 513-514.

Vila, M., Cooper, D.R., Davis, J.S., Standaert, M.L., Farese R.V. (1989) FEBS Lett. 244, 177-180.

Verghese, M.W., McConnell., R.T., Lenhard, J.L., Hammacher, L. and Jin, S-L.C. (1995) Mol. Pharmacol. 47, 1164-1171.

Vicini, E., Sette, C., Conti, M. (1994) FASEB J. 2132, A 369.

Vorherr, T., Knopfel, L., Hofmann, F., Mollner, S., Pfeuffer, T., and Carafoli, E.(1993) Biochemistry 32, 6081-6088.

Wada, H., Ohno, S., Kubo, K., Taya, C., Tsuji, S., Yonehara, S. and Suzuki, K. (1989) Biochem. Biophys. Res. Commun. 165, 533-538.

Wadzinski, B.E., Wheat, W.H., Jaspers, S., Peruski, L.F., Lickteig, R.L, Johnson, G.J., Klemm, D.J. (1993) Mol. Cell. Biol. 13, 2822-2834.

Wallach, J., Droste, M., Kluxen, FF.W., Pfeuffer, T., and Frank, R. (1994) FEBS Lett. 338, 264-266.

Wakelam, M.J.O., Murphy, G.J., Hruby, V.J. and Houslay, M.D. (1986) Nature 323, 68-71.

Walseth, T.F., Zhang, H-J., Olson, L.K., Schroeder, W.K. and Robertson, R.P. (1989) J. Biol. Chem. 264, 21106-21111.

Weiss, R.M., Hardman, J.G. and Wells, J.N. (1981) Biochem. Pharmacol. 30, 2371-2374.

Whipps, D.E., Armstron, A.E., Pryor, H.J. & Halestrap, A.P. (1987) Biochem. J. 241, 835-845

White, M.R. & Kahn, M.F. (1994) J. Biol. Chem. 269, 1-4.

Wilden, U., Hall, S.W. and Kuhn, H. (1986) Proc. Natl. Acad. Sci. USA 83, 1174-1178.

Williams, K.A., Murphy, W. and Haslam, R.J. (1987) Biochem. J. 243, 667-678.

Williamson, J.R., Hansen, C.A., Verhoeven, A., Coll, K.E., Johanson, R., Williamson, M.T. & Filburn, C. (1986) in "Cell Calcium and the Control of Membrane Transport", ch7 pp93-116, Rockefeller Univ. Press, NY.

Wilson, M., Sullivan, M., Brown, N. and Houslay, M.D. (1994) Biochem. J. 304, 407-415.

Wisnieski, B.J., and Bramhall, J.S. (1981) Nature 289, 319-321.

Woodworth, C.D., Secott, T., and Isom, G.C. (1986). Cancer Research 46, 4018-4026.

Xia, A., Choi, E.J., Wang, F., Blazynski, C. and Storm, D.R. (1993) J. Neurochem. 60, 305-311.

Yamamoto, T., Manganiello, V.C. and Vaughan, M. (1983) J. Biol. Chem. 258, 12526-12533.

Yamamoto, K.K., Gonzales, G.A., Briggs, W.H., Montming, M.R. (1988) Nature 334, 494-498.

Yatani, A., Codina, J., Imoto, Y., Reeves, J.P., Birnbaumer, L. and Brown, A.M. (1987) Science 238, 1288-1292.

Yatomi, Y., Arata, Y., Tada, S., Kume, S. and Ui, M. (1992) Eur. J. Biochem. 205, 1003-1009.

Yoshida, Y., Huang, F.L., Nakabayashi, H. and Huang, K.P. (1988) J. Biol. Chem. 263, 9868-9873.

Yoshimasa, T., Sibley, D.R., Bouvier, M., Lefkowitz, R.J. and Caron, M.G. (1897) Nature 327, 67-70

Yoshimura, M, and Cooper, D.M.F. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 6716-6720.

Yoshimura, M. & Cooper, D.M.F. (1993) J. Biol Chem. 268, 4604-4607.

Young, S., Parker, P.J., Ullrich, A. and Stabel, S. (1987) Biochem.J. 244, 775-779.

Yuan, N.Y., Friedman, J., Whaley, B.S. and Clark, R.B. (1994) J.Biol. Chem. 269, 23032-23038.

Zeng, L. and Houslay, M.D. (1995) Biochem. J. 312, 769-774.

Appendix

A role for protein kinase C-mediated phosphorylation in eliciting glucagon desensitization in rat hepatocytes.

Introduction

Exposure of receptors in intact cells or tissues to agonists often leads to a rapid loss of receptor responsivensess. This agonist-induced process is called desensitization (Lohse *et al.*, 1990). The desensitization processes can be subdivided along several lines, most importantly according to the causative stimulus ('heterologous' or 'homologous' or receptor-specific); according to the time-frame ('rapid' within minutes or 'slow' within hours or days) and, finally, depending on the type of mechanism (loss of receptor signalling 'function'-called 'uncoupling' or loss of receptor 'number'-called 'down-regulation') (Lohse, 1993). These subdivisions are complementary.

Desensitization is a process that occurs in response to a wide variety of stimuli and can be found in a multitude of organisms. The widespread occurrence of desensitization phenomena points to its importance in biological signalling. Desensitization may have two main purposes: it may serve to terminate the cellular response to an individual stimulus, or it may cause a general reduction in responsiveness without being responsible for the actual termination of a single signalling event. Possibly both effects may be achieved via the same mechanisms. In either case, the overall effect of desensitization is to reduce stimulatory effects and thereby maintain biological equilibria.

Several examples have been studied in detail in order to elucidate the mechanisms and biological roles of desensitization. Among them, G-protein-coupled receptors is one of the mechanisms which in order to reveal the receptor-specific processes of desensitization. Structurally, these receptors are characterized by seven transmembrane α-helices and functionally by their ability to transmit signals to effector molecules via GTP-binding regulatory proteins (Bourne *et al.*, 1990; Hepler and Gilman, 1992). Among the G_s-coupled receptors, the best studied class of receptors are the β-adrenoceptors. Three β-adrenergic receptor subtypes have been identified in mammalian tissues and

their cDNAs cloned; namely β_1 (Frielle *et al.*, 1987), β_2 (Dixon *et al.*, 1986) and β_3 recepters (Emorine *et al.*, 1989). The β_2-receptor subtype has been studied most extensively. It was the first receptor of this class that was pruified in significant quantities (Benovic *et al.*, 1984) and it was the first G-protein-coupled receptor whose primary sequence was elucidated (Dixon *et al.*, 1986). In addition, the above hypotheses about desensitization are based on studies of this receptor.

Desensitization of receptor function (receptor un-coupling)

Desensitization via receptor-specific kinases

Phosphorylation of receptors is a critical step in the rapid desensitization mechanisms which affect receptor 'function'. The most rapid and quantitatively most important desensitization mechanism is triggered by phosphorylation of the receptors by the specific β-adrenergic receptor kinases (Benovic *et al.*, 1989; Benovic *et al.*, 1991). These kinases phosphorylate only agonist-occupied, active receptors. Two mechanisms serve to maintain this very strict agonist dependence: (i) the cytosolic kinases need to bind to the G-protein βγ subunits in order to bind to the membrane and position themselves for receptor phosphorylation (Haga, and Haga, 1992; Pitcher *et al.*, 1992); and (ii) only agonist-occupied receptors are a substrate for the kinases (Benovic *et al.*, 1986).

Mechanisms similar to the effects of βARK on β_2 receptors have been observed in several other sensory and receptors systems. In fact, the visual system has served as a prototype in this respect. In this system, activation of rhodopsin, the 'light receptor', leads to its multiple C-terminal phosphorylation by rhodopsin kinase, followed by binding of the inhibitor protein arrestin (Wilden *et al.*, 1986).

Many other receptor systems appear to be regulated by specific receptor kinases which may be identical or homologous to βARK and show receptor-

specific desensitization was observed in isolated cell systems include G_s-coupled receptors such as the D1-dopamine receptor (Balmfort *et al.*,.1990; Barton *et al.*, 1990), the V_2-vasopressin receptor (Birnbaumer *et al.*, 1992), the 5-TH_4-serotonin receptor (Ansanay *et al.*, 1992) and parathyroid hormone receptors (Pun *et al.*, 1990). A similar homologous pattern of desensitization has been observed for several receptors that activate phospholipase C, such as 5-HT_2-serotonin receptors (Ivins and Molinoff, 1991), angiotensin II receptors (Abdellatif *et al.*, 1991).

The situation is less clear for G_i-coupled receptors, but some reports indicated that also in these receptor types a homologous pattern of rapid desensitization exists, albeit in many cases to a much smaller extent than for corresponding G_s-coupled receptors (Atkinson and Minneman, 1992).

Direct phosphorylation of receptors by βARK or by βARK-like kinases has been shown in reconstituted system for the β–2 and α–2 adrenergic receptors (Richardson and Hosey, 1992; Haga and Haga, 1992; Kwatra *et al.*, 1989). The phosphorylation sites have been shown to reside in the C-terminus of the β_2-adrenergic receptor, but in the third intracellular loop of the α_2-adrenergic receptor (Liggett *et al.*, 1992; Dohlman *et al.*, 1987). The presence of acidic amino acids located 2 or 3 positions N-terminal to a serine or threonine seems to be required for phosphorylation by βARK. However, in addition to homologous (βARK-like) effects, both PKA and PKC seem to be involved in mediating agonist-induced desensitization (Dawson et al., 1993; Boekhoff & Breer, 1992; Boekhoff et al., 1992).

Desensitization via the effector kinases

In addition to phosphorylation by their specific kinase, many G-protein-coupled receptors are also phosphorylated by their effector kinase, namely protein kinases A and C. This type of phosphorylation provides a direct negative feedback, whereby the effector enzyme (PKA) shuts off its own stimulation. At the same time, this pathway allows a generalized and not

receptor specific form of desensitization, which is termed heterologous desensitization. There are two consensus sites for these effector kinase-mediated phosphorylations in the β_2-adrenergic receptor sequence (Yuan *et al.*, 1994). One located in the third intracellular loop, RRSSK263 and the other in the carbxyl-terminal domain, RRSSSk348, could be involved in PKA and PKC-mediated desensitizations. These two domains are essential for coupling to G_s and thereby for stimulation of adenylate cyclase activity. Further evidence that at least one of the two consensus sites was important for the PKA (Hausorff *et al.*, 1989) and the PKC-mediated desensitization (Bouvier *et al.*, 1991(a)).

The effector kinase-mediated desensitization has also been observed in other G-protein-coupled receptor systems. For example, from characterization studies of the epinephrine, prostaglandin E1 (PGE1) and PMA-induced desensitization pathways in L cells (Yuan *et al.*, 1994).

In summary, two different patterns of rapid, phosphorylation-mediated desensitization of G-protein-coupled receptors can be distinguished: one which is mediated by specific receptor kinases and which is rapid, strictly homologous and possibly confined mainly to synaptic receptors, and a second which is mediated by the effector kinases, PKA or PKC, which is somewhat less rapid, generalized (heterologous) and much more sensitive to agonist concentrations. The degrees to which the two mechanisms will be operative in an individual cell and receptor will depend on a multitude of factors: the number of phosphorylation sites for the different kinases in an individual receptor, the cellular levels of the different kinases and β-arrestins, and the degree of receptor stimulation.

Glucagon induced desensitization of adenylyl cyclase in rat hepatocytes through protein kinase C-mediated phosphorylation

Glucagon is a 29-amino acid pancreatic hormone that affects the production and distribution of glucose by its target organ, ie liver. glucagon

functions to maintain usual concentrations of glucose and is a key hormone in the pathogenesis of diabetes. Glucagon binds to receptors in liver and activates two enzymatic pathways, glycogenesis and glyconeogenesis, which result in the production of glucose (Unger *et al.*, 1990). It is assumed that these actions of glucagon on the liver result when glucagon binds its hepatic receptor and activates adenylate cyclase, thereby increasing the intracellular concentration of cAMP. However, glucagon may also cause hydrolysis of inositol phospholipids (Wakelam *et al.*, 1986; Unson *et.al.*, 1989) and increase in intracellular calcium concentratons (Sistare *et al.*, 1985). Two types of hepatic glucagon receptor have been proposed and it has been suggested that they signal via two different intracellular messengers-cAMP and calcium. However, recently a complementary cDNA clone for the glucagon receptor (Jelinek *et al.*, 1993) was isolated by an expression cloning strategy and the receptor protein was expressed in several kidney cell lines. The cloned receptor bound glucagon and caused an increase in the intracellular concentration of cAMP. The cloned glucagon receptor also transduced a signal that led to an increased concentration of intracellular calcium. Thus a single receptor can couple to more than one transduction system.

The rat hepatic glucagon receptor is coupled to the activation of adenylate cyclase through a process that is mediated by the stimulatory guanine nucleotide-binding protein G_s and leads to a profound, but transient, increase in the intracellular levels of cyclic AMP (Houslay, 1991; Murphy *et al.*, 1987). This is governed by the rapid desensitization of adenylate cyclase to glucagon stimulation, which has been attributed to an uncoupling of the glucagon receptor from the stimulatory G-protein G_s (Houslay, 1991). This desensitization process is however independent of any increase in cyclic AMP concentration and it has been suggested that it may result from the action of protein kinase C (Murphy *et al.*, 1987). In this regard, the hormone vasopressin which does not increase intracellular cyclic AMP levels in hepatocytes but, rather mediate its

effects through phospholipid metabolism and protein kinase C activation, can also cause the rapid desensitization of glucagon-stimulated adenylate cyclase activity (Houslay, 1991).

Here, we have develop a model system, using hepatocytes immobilized on collagen plates, which allowed us to demonstrate that the glucagon-stimulated elevation of intracellular cyclic AMP levels can be desensitized (Savage *et al.,* 1995). However, we also show that desensitization elicited by glucagon and vasopressin can be inhibited differentially by protein kinase C-selective inhibitors and suggested that the desensitization of glucagon-stimulated adenylated cyclase activity involves a role for protein kinase C-mediated phosphorylation in rat hepatocytes.

Results & Discussion

Challenge of hepatocytes with glucagon leads to a transient rise in intracellular cyclic AMP concentrations (Houslay, 1991). It has suggested that this is primarily governed by the rapid desensitization of adenylate cyclase with the subsequent degradation of cyclic AMP through phosphodiesterase action (Houslay and Kilgour, 1990; Houslay, 1990). The criterion for desensitization was that in membranes isolated from cells which had been challenged with glucagon for various periods of time, a rapid attenuation in the ability of subsequent glucagon treatment to stimulate adenylate cyclase activity (by approx. 50%-60% (Housaly, 1991).) was observed. However, results here show for the first time that treatment of intact hepatocytes with glucagon can desensitize the ability of a subsequent challenge with hormone to elevate intracellular cyclic AMP levels **(Figure A.1).** This has done using hepatocytes attached to collagen plates such that they could first be challenged with glucagon (10nM; 6 min) then washed, both to remove the hormone and to allow intracellular cyclic AMP levels to re-attain those seen under basal (resting conditions) conditions, before re-challenge with glucagon. Thus the intact-cell cyclic AMP response to glucagon itself became desensitized **(Figure A.1).** As with studies performed to observe the desensitization of glucagon-stimulated adenylate cyclase activity in membranes (Murphy *et al.*, 1987), desensitization assessed using the intact-cell cyclic AMP response could also be elicited by pretreating intact hepatocytes with the hormone vasopressin. This occurred in a manner that was just as effective as that elicited by glucagon in achieving the desensitization of the intact-cell cyclic AMP response **(Figure A.2).** Vasopressin does not, however, increase intracellular cyclic AMP level in hepatocytes (Houslay, 1991; Crebe *et al.*, 1983). That it can elicit the desensitization of glucagon-stimulated adenylate cyclase activity **(Figure A.2),** as can angiotensin, tumour-promoting phorbol esters and synthetic

diacylglycerols (Houslay, 1991) supports the concept that the desensitization of glucagon-stimulated adenylate cyclase is a cyclic AMP-independent process (Houslay, 1991; Murphy *et al.,* 1987; Refnes *et al.,* 1989) which can be elicited through the activation of protein kinase C in rat hepatocytes (Houslay, 1994).

Both glucagon and vasopressin (Mauger *et al.,* 1985; Mine and Ogata, 1988; Bygrave and Benedetti, 1993) elicit major increases in intracellular Ca^{2+} levels. It is possible that such changes could be sufficient to trigger desensitization by stimulating the activity of either Ca^{2+}/calmodulin-dependent protein kinase activity or protein kinase C. But neither an increase in intracellular Ca^{2+} nor a flux of this bivalent cation across the plasma membrane is pivotal in mediating the desensitization of glucagon-stimulated adenylate cyclase that can be elicited by both vasopressin and by glucagon (Savage *et al.,* 1995).

It has been suggested (Houslay, 1991) that protein kinase C may play a pivotal role in mediating the desensitization of glucagon-stimulated adenylate cyclase. This was based upon observations which showed that not only did desensitization occur apparently independently of any increase in cyclic AMP but that it could be triggered by both phorbol esters (Murphy *et al.,* 1987) and synthetic diacylglycerols (Newlands and Houslay, 1991). In addition, the hormones vasopressin and angiotensin, which are known to stimulate inositol pospholipid metabolism and produce diacylglycerol in hepatocytes (Creba *et al.,* 1983; Boeckino *et al.,* 1985) elicited a similar uncoupling of the glucagon receptor from adenylate cyclase (Murphy *et al.,* 1987). Indeed, glucagon is regarded as utilizing such a mechanism, in that it too can elicit the production of diacylglycerol in hepatocytes (Boeckino *et al.,* 1985) with the stimulation of phosphatidylcholine metabolism which is believed (Pittner and Fain, 1991) have demonstrated that both vasopressin and glucagon can cause a transient activation of hepatocyte protein kinase C activity, the kinetics of which parallel those seen for desensitization.

In hepatocytes, under resting conditions, the level of cyclic AMP is well below the threshold for activation of protein kinase A that remains in its inactive tetrameric state (Houslay, 1991). In contrast to this, however, it would seem that in hepatocytes, under resting conditions, a residual protein kinase C activity can be observed which is able to act on the α-subunit of the inhibitory G-protein G_i-2, leading to a small fracton of this protein being phosphorylated (Bushfield *et al.*, 1990a; Bushfield *et al.*, 1991; Morris *et al.*, 1994). This G-protein appears to be the focus of an active phosphorylation/dephosphorylation cycle, in that its phosphorylation can be rapidly elicited by either activation of protein kinase C or inhibition of protein phosphatase activity (Bushfield *et al.*, 1990a; Bushfield *et al.*, 1991; Morris *et al.*, 1994). This latter action can be demonstrated by treatment of intact hepatocytes with the protein phosphatase inhibitor okadaic acid (Bushfield *et al.*, 1991). Treatment of hepatocytes, under basal conditions, with okadaic acid was able to mimic the degree of desensitization achieved by both glucagon and vasopressin (Savage *et al.*, 1995). Heance, desensitization can be achieved not only through the activation of protein kinase C (as with phorbol myristate acetate and diacylglycerols), but can also result as a consequence of protein phosphatase inhibition. This indicates that, in hepatocytes, the control of coupling of the glucagon receptor to adenylate cyclase is also at the centre of an active phosphorylation/dephosphorylation cycle which is determined by protein kinase C and protein phosphatase activity.

In order to explore further the possible role of protein kinase C in glucagon desensitization, the process was investigated using chosen selective protein kinase C inhibitors. One such compound is chelerythrine, which has been shown to exhibit some selectivity for inhibition of protein kinase C at low concentrations (Herbert *et al.*, 1990). Indeed, I found that a concentration of 1μM chelerythrine was sufficient to obliterate completely the ability of vasopressin to cause desensitization (**Table A.1**). However, intriguingly,

such a concentration of chelerythrine had no effect upon the ability of glucagon to cause desensitization; with a higher concentration of 5μM causing half maximal blockade **(Table A.1)**. This differential sensitivity to the action of a protein kinase C inhibitor was not limited to chelerythrine, however, we also analysed the action of two other selective protein kinase C inhibitors, namely staurosporine (Herbert *et al.*, 1990) and calphostin C (Hidaka *et al.*, 1984; Kobayashi *et al.*, 1989). Using these compounds I also noted that they expressed a greater susceptibility to block the vasopressin-compared with the glucagon-induced desensitization of adenylate cyclase **(Table A.1)**. One possible explanation for these observations is that vasopressin and glucagon show very different abilities to stimulate lipid signalling pathways in hepatocytes and can thus be expected to produce diacylglycerol species with different spectra of acyl chains. This might well give rise to a different profile of activation of the various isoforms of protein kinase C that are found in hepatocytes. Consistent with such a hypothesis are the analyses showing marked differences (Tang and Houslay, 1992) in the ability of both glucagon and vasopressin to increase protein kinase C activity in hepatocyte membrane and cytosolic compartments. If the spectrum of protein kinase C isoforms activated by these two hormones exhibited slightly different sensitivities to inhibition by chelerythrine then this might provide a basis for our observations. It is also possible, however, that additional regulatory effects may complicate the situation when glucagon is used to effect desensitiztion. For example, glucagon challenge will also lead to the activation of protein kinase A, which might affect the functioning of protein kinase C isoforms and of protein phosphatases. It is also possible, however, that in a similar fashion to that seen for the β-adrenoceptor (Lohse, 1993), glucagon may elicit additional desensitizing contributions by activating a receptor-specific kinase which shows a susceptibility to inhibition by these protein kinase C inhibitors, albeit at slightly reduced sensitivity.

On the assumption that a component of the glucagon-stimulated adenylate cyclase system becomes phosphorylated during desensitization, the process can be reversed by alkaline phosphatase treatment in membranes from cells that had been pretreated with either glucagon or vasopressin to achieve desensitization (Savage *et al.*, 1995). These results suggest that the phosphorylation of a membrane protein is pivotal to the desensitization process.

By analysing various adenylate cyclase ligand-stimulated activities I have been able to show that the desensitization lesion takes the form of an inability of the glucagon receptor to couple functionally to G_s. It does not occur as a result of the phosphorylation of G_s as this G-protein can be immunoprecipitated from desensitized hepatocytes in a non-phosphorylated form (Tang and Houslay, 1992; Pyne *et al.*, 1989). On such a basis it is believed that the glucagon receptor itself becomes phosphorylated in the desensitization process through the action of protein kinase C. Desensitization of glucagon-stimulated adenylate cyclase can be achieved in both a homologous fashion by glucagon and also in a heterologous fashion by vasopressin, with protein kinase C-selective inhibitors identifying subtle differences in the processes utilized by these two hormones.

Figure A.1 Desensitization of glucagon-stimulated cyclic AMP accumulation in intact hepatocytes

Desensitization of glucagon (10nM, 6 min)-stimulated cyclic AMP accumulation was studied in intact hepatocytes, attached to collagen plates, as described in the methods section 2.3.2 and 2.3.3. 1mM of IBMX was also present during the glucagon challange of intact cells. Intracellular cAMP was 0.3±0.2pmol/10^6 cells. When preteated with glucagon, cAMP levels were allow to return to the resting/basal state over the recovery period; the value of the glucagon-stimulated increase in the intracellular concentration of cyclic AMP following pretreatment was 11.7±0.5pmols/10^6 cells. In experimental sets the appropriate control value was taken as the native stimulation of pmol intracellular cAMP accumulation/10^6 cells (100%). Results are means±S.E. with similar results found in one other experiment.

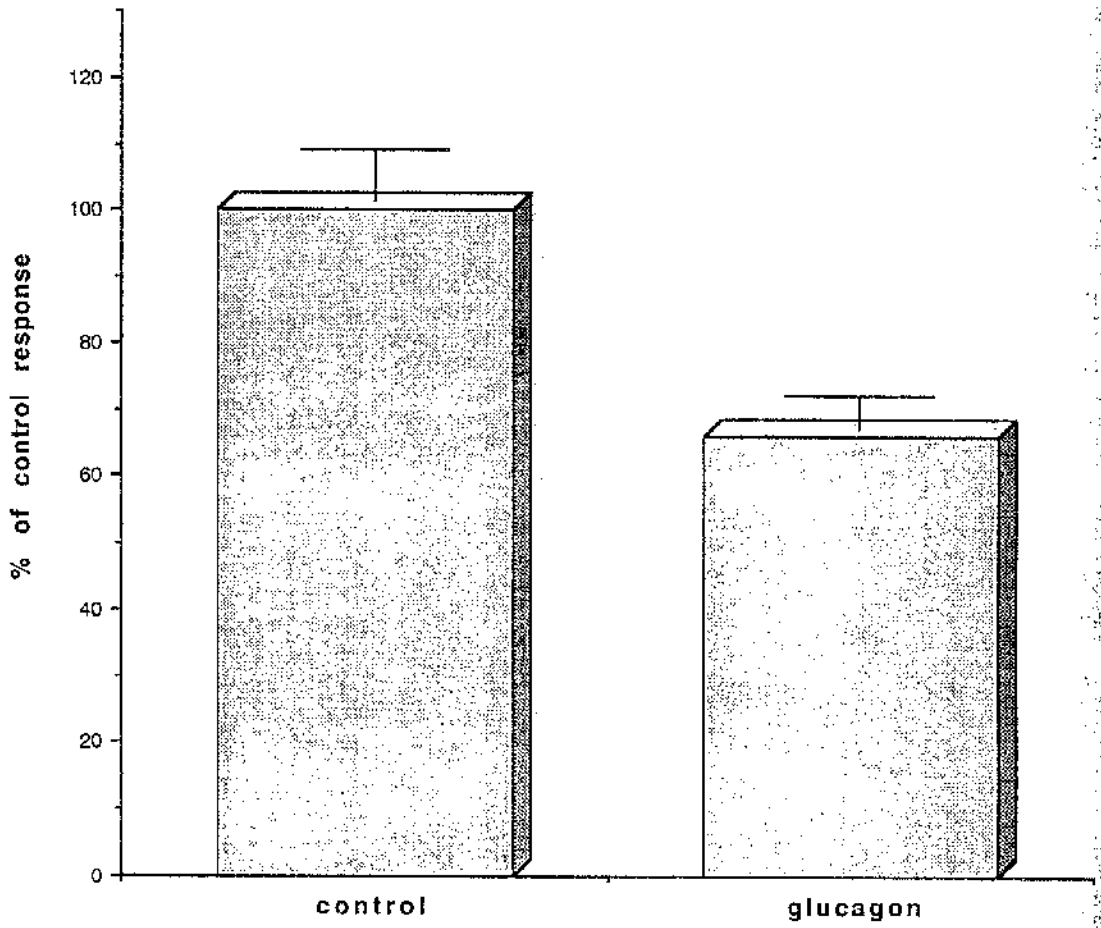

% of control response
120
100
80
60
40
20
0
control
glucagon

Figure A.2 Desensitization of glucagon-stimulated adenylyl cyclase in hepatocyte membranes

Intact hepatocytes were treated with either glucagon (10nM) or vasopressin (10nM) or were untreated (control) for 6 min. After this time cells were harvested, washed, disrupted and a washed membrane fraction obtained as described by methods section (2.3.1). Data shown is from a typical experiment with glucagon-stimulated adenylyl cyclase activity expressed in pmol of cyclic AMP produced/min/μg of membrane protein with means±S.E.for three determinations of activity.

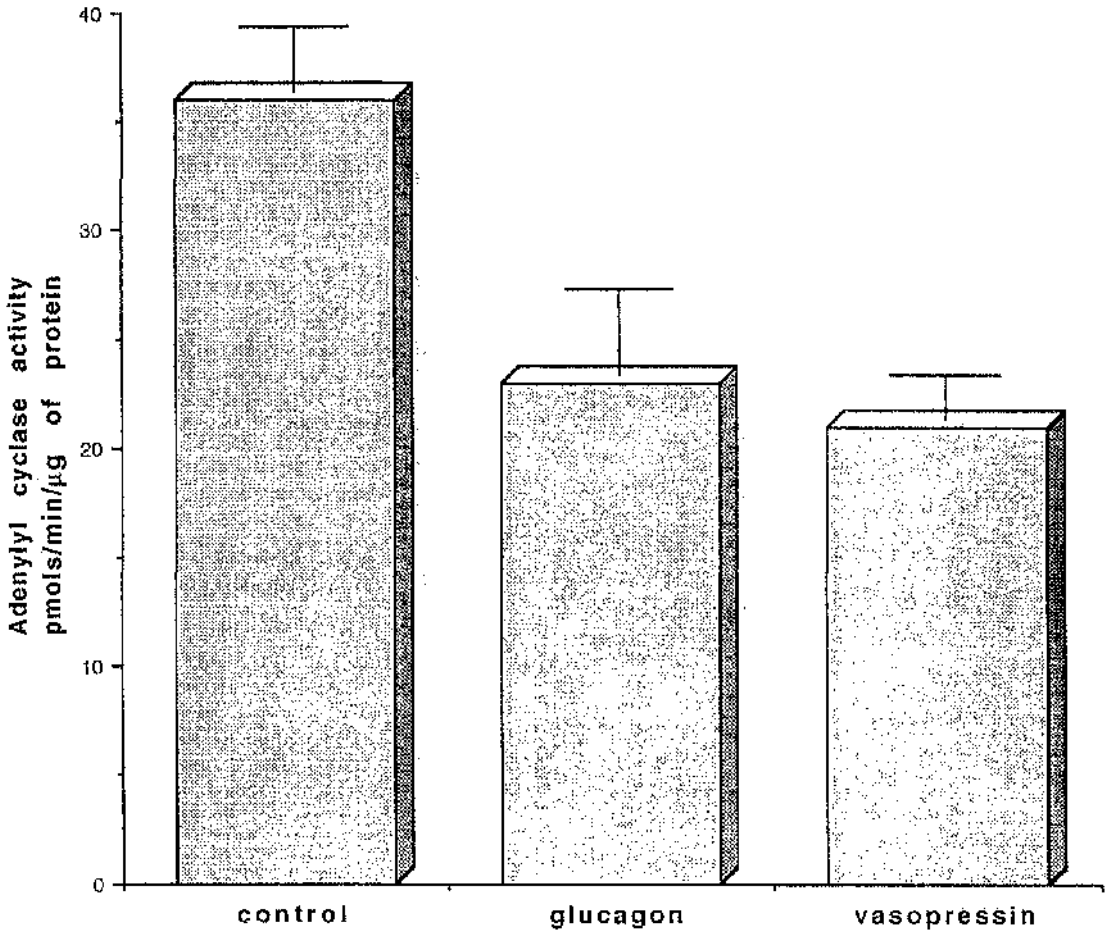

Adenylyl cyclase activity
pmols/min/μg of protein
40
30
20
10
0
control
glucagon
vasopressin

Table A.1 Protein kinase C involvement in the desensitization of glucagon-stimulated cyclic AMP accumulation in intact hepatocytes

In these experiments desensitization of glucagon-stimulated cyclic AMP accumulation was studied in intact hepatocytes attached to collagen plates, as described in the methods section (2.3.3). Protein kinase C inhibitors were added at the attachment stage so that a 40 min pre-incubation with these ligands was achieved. In experimental sets the appropriate control (100%) value was taken as the native stimulation of intracellular cAMP accumulation/10^6 cells. This was approximately 10.6pmols, with comparisons done using various ligands in the pre-incubation. The experiments described show data for n=3 with ±S.E.

Pre-incubation conditions	Cyclic AMP production by glucagon challenge (% of control response seen with no ligand pre-incubaton)
Control	100
Glucagon(10nM)	45±3
Vasopressin	51±3
Chelerythrine (1μM)	109±7
Chelerythrine (5μM)	91±3
Staurosporine (10nM)	98±6
Calphostin C (10nM)	98±4
Calphostin C (100nM)	96±5
Vasopressin (10mM)+chelerythrine (1μM)	106±5
Glucagon (10nM)+chelerythrine (1μM)	45±5
Glucagon (10nM)+chelerythrine (5μM)	73±4
Vasopressin (10nM)+staurosporine (10nM)	94±2
Glucagon (10nM)+staurosporine (10nM)	58±7
Vasopressin (10nM)+calphostin C (10nM)	71±5
Vasopressin (10nM)+calphostin C (100nM)	103±5
Glucagon (10mM)+calphostin C (10nM)	59±5
Glucagon (10nM)+calphostin C (100nM)	101±6