PROTEIN KINASE C ISOFORMS: INSULIN SIGNALLING, CYCLIC AMP METABOLISM AND DIABETES

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by
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Abbreviations

AA	Arachidonic acid
AC	Adenylate cyclase
ACTH	Adrenocorticotrophic hormone
ADP	Adenosine 5'-diphosphate
AMP	Adenosine 5'-monophosphate
APS	Ammonium persulphate
ATP	Adenosine 5'-triphosphate
Bis-acrylamide	N,N'-methylenebisacrylamide
BSA	Bovine serum albumin
C-terminal	Carboxy terminal
cAMP	Adenosine 3',-5'-cyclic monophosphate
CHO	Chinese Hamster Ovary
CTX	Cholera toxin
DAG	Diacylglycerol
DMSO	Dimethyl sulphoxide
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGTA	ethyleneglycol bis (β-aminoethyl ether)-N',N',N',N',-tetraacetic acid
ERK	Extracellular signal related kinase
ERT	EGF receptor threonine kinase
FCS	Foetal calf serum
GABA	Gamma amino butyric acid
GAP	GTPase activating protein
G-protein	Guanine nucleotide regulatory protein
GDP	Guanosine 5'-diphosphate

GNRP	Guanine-nucleotide releasing protein
GMP	Guanosine 5'-monophosphate
GTP	Guanosine 5'-triphosphate
Hepes	N-2-Hydroxyethylpiperazine-N'-2-ethane-sulphonic acid
HIR	Human insulin receptor
HRP	Horseradish peroxidase
IBMX	3-isobutyl-1-methylxanthine
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IP_3	Inositol-1,4,5-trisphosphate
IRS-1	Insulin receptor substrate-1
KLH	Keyhole limpet hemacyanin
LTC4/D4	Leukotriene C4/D4
MAP K	Mitogen activated protein kinase
MARCKS	Myristoylated alanine-rich C-kinase substrate
MBP	Myelin basic protein
NAD	Nicotinamide adenine dinucleotide
N-terminal	Amino terminal
NIDDM	Non-insulin-dependant diabetes mellitus
NP-40	Nonidet P-40
PA	Phosphatidic acid
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PC	Phosphatidylcholine
PCA	Perchloric acid
PDE	Phosphodiesterase
PDGF	Platelet-derived growth factor

PGE_1	Prostaglandin E_1
Pi	Inorganic phosphate
PI	Phosphatidylinositol
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PKC	Protein kinase C
PKM	Protein kinase M
PLC	Phospholipase C
PMSF	Phenylmethylsulphonyl fluoride
Ponceau-S	3-hydroxy-4-[2-sulfo-4-(sulfo-phenylazo)phenyl azo]-2, 7-naphthalene disulphonic acid
PPi	Pyrophosphate
PP-1	Protein phosphatase-1
PP-2A	Protein phosphatase-2A
PS	Phosphatidylserine
PTX	Pertussis toxin
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of mean
SH2 or SH3	src-homology
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
TPA	12-O-tetradecanoylphorbol 13-acetate
Tris	2-amino-2-hydroxymethyl-propane-1,3-diol
TXA2	Thromboxane A2
~	Approximately

Contents

Abstract

Protein kinase C has been implicated in the regulation of insulin signalling by many previous studies. This phenomenon was further investigated here using both established and novel experimental systems.

Using the streptozotocin-induced diabetic rat model, protein kinase C activity was assessed after partial purification of the enzyme from the liver of both normal and diabetic rats. It was found that inducing diabetes in rats using streptozotocin resulted in a decrease in total protein kinase C activity. Further analysis of protein kinase C activity showed that this difference was accounted for both by a reduction in activity of Ca^{2+}-dependent and Ca^{2+}-independent isoforms. To enable correlation of protein kinase C isoform expression with activity changes in these samples, antibodies were raised to specifically detect individual protein kinase C isoforms. Results from immunoblotting studies using isoform specific antisera suggested that, in rat liver, the reduced Ca^{2+}-dependent protein kinase C activity might be associated with alterations in the α and β_{II} isoforms, whilst modified activity of the PKC-ε species may have been responsible for the reduced Ca^{2+}-independent activity observed.

The availability of a set of Chinese hamster ovary cell clones overexpressing the human insulin receptor, both alone and combined with a specific protein kinase C isoform, allowed direct *in vitro* analysis of the interaction of insulin and protein kinase C. In this case their control of intracellular cyclic AMP metabolism was studied. Results obtained by directly measuring intracellular cyclic AMP concentration in cells suggest that insulin can attenuate agonist stimulated increases in intracellular cyclic AMP concentration. However, this effect of insulin was only evident in cells overexpressing both the human insulin receptor and PKC-ε

(CHO–ε cells).

Upon directly measuring cyclic AMP phosphodiesterase activity in all CHO cell clones available, it was observed that CHO-ε cells exhibited a higher basal phosphodiesterase activity than any other cell clone and that this activity was substantially increased by treating cells with insulin. Both of these observations were no longer apparent after cells had undergone long-term phorbol ester treatment to down-regulate protein kinase C. These results confirmed that PKC-ε plays a crucial role in regulating both basal phosphodiesterase activity and insulin induced changes in phosphodiesterase activity within these cells. They also suggested that insulin's ability to attenuate agonist induced increases in intracellular cyclic AMP concentrations in these cells was due, at least partly, to stimulation of cyclic AMP phosphodiesterase activity.

Chapter 1

Introduction

1.1. An introduction to signal transduction

The basic tenet of drug and hormone action is that they must exert some influence on one or more cells in such a way as to result in a biological response. Since in the majority of cases target cell number will vastly exceed the number of drug molecules, if these were merely distributed such that they interacted at random the chance of a response occurring would be negligible. We now know that to ensure accurate drug targetting, ligands bind to specific receptors located on or within the cell. The signal resulting from this interaction then needs to be amplified and apportioned to produce the desired response in the cell.

Events occuring after hormone-receptor binding can become extremely complex. They may, for example, simply alter membrane permeability directly via an ion channel or may involve a complex signal transduction process involving many second messengers. The main classes of receptor mediated signal transduction systems are summarized in Figure 1.1.

The sheer complexity of many systems, whereby a given cell contains a myriad of cell surface receptors each connected to a particular signal transduction system, begs the question how are these transduction mechanisms regulated and how might they relate to each other? The past two decades have seen expeditious progress in our understanding of signal transduction systems and their interaction with each other. Those of relevance to the work presented here will be discussed in further detail.

1.2. Insulin

1.2.1. Introduction

It is now more than sixty years since the initial discovery of insulin, yet the precise sequence of events whereby insulin triggers a cellular response is still unknown. This fact conveys the elaborate nature

of the programme of events involved in the insulin mediated signalling pathway, as in recent times no other single hormone has been the subject of such intense research interest. Whilst much has been learned, protein purification and molecular cloning studies, for example, have provided information on the structure and kinase activity of the insulin receptor, it is still not entirely clear as to how insulin propagates signals within the cell.

Information in Section 1.2.4 will provide an up to date summary of what is currently known about the signal transduction system for insulin.

1.2.2. Effects of insulin

Insulin regulates a wide range of effects, some of which are very rapid and seen in seconds, for example, transport processes and enzyme regulation. Others, affecting enzyme concentrations and cell growth, may be manifest only over a period of minutes or hours. This reinforces the fact that insulin may exert its effects via different signalling pathways.

Some idea of the pleiotropic nature of insulin's action is given by the following categories of response that are regulated by insulin:

a) Transport of molecules across the plasma membrane.

b) Control over intracellular levels of cyclic nucleotides.

c) Activities of key enzymes in intermediary metabolism.

d) Rates of protein synthesis.

e) Rates of DNA and RNA synthesis, including specific gene expression.

f) Cell growth.

g) Cell differentiation.

In relation to the discussion to follow concerning the condition of diabetes mellitus (Section 1.4) it is advantageous to examine more closely one of insulin's most important effects, namely glucose homeostasis.

Under normal circumstances, in response to increased glucose, pancreatic insulin secretion is stimulated and the combination of hyperinsulinaemia plus hyperglycaemia promotes glucose uptake by primarily liver, gut and muscle, in addition to suppressing hepatic glucose production. The initial step of insulin stimulated glucose metabolism involves activation of the glucose transport system, with increased transport of glucose into insulin sensitive tissues. The free glucose in the cell is subsequently phosphorylated by hexokinase and metabolized by a series of enzymatic steps under the control of insulin. Of these the most important are glycogen synthase (which controls glycogen synthesis) and pyruvate dehydrogenase (which regulates glucose oxidation).

Looking at this process in greater detail, whole body glucose homeostasis can be viewed as being dependent on the following three tightly coupled mechanisms:

a. Suppression of hepatic glucose production: After glucose ingestion, insulin is released into the portal vein and is transported to the liver where it binds to receptors on hepatocytes and suppresses hepatic glucose output. Failure of the liver to perceive this signal results in impaired suppression of hepatic glucose production and results in hyperglycaemia. In non insulin dependent diabetes mellitus (NIDDM) subjects, a consistent increase in hepatic glucose production has been demonstrated (Golay, *et al.*, 1988; Groop, *et al.*, 1989).

b. Augmentation of glucose uptake by splanchic (hepatic/gastrointestinal) tissues. All evidence suggests that there is no difference between control and diabetic subjects in the amount of glucose uptake in this area (De Fronzo, *et al.*, 1983). This illustrates that the splanchic tissues are fairly insensitive to insulin with very little glucose being taken up by these organs, even under hyperinsulinaemic conditions.

c. Peripheral (muscle) glucose uptake. As both examples given above are normal in NIDDM it is possible to assume by exclusion that peripheral tissues are the primary site of insulin resistance. There is infact an accelerated rate of glucose uptake in NIDDM (Gerich, *et al.*, 1990), explained by the mass action effect of hyperglycaemia that passively drives glucose into cells. However, the metabolic fate of the glucose taken up is abnormal as oxidation is impaired and there is no net flux of glucose into glycogen.

The numerous metabolic alterations that are characteristic of the diabetic state are becoming well defined, although the underlying lesions are proving more difficult to establish. Assessing the metabolic actions of insulin does however provide a useful estimation of insulin action in both normal and disease states.

1.2.3. The insulin receptor

1.2.3.1. Molecular biology

The insulin receptor gene is situated on the short arm of chromosome 19, far removed from the insulin gene on chromosome 11 (Yang-Feng, *et al.*, 1985). From this, a single proreceptor is synthesized and is cleaved in the endoplasmic reticulum to produce an alpha and beta subunit. A recent aspect of the insulin receptor to attract attention is the existence in most tissues of two isoforms, HIR-A and HIR-B (the latter lacking amino acid residues 720-731), produced by tissue specific alternative splicing at exon 11 of a primary proreceptor mRNA transcript (Mosthaf, *et al.*, 1990).

1.2.3.2. Structure

The holomeric insulin receptor is a symmetrical heterotetramer of M_r ~450 000 kDa, each heterodimer containing an extracellular,

hydrophilic α subunit (M_r ~135 000) linked to a membrane spanning β subunit (M_r ~95 000). Both subunits are glycosylated, which indicates the presence of extracellular domains in each. The heterotetramer ($\alpha_2\beta_2$) structure is maintained by disulphide bridges between both subunits with additional disulphide bridges between the α subunits.

The hydrophilic α-subunits are totally extracellular and provide the insulin binding site. They are anchored by means of their covalent attachment to the membrane spanning β-subunit. The β-subunit consists of an extracellular portion linked to its cytoplasmic domain by a single twist of α-helix across the plasma membrane. The above information is summarized in Figure 1.2.

The structure of the insulin receptor is such that dissection of signalling pathways at this stage is possible. Domains are being identified that are important in signalling one class of insulin action (metabolic responses) but are not involved in, and may even mask, the signalling of other types of responses (mitogenic).

1.2.3.3. Insulin binding

The insulin receptor was first identified by binding studies using mono-iodinated ^{125}I-insulin of high specific radioactivity many years before its molecular structure was determined (Kahn, 1976). These studies have shown that only one high affinity binding site occurs on each receptor molecule (Pang and Shafer, 1984) despite each receptor molecule possessing two putative binding sites. Binding of insulin to one such site is sufficient to cause receptor activation. Indeed, insulin is only transiently required to initiate insulin receptor activation, activity still being elevated after the liganded insulin has dissociated.

Such studies on the binding of insulin to target tissues and solubilized receptors is complex and difficult to reconcile with a homogenous population of non-interacting receptors. Possible explanations are that different receptor populations exist, each with differing affinities, or negative cooperativity between receptors. We now know that different receptor populations may exist and although theoretically each receptor could bind two insulin molecules, occupancy of one decreases the affinity of the other for insulin ~100 fold (Pang and Shafer, 1984).

The cysteine rich portion of the α-subunit seems important in ligand binding. It shows one of the lowest levels of sequence identity between the insulin and insulin-like growth factor 1 receptors, which is consistent with a role in defining the stereospecificity of ligand binding.

Insulin binding is followed by rapid internalization of the insulin-receptor complex, the insulin generally being passed to lysosomes to be degraded and the receptor mainly recycled. In the basal state approximately 10% of the receptor pool is intracellular, this figure increasing upon insulin exposure (Marshall, *et al.*, 1985). Each rat adipocyte, for example, can contain a total of around 200 000 insulin receptors.

It appears that the insulin receptor can also be activated by, for example, anti-receptor antibodies and trypsin to provoke insulin-like responses in the absence of tyrosine kinase activation and autophosphorylation (Sung, *et al.*, 1989). It has been proposed that this is achieved, perhaps in an analagous way to insulin, by relieving an inhibitory constraint, allowing the receptor to switch from an inactive to an active conformation.

1.2.4. The insulin signalling system

1.2.4.1. Tyrosine kinase activity

As recently as 1982 it was noted that the insulin receptor was a tyrosine protein kinase, a function now understood to be an integral part of the means by which insulin controls cellular function. Upon insulin binding to the extracellular domain of the receptor, activation of the intracellular domain incorporating the kinase activity ensues and initiates a unique sequence of tyrosine phosphorylations of both the receptor itself and specific cellular substrates. Prior to stimulation with insulin, the receptor is basally phosphorylated on serine and threonine residues (Kasuga, *et al.*, 1982).

We know that insulin receptor tyrosine kinase activity is initially stimulated by insulin binding and subsequently augmented by insulin-stimulated receptor autophosphorylation, although the means by which the insulin signal is transmitted through the short transmembrane region of the receptor is not understood. Whilst some mutations in this region have resulted in loss of receptor function (Longo, *et al.*, 1992), others have no effect (Frattali, *et al.*, 1991). Indeed several lines of evidence suggest that the extracellular and transmembrane domains of the receptor may not be an absolute requirement for effective receptor function; recombinant insulin receptors possessing only the kinase domain are active (Rosen, 1989) and trypsin activates the tyrosine kinase by removing most of the α-subunit. It seems that the unoccupied extracellular domain exerts tonic inhibition of the receptor kinase.

Establishing the theme of autophosphorylation and tyrosine kinase activity as central to any hypothesis regarding insulin's action is the fact that conservative mutations in the ATP binding site which abolish this activity appear to inactivate signal transduction. A lysine residue at position 1018 plays a critical role in the ATP binding site, the absence or

mutation of this amino acid having been documented to attenuate autophosphorylation and substrate phosphorylation (Chou, *et al.*, 1987).

Other important domains on the receptor β-subunit besides the active site described above include the C-terminal portion. Deletion of the 43 C-terminal amino acids results in a receptor exhibiting normal kinetics for the binding of insulin, receptor autophosphorylation, receptor endocytosis and recycling as well as similar substrate phosphorylation as native receptor. When expressed in intact fibroblasts, unlike the native insulin receptor its function was lost, suggesting that this domain may be involved in associating with a downstream effector molecule in the signalling pathway (Maegawa, *et al.*, 1988). The significance of phosphorylation at tyrosine 960 and at the principal sites of autophosphorylation (tyrosines 1146, 1150 and 1151) will be discussed below.

1.2.4.2. Autophosphorylation.

Autophosphorylation appears to occur through a trans-mechanism in which insulin binding to the α-subunit of one α–β dimer stimulates the phosphorylation of the adjacent covalently linked β-subunit (Lammerst, *et al.*, 1990). Several of the parameters associated with the phenomenon of insulin receptor autophosphorylation are outlined briefly below:

a) Ligand specificity. *In vitro* the ED_{50} for insulin stimulated autophosphorylation is 2-5nM, corresponding to half-maximal insulin binding. Maximal stimulation usually occurs at 100nM insulin, with a small inhibition (~15%) seen above this concentration. Insulin analogues promote autophosphorylation directly in parallel with their receptor binding affinity, for example IGF-I has a very low binding affinity and is about 5% as effective as insulin in its action.

b) Metal ion requirements. Divalent metal cations are necessary for insulin receptor kinase activity, serving as substrate cofactors and/or allosteric activators. Mn^{2+} and Mg^{2+} are the most effective.

c) Nucleotide dependency. ATP is the only phosphate donor known to be accepted by the insulin receptor kinase with a K_m highly dependent on assay conditions but ranging from 30 - 150μM. Autophosphorylation appears to increase V_{max} but not alter K_m for either ATP or substrate.

d) Inhibitors. Certain divalent metal ions (Cu^{2+}, Zn^{2+}) inhibit the insulin receptor. Specific inhibition has also been attained with antibodies. An antibody directed to a sequence surrounding the tyrosine-960 residue binds to the receptor, blocking autophosphorylation, although once autophosphorylated the receptor is no longer open to such inhibition.

e) Activators. Trypsinisation, hydrogen peroxide (H_2O_2), anti-receptor antibodies and sodium vanadate can all activate the insulin receptor.

1.2.4.3. Sites of autophosphorylation

Upon receptor activation, phosphotyrosines first appear within the tyrosine kinase domain at residues 1146, 1150 and 1151. This is generally found to be sufficient to cause maximal (10-20 fold) stimulation of receptor kinase activity (White, *et al.*, 1988). Concomitant with the appearance of the third phosphotyrosine at residue 1150 or 1151, phosphotyrosine also appears in residues 1316 and 1322 at the C- terminal tail of the receptor. These seem to play a regulatory role but are not essential for insulin signalling as their mutation or deletion does not alter receptor autophosphorylation or biological activity (Yamamoto-Honda, *et al.*, 1993). The intracellular juxtamembrane region of the receptor, however, is essential for signal transduction. It contains at least one autophosphorylation site (tyrosine 960), where if tyrosine is replaced by phenylalanine or alanine signal transmission is inhibited despite

autophosphorylation and kinase activity being unaffected (Kaburagi, *et al.*, 1993). It is proposed then that this region is involved in substrate, for example IRS-1, selection as overexpression of IRS-1 can rescue certain biological effects lost upon tyrosine 960 mutation (White and Kahn, 1994).

Proof that autophosphorylation alters conformation of the receptor comes from the fact that antibodies raised against a sequence containing the major autophosphorylation sites will immunoprecipitate receptors only after autophosphorylation and *vice versa*. It has been proposed that, for at least some forms of insulin mediated signal transduction, the altered conformation caused by autophosphorylation is required simply to allow physical interaction with a component of the signalling system allowing signal propagation.

1.2.4.4. The role of SH2 and SH3 domains

An intriguing question is how do growth factors exhibiting tyrosine kinase activity select their targets? Recent years have seen this clarified via the identification of *Src* homology 2 (SH2) domains (Pawson and Gish, 1992). These protein sequences of approximately 100 amino acids are conserved in even the most diverse cytoplasmic signalling proteins. Most of these proteins will also contain a distinct sequence of about 50 residues, the SH3 domain, which is also involved in regulating protein-protein interactions during signal transduction. SH2 and SH3 domains specifically recognize and bind phosphotyrosine, albeit only when implanted within a specific amino acid sequence.

In the case of the insulin receptor there is no evidence of direct association with signalling proteins. Instead, the best characterized substrate, insulin receptor substrate 1 (IRS-1), contains multiple potential tyrosine phosphorylation sites. Tyrosine phosphorylation of these sites in

insulin stimulated cells has been shown to allow binding of IRS-1 to, for example, PI 3'-kinase (Sun, *et al.*, 1991). This suggests that the insulin receptor, rather than having SH2 binding domains as part of the receptor, uses its direct tyrosine phosphorylation substrate, IRS-1, as an SH2 docking protein.

1.2.4.5. Substrates of the insulin receptor kinase

Many cellular components involved in insulin signalling have been proposed as substrates for phosphorylation. These include, protein kinases and phosphatases, G-proteins, calmodulin, enzymes involved in glucose metabolism or enzymes catalyzing the production of mediators such as PIP_2 (Kasuga, *et al.*, 1990).

Several approaches have been used to confirm cellular substrates of the insulin receptor. These include, phosphorylation of candidate proteins in cell free systems, immunoprecipitation of candidate substrates with specific antibodies, SDS-PAGE separation of labelled proteins from cells incubated with ^{32}P and immunoprecipitation of cellular proteins with anti-phosphotyrosine antibodies (White, *et al.*, 1985). Substrates determined using these techniques include MAP-2 (Kadowaki, *et al.*, 1985b), the α-subunit of tubulin (Kadowaki, *et al.*, 1985a), calmodulin (Graves, *et al.*, 1986), and protein phosphatase 2A (Chen, *et al.*, 1992), all identified using purified receptor preparations. Few proteins have been conclusively established to be substrates in the intact cell. One such protein, however, is HA4, identified as an ATPase on hepatocyte plasma membranes (Lin and Guidotti, 1989). Immunoprecipitation of a 185kDa protein (pp185) with anti-phosphotyrosine antibodies following insulin stimulation of Fao hepatoma cells first highlighted this important cellular

substrate (White, *et al.*, 1985). Subsequently pp185 has been found in all cell types studied, being immediately phosphorylated after insulin stimulation, and is now known as IRS-1 (Sun, *et al.*, 1991).

IRS-1 contains no sequence motifs to suggest it is a protein kinase and contains 21 potential tyrosine phosphorylation sites, including 6 in YMXM motifs, 3 in YXXM motifs and 12 in other hydrophobic motifs as well as 30 potential serine/threonine phosphorylation sites in motifs recognized by various kinases (White and Kahn, 1994). At least 8 of the tyrosine residues are phosphorylated by the activated insulin receptor (Sun, *et al.*, 1993).

IRS-1 is highly conserved between species, rat and human being 90% identical, with the tyrosines totally conserved (Nishiyama and Wands, 1992) and is essential for most, if not all, of insulin's biological effects.

1.2.4.6. Signal transduction via IRS-1

Activity of PI-3' kinase, an important mediator of cellular growth and metabolism, increases following insulin stimulation, suggesting that it is somehow activated by the insulin receptor. IRS-1 is known to bind strongly to this enzyme (Sun, *et al.*, 1991), this being attributed to binding of tyrosine phosphorylated YMXM motifs of IRS-1 to SH2 domains present in the 85 kDa subunit of PI-3' kinase.

These findings established a new mechanism of allosteric enzyme regulation which may be generalized to other SH2 domain-containing proteins, such as SH-PTP-2 (Kuhne, *et al.*, 1993), GRB-2 (Skolnik, *et al.*, 1993) and Nck (Li, *et al.*, 1994), with IRS-1 as a central molecule in the insulin transducing pathway. SH-PTP-2 (or Syp) is a protein tyrosine phosphatase containing two SH2 domains which is activated when these bind to IRS-1. GRB-2 contains two SH3 domains and one SH2 domain

and acts to link the guanine nucleotide exchange factor for p21ras (mSOS) to IRS-1, the GRB-2/mSOS complex activating p21ras by stimulating GTP binding. In the future many more cellular elements interacting with IRS-1 will be identified, and indeed molecules similar to IRS-1 may still await discovery.

1.2.4.7. Insulin signal transduction via protein kinase cascades

In common with many other growth promoting hormones, compelling evidence gathered over recent years points to the involvement of a phosphorylation cascade in insulin action. Although this originates from receptor tyrosine kinase activity, serine and threonine phosphorylation play the major role. Indeed, tyrosine phosphorylation represents only 0.03% of all protein phosphorylation in cells.

Several protein kinases are activated in cells by insulin, including casein kinase II, MAP kinases, raf-1 kinase, acetylCoA carboxylase kinase and ribosomal S6 kinases. Conversely, many actions of insulin are brought about by dephosphorylation of proteins, including glycogen synthase, pyruvate dehydrogenase, triacylglycerol lipase and phosphorylase kinase (Denton, 1990). Protein phosphatases, such as PP1 are, however, in turn activated by insulin-sensitive protein kinases (ISPK) such as S6 kinase ($p90^{rsk}$) in the case of PP1 (Lavoinne, *et al.*, 1991), whose activation is mediated by yet another ISPK known as MAP-2 kinase (Sturgill, *et al.*, 1988).

It is now known that the upstream activator of MAP kinase, the MAP kinase kinase, is activated by c-raf-1 kinase mediated phosphorylation and inactivated by phosphatase 2A (Kyriakis, *et al.*, 1992). A putative signal transduction sequence might thus involve insulin binding - receptor activation - phosphorylation of IRS-1 - binding of a GRB-2/mSOS complex to IRS-1 - activation of p21ras - activation of raf-1

kinase - activation of MAP kinases - activation of S6 kinases and ultimately activation of PP1. The components of such a pathway will now be discussed in more detail.

Mitogen Activated Protein Kinases

MAP kinase was infact identified as an insulin stimulated kinase in 3T3-L1 fibroblasts that had been converted to adipocytes (70-80%) by treatment with dexamethasone and isobutylmethylxanthine (Sturgill and Ray, 1986). Over the last two years these proteins have acquired a number of names and acronyms. MAP kinase is now generally used to mean mitogen activated protein kinase although confusingly is still often used to imply microtubule associated protein. Other members of the same family were first, and are still often described by other names such as ERK (extracellular-signal related kinase), MBP (myelin basic protein) kinase and ERT (EGF receptor threonine) kinase.

Many potential substrates for MAP kinase have been proposed, for example, raf-1 kinase (Anderson, *et al.*, 1991), S6 kinase (Sturgill and Wu, 1991) and PP1 (Lavoinne, *et al.*, 1991), and identified following the identification of a consensus sequence, namely Pro-X-Ser/Thr-Pro, for MAP kinase mediated phosphorylation (Alvarez, *et al.*, 1991). This will hopefully further aid identification of physiologically important downstream components in the signalling pathway which interact with MAP kinase. Upstream regulation of MAP kinase involves tyrosine or serine/threonine kinases, including protein kinase C, and possibly a cyclin like molecule which stimulates MAP kinase autophosphorylation (Ahn, *et al.*, 1991).

Protein kinase C

Another possible component of the insulin signalling pathway is protein kinase C. In BC3H-1 myocytes diacylglycerol levels increased within 30 seconds of stimulation by insulin. Membrane protein kinase C

activity increased two-fold within 60 seconds, and cytosolic protein kinase C activity increased by 80% over 20 minutes (Cooper, *et al.*, 1987). Further recent studies suggest that diacylglycerol produced in response to insulin is synthesized mainly from glycerol through phosphatidic acid and by hydrolysis of phosphatidylcholine by an insulin-dependent phospholipase D (Baldini, *et al.*, 1992). Several other groups have demonstrated insulin stimulated increases in both cytosolic and membrane bound activity (Cooper, *et al.*, 1990; Ishizuka, *et al.*, 1989). However, this was not a consistent observation (Vaartjes, *et al.*, 1986; Blackshear, *et al.*, 1991), thus making this an area of considerable controversy. Interactions between protein kinase C and insulin are discussed in much greater detail in Section 1.5.

Phosphatases

Introduction of phosphoryl groups from ATP onto intracellular proteins by protein kinases is recognized as a fundamental mechanism in the signal transduction process. Protein phosphatases reverse the action of protein kinases by hydrolyzing phosphoryl groups from proteins. Maintenance of steady state basal phosphorylation levels and inactivation or activation of proteins regulated by their phosphorylation state requires finely tuned phosphatase activity as the basal phosphorylation state of a protein is determined by a dynamic equilibrium between the activities of the kinase(s) and phosphatase(s) that catalyze the phosphorylation and dephosphorylation reactions respectively. The protein phosphatase family is extensive (Brautigan, 1992) although its members seem more related through action than by heredity. Several diverse molecules have apparently converged on a function, utilizing different protein backbones to exhibit a critical surface nucleophile and small supporting cast of sidechains to carry out hydrolysis.

Insulin is capable of dephosphorylating and activating glycogen synthase or dephosphorylating and inactivating glycogen phosphorylase by activation of protein phosphatase 1 via serine phosphorylation, an effect antagonized by adrenaline (Cohen, 1992). In insulin-dependant diabetic subjects the inability of glycogen synthase activity to be stimulated by a glucose load has been shown to be due to a decreased synthase phosphatase activity (Langdon and Curnow, 1983). This is not seen in NIDDM suggesting that actual lack of insulin, rather than increased blood glucose, is responsible for this decreased synthase phosphatase activity.

p21 ras

The insulin receptor appears to be able to interact with the GDP-bound form of the ras oncogene product p21 (O'Brien, *et al.*, 1987), a G-protein which may be involved in certain processes whereby insulin controls cellular events (Korn, *et al.*, 1987).

In mammals there are at least three distinct ras proteins; H-ras, K-ras and N-ras. A variety of related gene products displaying ~50% homology to ras, including rho, ral and YP2, have been identified. With the discovery of mutant ras alleles in human tumours it became clear that p21ras had an important role in signal transduction pathways of growth promoting hormones, including insulin. The p21ras contains a GDP/GTP binding site and a low intrinsic GTPase activity. This allows ras to serve as a biological 'switch' that is 'on' when GTP is bound and 'off' when GDP is bound. This feature is in turn regulated by two accessory proteins, GTPase activating protein (GAP) and guanine nucleotide releasing protein (GNRP).

The p21ras has been implicated as part of the insulin signal transduction pathway leading to the expression of certain genes (Haubruck and McCormick, 1991; Burgering, *et al.*, 1991; Korn, *et al.*, 1987). Whilst still somewhat unclear, it may be that GAP links the insulin receptor to p21ras, either directly or via IRS-1, since it contains two SH2 domains. More recently, GRB-2 has been identified as another possible linking molecule since it binds ras and contains one SH2 and two SH3 domains. Subsequently ras has been shown to bind directly to raf-1 serine/threonine kinase which in turn activates MAP kinase by phosphorylation and activation of MAP kinase kinase (Crews and Erikson, 1993).

Insulin induced maturation of oocytes was blocked using anti-ras antibodies (Korn, *et al.*, 1987). Subsequent similar experiments highlighted that ras inactivation blocked growth factor receptor tyrosine kinase stimulated phosphorylation and activation of MAP kinase and protein kinase C (Thomas, *et al.*, 1992) Therefore, it seems that ras and protein kinase C can function upstream or downstream from each other in distinct signalling pathways. A further intriguing aspect of ras involvement in signalling is that protein kinase C activation also induces a rapid activation of p21ras.GTP (Downward, *et al.*, 1990). Dominant-negative ras alleles can block growth factor activation of Raf-1 kinase and MAP kinase, placing ras upstream of both kinases (Itoh, *et al.*, 1988).

1.2.4.8. The role of phosphoinositol glycans

There seems little doubt that insulin provokes rapid changes in phospholipid metabolism in many of its target tissues. Possible routes by which this is mediated include via:

a) hydrolysis of phosphoinositol glycan or other glycolipids. One possible means of signal transduction is release of a phosphoinositol glycan and DAG by PLC action.

b) activation of *de novo* PA synthesis. Insulin increases PA synthesis by increasing activity of glycerol-3-phosphate acyltransferase, apparently by decreasing its K_m. This appears to be PTX sensitive and requires activation of a PLC.

c) PC hydrolysis. Increased incorporation of ^{3}H-glycerol into lipids (eg. PC (Nair, *et al.*, 1988)) during insulin action has been shown in a number of cell types.

All three possibilities can result in DAG production. In cells where both PI-glycan hydrolysis and PA synthesis have been blocked by PTX treatment (leaving PC hydrolysis intact), insulin stimulated increases in DAG levels are initially intact. However, increases after 1 minute are diminished (Luttrell, *et al.*, 1988), suggesting that individual pathways are responsible for different phases of DAG production. The production of structurally distinct DAG, for example, lacking the usual arachidonate, by these means may also be an underlying factor in the selective regulation of protein kinase C isoforms.

1.3. Clinical syndromes associated with defects in insulin signalling: diabetes mellitus and associated molecular defects.

Insulin resistance of skeletal muscle, liver and fat combined with an abnormality of insulin secretion characterizes type 2 non-insulin dependant diabetes mellitus (NIDDM) . This condition affects at least 5% of the general population. It must be stressed that insulin resistance is only seen as it is partial, complete resistance leaving the subject with almost zero chance of survival.

Whilst much attention is focussed on the genetic basis of insulin resistance in NIDDM, acquired factors such as obesity and decreased physical activity can also contribute significantly to the development of

impaired insulin action in NIDDM.

NIDDM results from an imbalance between insulin sensitivity and insulin secretion with the earliest detectable abnormality being an impairment in the body's ability to respond to insulin. Initially, the pancreas can correspondingly augment its secretion of insulin to offset any resistance such that glucose tolerance remains normal. With time however, the β-cells fail to maintain this high output of insulin and glucose tolerance is impaired, leading eventually to overt diabetes mellitus. This implies therefore, that full-blown NIDDM initially requires two major defects, insulin resistance and impaired β-cell function.

It is certainly fashionable to assume that insulin resistance is the primary defect in NIDDM with hyperinsulinaemia being regarded solely as a compensatory response. It must be stressed however, that while this is almost certainly the case, it is plausible that increased insulin secretion represents the primary disturbance. Insulin resistance may then arise as a downregulation of both the receptor and postreceptor events caused by chronic sustained hyperinsulinaemia, as seems to be the case in at least one study group (Lillioja, *et al.*, 1991).

In some cases of NIDDM the primary defect can be at the level of the β-cell with resultant impaired insulin secretion and subsequent development of insulin resistance. Such individuals are relatively rare and are represented by the lean diabetic.

Normal insulin secretion is biphasic (De Fronzo, *et al.*, 1979) with an early burst of release within the first ten minutes after stimulation followed by progressively increasing pulsatile secretions (Lang, *et al.*, 1979). Evidence suggests that pulsatile insulin secretions or delivery is more effective in promoting glucose disposal than continuous administration (Paolisso, *et al.*, 1987). Recently one study has suggested

that as loss of oscillatory insulin secretion is a characteristic feature of relatives of patients with NIDDM, and this could represent the earliest natural lesion in NIDDM (Gulli, *et al.*, 1990).

Many recent studies have shown that the onset of type 2 diabetes in different high-risk populations is preceded by an undefined time period when insulin resistance may be detected through exhibited symptoms including increased abdominal fat, obesity, hypertension, elevated very low density lipoproteins and decreased high density lipoproteins as well as increased fasting (Modan, *et al.*, 1985; Haring and Mehnert, 1993). Diabetes mellitus manifests itself by being associated with blindness, end stage renal disease, and a variety of atherosclerotic cardiovascular disorders. It is likely that disorders of intracellular signalling lie at the heart of clinical problems of insulin resistance. Several candidates for the location of such a lesion will now be discussed.

1.3.1. Possible defects causing insulin resistance in NIDDM

1.3.1.1. Insulin receptor

As the first element of the insulin mediated signal transduction pathway, the insulin receptor is a strong candidate for the location of any defect. Decreases in insulin binding in NIDDM are observed, reflecting either decreased receptor number or affinity (Olefsky and Reavan, 1977). These results are dubious, however, since they were performed on studies showing hyperinsulinaemia which naturally would lead to receptor down-regulation. In general, most studies suggest that decreased receptor number is not a prevalent phenomenon in NIDDM cases.

Can analysis of the insulin receptor gene highlight any associated defect? In studying cases of severe insulin resistance several point mutations of the insulin receptor have infact been found to occur (Taylor, *et al.*, 1990). However, in testing over 100 type 2 diabetic patients only a

few showed mutations of the insulin receptor gene, and for these there was no proof that they affected signalling of the resulting receptor (Cocozza, *et al.*, 1992). Such studies do not allow for the fact that, while the insulin gene itself may be normal, the possibility exists for functionally significant aberrations to occur during processing.

Alterations in receptor kinase activity in normal and diabetic states has also been assessed. In a type 2 case, kinase isolated from skeletal muscle was found to have an altered autoactivation process resulting in higher levels of inactivity (Obermaier-Kusser, *et al.*, 1989). In general it appears that receptor tyrosine kinase activity is diminished in disease states (Considine and Caro, 1993; Comi, *et al.*, 1987)

In conclusion, evidence suggests that a genetically determined, inherent defect of the receptor is unlikely. This infact, only accounts for a fraction of the NIDDM population. Rather, it appears that either altered receptor kinase activity or a secondary defect in the signalling cascade, diminished glucose transport or an abnormality in some critical enzyme step during glucose utilization seems more plausible.

1.3.1.2. Protein kinase C

Since decreased tyrosine kinase activity is exhibited by insulin receptors from NIDDM patients and protein kinase C can be responsible for attenuating receptor function it is tempting to speculate that the two are related. Supporting this hypothesis is data showing increased PIP_2-PLC activity in diabetic rat liver (Thakker, *et al.*, 1989), increased protein kinase C activity in the liver of rats made diabetic by starvation (Karasik, *et al.*, 1990), increases of 82% in the diacylglycerol content of insulin-resistant rat liver (Turinsky, *et al.*, 1990), and elevated glucose stimulated *de novo* diacylglycerol synthesis (Wolf, *et al.*, 1991) as a direct result of glucose conversion (Lee, *et al.*, 1989).

These results strongly implicate protein kinase C in the pathogenesis of insulin resistance, although these findings, once again, are not always apparent when assessed. In addition, protein kinase C has also been shown to have 'insulin-like' effects in increasing glucose transport (Henriksen, *et al.*, 1989) and lipogenesis (Van de Werve, *et al.*, 1985).

Note that as well as being able to attenuate insulin receptor activity, protein kinase C may also cause insulin resistance by phosphorylating a target enzyme, such as glycogen synthase, either directly or via other protein kinases downstream of protein kinase C.

1.3.1.3. Phosphatases

Phosphatases are a vitally important target for insulin action, as seen, for example, in the regulation of glycogen synthase activity (Cohen, 1992). If we consider the proposed phosphorylation cascade initiated by insulin then phosphatases have the potential to regulate insulin mediated activity at every stage from basal receptor phosphorylation state, through phosphorylation state of components in the signalling pathway to the phosphorylation state of the ultimate target protein.

Evidence has emerged recently for an altered type-1 protein phosphatase (PP-1) activity in insulin resistant individuals, a decreased basal and insulin stimulated activity of this serine kinase now having been well documented (Freymond, *et al.*, 1988; Kida, *et al.*, 1992).

1.3.1.4. Loss of Gi function

A loss of tonic inhibitory G-protein mediated inhibition of adenylate cyclase leads to enhanced activity in the presence or absence of any hormonal stimulation, as is the case in streptozotocin-induced diabetic rats (Strassheim, *et al.*, 1990). Since this effect can readily be reversed by

insulin therapy it is suspected that it may be of consequence in the pathogenesis of diabetes.

1.3.1.5. Glycogen synthase

A pivotal role for glycogen synthase is suggested by the observation that in type 2 diabetes both oxidative and non-oxidative glucose disposal is impaired (De Fronzo, *et al.*, 1992), whereas in the pre-diabetic state only non-oxidative disposal is affected (Warram, *et al.*, 1990).

Activity of this enzyme is regulated by phosphorylation (serine kinases) and dephosphorylation (at least two phosphatases), the latter converting the enzyme to a glucose 6-phosphate independent form. Insulin activates the enzyme by promoting dephosphorylation (Lamer, 1983), this effect of insulin being reduced in type 2 diabetic patients (Damsbo, *et al.*, 1991; Nyomba, *et al.*, 1990).

Collectively, the data do not suggest that glycogen synthase itself is defective, rather that the defect occurs at a discrete point somewhere along the signal transduction pathway between the insulin receptor and glycogen synthase.

1.3.1.6. β-cell number

This factor is a critical determinant of insulin secretion volume. Most studies (Clark, *et al.*, 1988; Westermark and Wilander, 1978) demonstrate a loss of up to 40% in β-cell mass in patients with well established NIDDM, although this figure is qualified by the observation that a decrease of around 80% is required to induce overt diabetes mellitus (Eisenbarth, *et al.*, 1987).

1.3.1.7. Glucose toxicity

Evidence suggests that chronic hyperglycaemia is responsible, at least in part, for the β-cells' inability to respond to an acute hyperglycaemic challenge (Rossetti, *et al.*, 1990) This means that hyperglycaemia is not only to be viewed as a symptom of diabetes mellitus but also a pathogenic factor perpetuating the diabetic state. In addition, drug treatments to lower blood glucose, such as insulin or sulphonylureas, will alleviate symptoms somewhat and are associated with a concomitant increase in insulin secretion (Kosaka, *et al.*, 1980).

1.3.1.8. Amylin

Amylin is a novel pancreatic hormone, similar in structure to calcitonin gene related peptide, co-secreted with insulin by the β-cell. It is the precursor for the amyloid deposits that frequently are observed in patients with NIDDM (Nishi, *et al.*, 1990) and at very high doses can inhibit insulin secretion (Ohsawa, *et al.*, 1989). Originally called diabetes-associated peptide, amylin has now been isolated and completely characterized as a 37 amino acid peptide. At this time however, the way in which amylin causes insulin resistance is unclear. Another similar peptide, galanin, has also been implicated in inhibiting insulin secretion (Dunning and Taborsky, 1988).

1.3.1.9. Glucose transporter

Insulin promotes increased glucose transport by inducing the translocation of a large intracellular pool of glucose transporters to the plasma membrane. GLUT1 represents the predominant glucose transporter and regulates basal glucose uptake. GLUT2, the glucose sensitive transporter, predominates in liver and pancreatic β-cells, allowing these cells to act as glucose sensors. Of the seven facilitative glucose

transporters characterised to date, only GLUT4 has been identified as being insulin sensitive.

Although the molecular mechanism is unclear, glucose transport has been extensively studied and found to be consistently decreased in, for example, adipocytes and muscle cells of NIDDM sufferers (Rothmann, *et al.*, 1992; Dohm, *et al.*, 1988; Garvey, *et al.*, 1988). Available evidence points to no primary defect at the level of the glucose transporter itself (Pedersen, *et al.*, 1990; Eriksson, *et al.*, 1992). Despite obvious interest no phosphorylaton or dephosphorylation of glucose transporters in response to insulin has yet been described. Hence other mechanisms of regulation by insulin must be sought.

There are other explanations for insulin resistance, such as the development of auto-inhibitory antibodies to insulin or the insulin receptor, the overproduction of counter-regulatory hormones and the synthesis of mutant insulin. Genetic abnormalities only account for a very small percentage of cases and are likely to differ among different population groups. Such conditions appear to be multigenic, the NIDDM phenotype manifesting itself only when more than one gene defect is apparent.

1.4. Protein kinase C

1.4.1. Background.

Protein Kinase C was first discovered in 1977 as an undefined protein kinase found in almost every cell studied, which was activated by limited proteolysis with the neutral protease calpain (Takai, *et al.*, 1977). Infact the co-factor independent kinase, protein kinase M (PKM), generated upon proteolysis was at first thought to be the important molecule in terms of kinase activity. However, it is thought that generation

of PKM is a transient step on the pathway to complete proteolysis and inactivation of protein kinase C.

Protein kinase C is now known to represent a structurally homologous group of proteins similar in size, structure and mechanism of activation which can elicit a variety of cellular responses by phosphorylating target proteins, most of which remain unknown, on serine and threonine residues. Full understanding of protein kinase C function requires identification and characterisation of physiological substrates for the enzyme as well as elucidation of the function of these target proteins.

Interest in the enzyme accelerated greatly upon the observation that it was activated by tumour promoting phorbol esters and the subsequent realization that the structurally similar diacylglycerol was indeed the physiological activator of protein kinase C. The fact that diacylglycerol production could be elicited by a variety of hormones that caused receptor mediated hydrolysis of membrane phospholipids opened up a whole new branch of the complex intracellular signalling network mediated via protein phosphorylation.

The enzyme is synthesized in membranes as a non-phosphorylated and inactive form that is converted to the active protein kinase C molecule by post-translational modification (Borner, *et al.*, 1989). At least ten isoforms of protein kinase C have now been identified, all of which are encoded by a large gene family. These have been termed PKC- α, β_I, β_{II}, γ, ε, ζ, δ, θ, η, (L) and λ, as described in Table 1.1. These isoenzymes are often divided broadly into two categories, namely those which are calcium sensitive and those which are not. A great deal of work has been carried out on their structure, biochemical properties, expression, localization and activation within resting, proliferating and differentiated cells.

A question we must ask is to what extent do the protein kinase Cs that have been identified through cDNA cloning studies represent a discrete family of kinases, and if so, how are the limits for such a family defined. One would have to say that the protein kinase C family is primarily bound together by their regulatory properties as they can clearly differ with respect to reactions catalyzed. Indeed the possibility that protein kinase C subspecies have specific cellular substrates allows for diverse and specific responses from cells in response to protein kinase C activation. This explains how in some instances protein kinase C can be involved in mediating the mitogenic responses of cells while conversely may also inhibit cell proliferation and induce differentiation. Protein kinase C has been implicated in diverse effects, including regulation of ion channel conductance, exocytosis, secretion, receptor down-regulation and cross-talk with other signalling systems.

1.4.2. Structure

The primary structure of the protein kinase C isoenzymes consists of a single polypeptide chain showing conserved structural motifs with a high degree of sequence homology. This sequence can be considered as having four conserved (C) regions separated by five variable (V) regions. The N-terminal half (regions C1, C2, V1 and V2) constitutes the regulatory domain while the C-terminal half (regions C3, C4, V4 and V5) represent the catalytic domain, the two domains being separated by the variable region V3.

The so-called conventional protein kinase Cs (cPKCs - α, β_I, β_{II}, and γ) have a structure similar to that described above, but the non-conventional (nPKCs - δ, ε, and ζ) which are calcium independent lack the C2 region.

As the name implies, the regulatory domain interacts with calcium ions, phospholipids and diacylglycerol. The catalytic domain contains the active site and shows homology to many other protein kinases. Also in the N-terminal half is the pseudosubstrate region, an amino acid sequence which closely resembles protein kinase C substrate recognition sites. The following sections focus on individual areas in greater detail.

1.4.2.1. C1-region

The C1 domain of all protein kinase Cs except PKC-ζ contains a tandem repeat of a cysteine rich sequence (Parker, *et al.*, 1986):

$Cys\text{-}X_2\text{-}Cys\text{-}X_{13(14)}\text{-}Cys\text{-}X_2\text{-}Cys\text{-}X_7\text{-}Cys\text{-}X_7\text{-}Cys$

Where X = Any amino acid.

The sequence outlined above resembles the consensus of a so-called zinc-finger. A zinc-finger is comprised of an approximately thirty amino acid repeat with two zinc ions tetrahedrally co-ordinated between six cysteine residues. Studies on purified PKC-β_I from insect cells suggest the existence of four zinc ions per molecule (Hubbard, *et al.*, 1991). In the case of most protein kinase Cs, the C1 domain consists of two zinc-finger like motifs, although as previously mentioned PKC-ζ does not bind PS or DAG and contains only one cysteine rich sequence (Ono, *et al.*, 1987). This correlates with this isoform exhibiting, at least *in vitro*, constitutive activity (Liyange, *et al.*, 1992).

This region appears to be involved in phorbol ester and diacylglycerol binding since activity stimulated by these agents is abolished by deletion and point mutations within this region (Ono, *et al.*, 1989). It has recently been proposed that phorbol esters and diacylglycerol bind by virtue of hydrogen bonding to the cysteine sulphydryl groups prominent in this region (Gschwendt, *et al.*, 1991).

1.4.2.2. C2-region.

The Ca^{2+} binding site on protein kinase C is unknown, although the C2 region is thought to confer calcium dependence on the α, β and γ isoforms. Those protein kinase Cs lacking C2 are insensitive to Ca^{2+}, showing significant catalytic activity in its absence (Ono, *et al.*, 1988). In addition, deletion of C2 by mutagenesis makes the kinase activity and phorbol ester binding independent of calcium (Ono, *et al.*, 1989). Despite this, the C2 region has no obvious calcium binding structure such as an E-F hand or calelectrin-like-sequence (Parker, *et al.*, 1986).

1.4.2.3. C3 / C4-region.

The protein kinase domain covers regions C3 and C4 which are highly homologous to other protein kinases (Parker, *et al.*, 1986). The ATP binding sequence Gly-X-Gly-X-X-Gly.....Lys is to be found in both C3 and C4 regions, although why two should exist is unclear. Deletion of C3 does indeed produce a protein kinase C which has no kinase activity (Kaibuchik, *et al.*, 1989) and a similar effect is produced by a point mutation in the C3 ATP binding site sequence (Ohno, *et al.*, 1990).

1.4.2.4. V1-region.

This region is most likely to represent the sequence regulating substrate selection. The fact that it varies considerably between the various isoenzymes allows for the possibility that isoenzymes can have specific substrates not phosphorylated by others.

1.4.2.5. V5-region.

The variable C-terminal region is divergent among all the protein kinase Cs and also covers the splice variation between the β_I and β_{II} isoforms.

1.4.2.6. Pseudosubstrate

Autoinhibitory domains have been detected and characterized for many protein kinases and are thought to inhibit basal kinase activity by interacting with the catalytic domain, for example, at the substrate binding site. Such a region in protein kinase C was first identified as residues 19 - 36 of PKC-α (House and Kemp, 1987) In all protein kinase Cs so far examined an analagous sequence is conserved and localized just N-terminal, about 13 - 30 residues preceding the C1 region.

This region contains one or more basic residues with a sequence paralleling the natural substrate recognition sequence for the kinase, but lacking the phosphorylatable serine or threonine residue which is replaced by alanine.

A synthetic peptide representing this region is a potent inhibitor of protein kinase C *in vitro* (House and Kemp, 1987), and substitution of alanine with serine transforms this same peptide into an excellent substrate for the enzyme. In keeping with the proposed model of pseudosubstrate autoinhibition the complete deletion of this sequence from protein kinase C led to enhanced constitutive kinase activity. The pseudosubstrate model is summarized in Figure 1.3.

While the pseudosubstrate model is widely accepted and supported it does not predict how co-factors relieve the autoinhibition or explain the co-factor independent activity observed with some substrates.

1.4.3. Heterogeneity

As mentioned previously, molecular cloning analysis and enzymatic studies have shown that a family of protein kinase C molecules exist. At least ten members of the family are now known to exist, being derived from multiple genes and alternative splicing of a single RNA

transcript. Initially four cDNA clones (encoding the α, β_I, β_{II}, and γ isoforms) were identified (Parker, *et al.*, 1986), and these were later shown to be derived from three distinct genes on human chromosomes 17(α), 16(β) and 19(γ) (Coussens, *et al.*, 1986). Subsequently homologous cDNAs for a further three isoforms, designated δ, ε and ζ, were isolated from a rat brain library using a mixture of α, β_{II} and γ cDNAs as probes under low stringency conditions (Ono, *et al.*, 1987). More recently two further cDNA clones encoding η-PKC and L-PKC have been identified (Osada, *et al.*, 1990; Backer, *et al.*, 1991), L-PKC being the human homologue of rat η-PKC.

Partial genomic analysis established that β_I and β_{II} are derived from differential splicing at the 3' end of the PKC-β gene giving proteins identical for 621 residues then 50 (β_I) and 52 (β_{II}) residues showing around 50% homology. There is also evidence for alternative splicing of PKC-ε (Schaap, *et al.*, 1990a)

Due to this extensive variation within the protein kinase C family, it is tempting to speculate that protein kinase C subspecies may have distinct roles in the processing and modulation of a variety of physiological and pathological cellular responses. However, we are still far away from a clear cut picture of the distribution and physiological function of protein kinase C isoforms within distinct signalling pathways in different cells.

Several cases of 'selectivity' in the behaviour of individual isoforms are apparent; for example PKC-γ (when compared with PKC α and β) is much less sensitive to stimulation by TPA but more sensitive to arachidonic acid (Marais and Parker, 1989) and within the group of calcium dependent protein kinase Cs the β isoform is much less dependent on the cation than the α or γ isoforms. In addition, PKC-ζ is the only isoform which has not been shown to down-regulate in response to TPA

(Ways, *et al.*, 1992). However, since the properties (or lack of) defined to date for PKC-ζ contrast with other isoforms it may be appropriate to question its alignment within the protein kinase C family. Other brief examples include the fact that protein kinase C expression in B cells is dependent on the stage of differentiation (Mischak, *et al.*, 1991) and that thyrotropin-releasing-hormone selectively down-regulates PKC-ε with no effect on PKC α or β (Kiley, *et al.*, 1990), selective compartmentalization having been suggested as an explanation for this.

Such differential signalling via individual protein kinase C isoforms would explain how from cell type to cell type, certain effects of hormones , for example insulin, can be impaired by chronic phorbol ester treatment whereas in others the same phenomenon is unaffected. This is reviewed in detail by Farese (Farese, *et al.*, 1992a), and underlines the substantial difficulties in considering together studies on protein kinase C performed using different cell systems or even from lab to lab where results can also vary significantly in this area of research.

Finally, as well as leading to the discovery of new members of the protein kinase C family, further low stringency screening of cDNA libraries with full length PKC probes may lead to the isolation of a spectrum of 'related' kinases. Indeed, the close similarity of the rac-family kinase domain to that of protein kinase C (Coffer and Woodgett, 1991) already indicates a close evolutionary relationship.

1.4.4. Expression

Since it is possible that specific effects observed with particular cell lines and not others are mediated via protein kinase C isoforms, it is important to establish the relative activity and individual pattern of expression of multiple protein kinase C isoenzymes in various cells and tissues. This has been done to a certain extent with the relatively recent

emergence of isoenzyme specific antibodies. Results obtained to date are summarized in Table 1.1. Such work will help lead towards elucidating a role for individual protein kinase C isoforms in cellular signalling, although equally if not more important than protein kinase C isoform expression, is the availability within the cell (or indeed within a distinct cellular compartment) of the required substrate. Intracellular protein kinase C receptors (RACKs) have been proposed to contribute towards control of the subcellular localization of protein kinase C isoforms (Mochly-Rosen, *et al.*, 1991).

1.4.5. Regulation of activation

The initial event in the activation of protein kinase C is the formation of an enzyme/phospholipid/(calcium) complex. The second step involves the association of diacylglycerol which promotes a conformational change resulting in activation. Although diacylglycerol may be formed in various ways, it is adequate at this stage to state that an overwhelming amount of evidence supports the idea that agonist induced phospholipid breakdown to produce diacylglycerol results in activation of protein kinase C. The precursor role of, for example, phosphatidylinositol-4,5-bisphosphate cannot be over-exaggerated as the inositol-1,4,5-trisphosphate produced in conjunction with diacylglycerol causes liberation of Ca^{2+} within the cell which will influence the activity of the α, β and γ protein kinase C isoforms as they display calcium dependence. For these isoforms, calcium acts by increasing their membrane occupancy, where they will be more effectively activated by the limiting amount of diacylglycerol produced, and elevated Ca^{2+} can infact prime protein kinase C responses in the cell (Akers and Routtenberg, 1987).

1.4.6. Substrate specificity

In vitro assay of protein kinase C originally involved measurement of ^{32}P transfer from the γ position of ATP onto histones in the presence of sonicated lipid vesicles (Kikkawa, *et al.*, 1983). Many cationic, water-soluble proteins have since been used as substrates for protein kinase C. Histone, however, remains the most extensively used artificial substrate as, unlike, for example, protamine sulphate, it displays classical dependance on anionic lipid and Ca^{2+} for protein kinase C activation. Specifically designed peptides, such as that representing part of the sequence in the pseudosubstrate domain, make ideal substrates and are now becoming more widely employed.

Results of protein kinase C activity assays are largely dependent on the choice of substrate. It seems that whilst a given substrate may be effectively phosphorylated by some isoforms, it may not be by others. However, data regarding this is often contradictory, with histone III-S, for example, having been shown to be very poorly phosphorylated by PKC-ε (activity ratio of 1:68 *cf* peptide-ε) (Schaap, *et al.*, 1989; Schaap and Parker, 1990b). Conversely, other data (M.G. Kazanietz, personal communication) suggest that histone, as well as being a good broad spectrum substrate, is one of the better PKC-ε substrates. Protamine is also used extensively as it is a good broad spectrum substrate for many protein kinase Cs, especially PKC-ε and PKC-γ.

1.5. Cross-talk between insulin and protein kinase C.

The insulin receptor can be phosphorylated by protein kinase C (Jacobs, *et al.*, 1983; Hunter, *et al.*, 1984; Bollag, *et al.*, 1986; Takayama, *et al.*, 1984). Note moreover that the insulin receptor also appears to contain additional serine / threonine residues that are substrates for other protein kinases, such as cyclic AMP dependent kinase (Roth and

Beaudoin, 1987). This, therefore, represents an important point of integration between the serine/threonine kinase dominated and tyrosine kinase mediated signalling pathways.

Phorbol ester treatment of intact cells gives a dramatic increase in phosphorylation of the insulin receptor β-subunit (Jacobs and Cuatrecasas, 1986; Takayama, *et al.*, 1984).This has been shown in hepatoma cells and adipocytes to lead to a decrease in receptor tyrosine kinase activity (Haring, *et al.*, 1986; Takayama, *et al.*, 1988). More recently, decreased insulin stimulated tyrosine phosphorylation of IRS-1 subsequent to TPA treatment of cells transfected with PKC-α has been observed (Chin, *et al.*, 1994). There is also evidence that purified protein kinase C itself can elicit the direct phosphorylation of the insulin receptor *in vitro* , with associated attenuation of insulin stimulated tyrosine kinase activity (Bollag, *et al.*, 1986). In these experiments and others, no change in receptor binding was observed. However, in some cases phorbol ester treatment of cells does appear to decrease insulin binding and there is some evidence suggesting this is due to increased internalization of the receptor (Haring, *et al.*, 1986; Grunberger and Levy, 1990).

Several distinct sites on the insulin receptor β-subunit appear to become phosphorylated after phorbol ester treatment, the phosphorylated serine residues appearing to be identical to those whose phosphorylation is promoted by insulin itself (Lewis, *et al.*, 1990). A distinctive difference is the prominent increase in phosphothreonine achieved by phorbol ester treatment.

Inspection of the insulin receptor β-subunit allows for the identification of possible sites where protein kinase C might phosphorylate. These include:

Ser 1315 - In the autophosphorylation domain B in the C-terminal region,

Ser 1294 - In the functionally sensitive C-terminal region, and

Thr 1336 - In the extreme C-terminal region.

There are also four serine residues at 951-964 (in tyrosine 960 domain) on the insulin receptor β-subunit although none of these has a basic residue one removed on the N-terminal side as is usual for C-kinase substrates, hence these residues are not phosphorylated by protein kinase C.

From studies employing radiosequencing, comparison with synthetic peptides and use of monoclonal antibodies (Kasuga, *et al.*, 1989), threonine 1336 seems to be preferentially phosphorylated (Lewis, *et al.*, 1990). This residue appears to be of crucial functional importance as deletion of the last 43 C-terminal amino acids of the β-subunit (incorporating threonine 1336), although failing to affect kinetics of binding, endocytosis, recycling or auto / substrate phosphorylation, did fail to mediate insulin's ability to stimulate either glucose uptake or glycogen synthase (Maegawa, *et al.*, 1988; McClain, *et al.*, 1988) upon transfection into fibroblasts.

Several studies have suggested insulin may exert its actions on target cells by activating protein kinase C (Considine and Caro, 1993), although this is an area of considerable controversy. This possibility grew from the realization that insulin was intimately involved in controlling similar key physiological processes in both growth and development as protein kinase C, hence it was natural to focus on possible interactions between their signalling pathways.

Although insulin and protein kinase C both phosphorylate, for example, the MARCKS protein (Roth and Beaudoin, 1987) the pattern of substrate phosphorylation elicited by insulin and TPA shows some differences (Farese, *et al.*, 1992). Thus we must ask why they should induce different patterns of substrate phosphorylation? The following possibilities are proposed as explanations -

a. Insulin activates other protein kinases in addition to protein kinase C.

b. Insulin activates protein phosphatases.

c. Diacylglycerols formed from PIP_2 hydrolysis as opposed to PC hydrolysis differentially activate protein kinase Cs.

d. Insulin increases diacylglycerol levels in different subcellular compartments.

e. Protein kinase C isoforms differ in their activation by insulin, diacylglycerol, TPA, and other activators.

The insulin receptor cannot activate inositol phospholipid metabolism by causing breakdown of phosphatidylinositol-4,5-bisphosphate to IP_3 and diacylglycerol. Insulin does however rapidly stimulate diacylglycerol production, as measured by ^{3}H-glycerol labelled diacylglycerol formation (Iskizuka, *et al.*, 1990). Insulin can elicit this increase in diacylglycerol levels in some cells apparently through increasing *de novo* PA synthesis (from glycerol 3-phosphate and fatty acylCoA) (Farese, *et al.*, 1987) and phospholipid hydrolysis (Farese, *et al.*, 1985; Nair, *et al.*, 1988) as well as hydrolysis of inositol glycolipids (Saltiel, *et al.*, 1986). Once again, controversy surrounds the question of whether these insulin induced increases in diacylglycerol levels result in protein kinase C activation (Cooper, *et al.*, 1987; Blackshear, *et al.*, 1991). A major reason for the prevailing controversy is that results can vary greatly from system to system and even between different research groups. It is likely that the actions of insulin mediated by protein kinase C are restricted to certain cells.

More and more evidence would suggest that protein kinase C is intimately involved in insulin signalling. For example, elevated glucose has previously been reported to activate protein kinase C, consistent with a potential role of protein kinase C in modulating insulin's action in diabetes (Lee, *et al.*, 1989). Recent data show that glucose induces an increased serine phosphorylation and subsequent decreased tyrosine kinase activity

of the insulin receptor as well as decreased phosphorylation of IRS-1 (Berti, *et al.*, 1994). This effect is paralleled by an overall increase in protein kinase C activity and translocation of the α, δ, ε and ζ isoforms to the membrane, and can be inhibited by H7 (Berti, *et al.*, 1994).

Other examples include recent work by Farese (Farese, *et al.*, 1992) where insulin stimulates the translocation of PKC- α, β, γ, ε and ζ in various tissues studied, as does TPA, although differences do occur; insulin seemingly having a higher affinity for PKC-β. Secondly, studies using HL-60 cells highlight the fact that TPA at least partially replaces the requirement for insulin in cell proliferation (Trayner and Clemens, 1992). Thirdly, using TPA to down-regulate protein kinase C inhibits (60 - 87%) subsequent insulin stimulated deoxyglucose uptake (Tanti, *et al.*, 1989).

1.6. Cyclic AMP metabolism

As cyclic AMP is an important intracellular second messenger, this necessitates that its metabolism must be tightly regulated. Systems for both synthesis and degradation have evolved to highly sophisticated levels. Controlling the synthesis of cyclic AMP from adenosine are the family of adenylate cyclase isoforms, degradation of cyclic AMP to 5'-AMP being managed by a series of phosphodiesterase isoenzymes. These are now discussed in further detail.

1.7. Adenylate cyclase

Adenylate cyclase exists as a series of isoenzymes, at least eight of which are now known, there being 70-75% sequence homology between them. All adenylate cyclase isoforms have similar overall structure, with 12 hydrophobic membrane spanning sequences, two hydrophilic intracellular domains essential for activity and both N- and C-termini intracellularly located (Kelley Bentley and Beavo, 1992). These

isoenzymes are subdivided into those whose activity is stimulated by calcium-calmodulin and those whose are not. They can also differ in their sensitivity to the α-subunit of G_s as well as to βγ-subunits.

The receptor regulated adenylate cyclase system can be considered as being composed of several functional units: i) a receptor which binds the stimulatory (eg. β-adrenoceptor) or inhibitory (eg. α_2-adrenoceptor) hormone or neurotransmitter. ii) G-Proteins. The stimulatory G-protein (G_s) or the inhibitory G-protein (G_i). The α-subunits of G_s and G_i are highly homologous, as expected, in the GTP binding domain but have divergent sequences in C-terminal regions which participate in G-protein receptor and effector interactions, with the βγ-subunits being so similar as to be interchangeable. iii) the catalytic unit (C) of the enzyme which is a hydrophobic protein having 12 transmembrane domains with the catalytic site situated on the cytoplasmic side. The catalytic unit is highly conserved between different tissues and species as evidenced by the equipotent association of individual components of the system from different sources (Feder, *et al.*, 1986).

There are two proposed mechanisms whereby adenylate cyclase is inhibited by G_i. The first is via a direct effect of α–i on the catalytic unit of the enzyme, while the second is via release of excess βγ-subunits upon G_i dissociation which inhibits G_s dissociation and activation.

As would be expected from sequence inspection, it also appears that phosphorylation of a number of forms of adenylate cyclase can be mediated by protein kinase C (Yoshimasa, *et al.*, 1987), although the regulatory function, if any, of such phosphorylation has yet to be established. Similarly, phorbol ester treatment of cells (Simmoteit, *et al.*, 1991) has also been shown to increase the incorporation of ^{32}P into the catalytic unit of adenylate cyclase 5-fold, an effect again resulting in activation of the enzyme. Phorbol ester-mediated activation of adenylate

cyclase, however, is not a universal observation. This might reflect current awareness of multiple forms of both protein kinase C and adenylate cyclase. Indeed, futher recent studies suggest that adenylate cyclase isoforms do differ in their sensitivity to phosphorylation by protein kinase C, with types AC-I, AC-II and AC-III being most susceptible (Jacobowitz, *et al.*, 1993).

1.8. G-proteins

The family of heterotetrameric guanine nucleotide-binding proteins play an essential role in transducing receptor mediated signals for many ligands. G-proteins are composed of three distinct subunits termed α (M_r 39 - 46 kDa), β (37 kDa) and γ (8 kDa). The α-subunits have a single, high affinity binding site for guanine nucleotides such that when GDP is bound the α-subunit binds tightly to βγ and is inactive. The GTP-bound form of α dissociates readily from βγ and becomes active as an effector in a given system. All α-subunits possess intrinsic GTPase activity, allowing hydrolysis of the terminal phosphate on bound GTP to return the system to an inactive state. To date cDNAs for 21 distinct α-subunits (the products of 17 genes) have been cloned.

Bacterial toxins act by virtue of their effect on G-proteins, a fact that has been well exploited as an experimental tool. Cholera toxin catalyses the transfer of the ADP-ribose moiety of NAD to a specific arginine residue in certain α-subunits (eg. G_s). This modification activates these G-proteins by inhibiting their GTPase activity. Pertussis toxin ADP-ribosylates specific cysteine residues near the C-terminus on some α-subunits (eg. G_i), which prevents receptor mediated activation of these G-proteins.

1.9. Phosphodiesterases.

1.9.1. Introduction and background.

Some of the oldest and best understood signal transduction pathways use cyclic nucleotides as second messengers. Compared with our understanding of cyclic nucleotide synthesis, much less is known about the complex regulation of degradation, despite the fact that phosphodiesterase activity was first identified and characterised over twenty years ago, almost immediately after the discovery that cyclic nucleotides existed in cells. It was only more recently that the occurrence of a large number of isoenzymes was established along with the fact that these were differentially expressed and regulated from cell to cell.

Cyclic nucleotide phosphodiesterases show specificity for purine cyclic nucleotide substrates and, as the majority of cells are unable to extrude cyclic AMP, represent the only known means of lowering intracellular cyclic AMP and cyclic GMP levels by catalyzing their hydrolysis. This activity plays a crucial role in determining steady state cyclic nucleotide levels within cells, hence these enzymes are of interest therapeutically as a means of manipulating cellular function.

The development of a sensitive assay to measure phosphodiesterase activity (Thomson and Appleman, 1971) has facilitated advances in the understanding of this class of enzymes. The basis of the assay is to use a radioactive tracer, allowing assay of enzyme activity at subsaturating concentrations of substrate. An initial incubation allows sample phosphodiesterase to catalyse conversion of cyclic-3',5'-AMP to 5'-adenosine monophosphate. A second incubation uses an excess of the enzyme 5'-nucleotidase, present in snake venom, to convert 5'-adenosine monophosphate to 5'-adenosine. This step allows more efficient separation of reaction product from unreacted substrate, making quantification more accurate. Separation is achieved using Dowex-1-chloride anion exchange

resin which selectively binds the cyclic nucleotide and quantification is by liquid scintillation counting of the isolated product, namely [^{3}H]5'-adenosine.

Phosphodiesterases are found in cytoplasmic fractions as well as in association with cellular membranes, these enzymes differing in their kinetic characteristics, substrate affinities, responsiveness to effectors and inhibitors and mechanisms of regulation. The fact that cyclic nucleotide phosphodiesterases are localized in distinct subcellular fractions in most cells is a feature which greatly influences both enzyme action and inhibitor effectiveness. As an example, differences in subcellular localization of phosphodiesterases in cardiac sarcoplasmic reticulum between species has been proposed as an explanation for the variable contractile responses to milrinone in different species (Weishaar, *et al.*, 1987).

Some suggestions propose that while there can be no arguing about the role of phosphodiesterases in controlling cyclic nucleotide levels within cells they may have another, perhaps even more important role. Since we know that cyclic AMP and cyclic GMP turn over very rapidly in most cells (Goldberg, *et al.*, 1983) and that the enthalpy associated with their hydrolysis is very large (10 - 14 kcal/mol) (Rudolph, *et al.*, 1971) it has been proposed that this source of energy is of crucial importance in allowing the cell to do work. The analogy drawn is that a similarly large number of ATPase isoenzymes have evolved to couple the high energy of ATP hydrolysis to many different processes so why not a similar story for phosphodiesterases?

1.9.2. Nomenclature and classes.

All classes of phosphodiesterase isoenzymes are thought to be coded for by related but distinct members of a larger supergene family as they consist of highly conserved and homologous catalytic domains yet

have multivariant regulatory (e.g. calmodulin or cyclic GMP binding) and functional domains.

We still do not know precisely how many different phosphodiesterase isoenzymes are present in most cells. At present there are at least 25 tentatively identified phosphodiesterase isoenzymes, a number that is sure to expand. Information gained using selective drugs and from cloning and sequencing experiments suggests that a large family of at least five distinct isoenzymes exists.

The classification of phosphodiesterase isoenzymes used here is that proposed by Beavo (Beavo and Reifsnyder, 1990) and is as follows:

Class I - Calcium / Calmodulin-dependent phosphodiesterases.

Class II - Cyclic GMP stimulated phosphodiesterases.

Class III - Cyclic GMP inhibited phosphodiesterases.

Class IV - Cyclic AMP specific phosphodiesterases.

Class V - Cyclic GMP specific phosphodiesterases.

Each of the above classes is composed of varying numbers of subfamilies with further subdivision of these also being necessary due to alternative splicing. As a guide it appears that any of the above classes are related to each other by approximately 15 - 20% sequence identity, the bulk of which occurs in one catalytically active domain. Within classes all enzymes show greater than 70% identity to each other (Hall, 1993).

Currently available drugs and antibodies are able to distinguish between the five main classes and to a lesser extent within these classes, although with the massive potential for therapeutic exploitation of such drugs more and more effort is being directed towards this goal.

Many phosphodiesterases now appear to be regulated by cyclic nucleotide- or Ca^{2+}-dependent phosphorylation. In this way they represent

a vital stage where integration of cellular responses mediated by a variety of signals (ie. cross-talk) can occur.

1.9.3. Structure-function relationships among cyclic nucleotide phosphodiesterases

The multiple intracellular phosphodiesterase enzymes listed above account for the total cyclic nucleotide phosphodiesterase activity measured in mammalian cells. The different classes can be distinguished by their molecular weight, substrate preference, sensitivity to drugs, amino acid sequence and mode of regulation. The relatively large size of these isoenzymes and the complexity of their regulatory features suggest that most phosphodiesterases are composed of multiple domains. This multidomain character and the diversity of regulatory features provide intriguing questions at the molecular level and at the level of control of cyclic nucleotide metabolism.

The homology among different phosphodiesterase isoenzymes was first demonstrated by comparisons of partial amino acid sequence data from two mammalian isoenzymes, the cyclic GMP stimulated phosphodiesterase and the 61kDa Ca^{2+}/calmodulin dependent phosphodiesterase (Charbonneau, *et al.*, 1986). Since these studies, the sequence of several different phosphodiesterase isoenzymes have been deduced from the nucleotide sequence of cDNA clones (Charbonneau, 1990). The bulk of phosphodiesterases are related by bearing a 250 residue conserved segment representing the catalytic domain (Charbonneau, *et al.*, 1986). This conserved region appears to be a unique feature of phosphodiesterases since searches of sequence databases reveal no other proteins with significant similarities.

1.9.4. Class I

Calcium/calmodulin dependent phosphodiesterases

Calmodulin-stimulated cyclic nucleotide phosphodiesterase was discovered in close parallel with calmodulin itself during the late 1960's and early 1970's (Appleman, *et al.*, 1985). Initially a calcium stimulated phosphodiesterase activity was established which was in turn seen to be enhanced by a protein-activating factor. Subsequently, in the late 1970's Cheung and co-workers termed this protein-activating factor calmodulin (Cheung, *et al.*, 1978).

This class of phosphodiesterase, the first to be purified and characterized, is now one of the most extensively studied and best characterized. A lot of attention was focussed on it simply because it was one of the first known functions of calmodulin (Cheung, 1980). Originally, it was thought that this was an enzyme of ~60kDa subunit molecular weight, which appeared to exist as a homodimer in the native state (Morrill, *et al.*, 1979). More recently, with the advent of specific monoclonal antibodies allowing the use of immunoaffinity chromatography, we know that Ca^{2+}/calmodulin-stimulated phosphodiesterases exist as tissue specific and immunologically distinct isoenzymes (Rossi, *et al.*, 1988; Sharma and Wang, 1986). Despite exhibiting clear differences, bovine brain and bovine heart class I phosphodiesterases, for example, have sequences that are almost identical, excepting a small insertion proximal to the N-terminus in the brain enzyme (Charbonneau, *et al.*, 1986). This ratifies the fact that these, along with other varying forms of this class, are isoenzymes arising from differential splicing of mRNA.

This class of enzyme has a widespread, yet not ubiquitous, distribution although is generally present in very low abundance. By far the best source is mammalian brain, yielding approximately 10mg/kg

tissue. In most studies the bulk of class I phosphodiesterase activity is cytosolic although small amounts can be found associated with particulate fractions (Grab, *et al.*, 1981). On the basis of kinetic criteria, the calcium/calmodulin-stimulated phosphodiesterase isoforms may be divided into two general groups; a group showing high affinity for cyclic GMP but low affinity for cyclic AMP, and a group showing high affinity for both cyclic nucleotides.

Although these enzymes are usually referred to as Ca^{2+}/calmodulin-stimulated it is important to note that other metal ions such as Mn^{2+}, Hg^{2+}, Pb^{2+} and Cd^{2+} can substitute for calcium in this role. Because of its well established signalling role calcium is considered the physiological metal activator; however the possibility that heavy metal toxicity is mediated by calmodulin has been suggested (Chao, *et al.*, 1984). In most tissues the amount of calmodulin (estimated at 22μM, for example, in bovine brain) is in excess of the quantity of phosphodiesterase (Hansen and Beavo, 1986), such that the limiting factor for stimulation is the concentration of free calcium. It is also interesting to note that phosphatidylserine (viz. protein kinase C story) has been shown to activate this PDE, albeit in a calcium independent manner. However, the effective concentration of phosphatidylserine required to stimulate the enzyme is above the critical micellar concentration (Gietzen, *et al.*, 1982).

Looking at how broadly used the inhibitors of Ca^{2+} / calmodulin-stimulated phosphodiesterases are (see over) gives a clear indication of the importance of this particular class of enzyme (Prozialeck, 1983; Asano and Hidaka, 1984).

Inhibitor	Clinical Use
Trifluoperazine	Antipsychotic agent
Vinblastin	Antitumour agent
W7	Muscle relaxant
Vinpocetine	Coronary artery relaxant
Imipramine	Antidiarrheal agent
Cyclosporin	Immunosuppressant.

1.9.5. Class II

Cyclic GMP stimulated phosphodiesterases

This class of enzyme was originally described in rat liver, although further investigation and characterization has shown it to be most abundant in adrenal glands (Beavo, *et al.*, 1970). It is named due to the fact that relatively low concentrations of cyclic GMP will increase the rate of cyclic AMP hydrolysis. It represents a potentially important point of interaction between two second messenger systems, namely cyclic AMP and cyclic GMP. In this respect it resembles the Ca^{2+}/calmodulin-stimulated phosphodiesterase which impinges on calcium and cyclic nucleotide utilizing systems. In this class of phosphodiesterases there exists a tandem repeat sequence within the N-terminal portion which represents the site of cyclic GMP binding (Trong, *et al.*, 1990).

Allosteric regulation of both cyclic AMP and cyclic GMP hydrolysis is the main feature of this class of phosphodiesterase. Hydrolysis of both cyclic nucleotides displays positive co-operative behaviour (Yamamoto, *et al.*, 1983). As well as being the most potent activator, cyclic GMP is the preferred substrate (Erneaux, *et al.*, 1981) for this phosphodiesterase (ie. K_m cGMP (10-15μM) < K_m cAMP (33-40μM)), although in early studies the enzyme was initially on occasion called 'cyclic GMP stimulated cyclic AMP phosphodiesterase'. The

contribution of membrane and cytosolic forms to total activity has been found to differ significantly between tissues, soluble activity accounting for 80% of total in adrenal tissue, 60% in spleen, 40% in heart and 25% in testis (Hurwitz, *et al.*, 1984).

The enzyme isolated from bovine sources appears to have a subunit molecular weight of between 102-107kDa (Yamamoto, *et al.*, 1983) whilst the rat liver enzymes have smaller subunit molecular weights of ~67kDa (Pyne, *et al.*, 1986). Native molecular weight determinations indicate that all bovine and rat forms are similar in that they exist as dimers.

Relatively little is known concerning the regulation of the cyclic GMP stimulated phosphodiesterase or its physiological role in the control of cyclic nucleotide metabolism in intact cells. One suggested role for this enzyme is being the means by which insulin lowers intracell ular cyclic AMP in hepatocytes (Pyne and Houslay, 1988). It is also appealing to suggest these enzymes represent the main cellular receptor for cyclic GMP and can act as a point of crossover between cyclic AMP and cyclic GMP metabolism, one nucleotide regulating the metabolism of the other. Established roles for which evidence does exist include regulation of cardiac calcium channels (Fischmeister and Hartzell, 1991) and regulation of ACTH induced aldosterone secretion in glomerulosa cells (MacFarland, *et al.*, 1991).

Specific inhibitors for this class of enzymes have not yet flourished, although AR-L57, for example, exhibits a somewhat greater inhibitory effect on cardiac PDE-II than on PDE-I or PDE-III (Quade, *et al.*, 1984).

1.9.6. Class III

Cyclic GMP inhibited phosphodiesterase

This class of phosphodiesterase was one of the first to be classified. It exhibited a low K_m for both cyclic AMP and cyclic GMP but a much greater V_{max} for cyclic AMP than for cyclic GMP (Appleman and Terasaki, 1975), such that cyclic GMP was hydrolyzed at less than 1% of the rate of cyclic AMP by these enzymes. Hence, they are generally referred to as cyclic AMP specific.

This class of phosphodiesterase have been purified from many tissues, such as adipose (Degerman, *et al.*, 1987) and liver (Pyne, *et al.*, 1987). These enzymes are represented by single bands upon non-denaturing gel electrophoresis, and appeared as single peaks of ~110kDa upon Sephadex G-200 chromatography (Degerman, *et al.*, 1988; Degerman, *et al.*, 1987). Considering all tissues so far examined, this M_r value ranges from 105-135kDa, with these polypeptides in turn being proteolyzed to produce 30-80kDa fragments upon SDS-PAGE.

The apparent subcellular localization of the phosphodiesterase varies depending on the tissue, being cytosolic in human platelets (MacPhee, *et al.*, 1986) and membrane bound in rat liver (Heyworth, *et al.*, 1983a). Localization also varies within tissue depending on the species in question, being membrane bound in humans and soluble in guinea pig (Reeves, *et al.*, 1987).

The activity of this class of enzymes may be modulated by hormones in, for example, liver (Heyworth, *et al.*, 1983a) and adipocytes (Degerman, *et al.*, 1987). In platelets it is the target of many pharmacological agents that inhibit platelet aggregation (MacPhee, *et al.*, 1986). In addition, a number of cardiotonic agents such as milrinone and amrinone, which act as positive inotropes, are potent and selective inhibitors of this class of phosphodiesterase (Harrison, *et al.*, 1986).

1.9.7. Class IV

Cyclic AMP specific phosphodiesterases

Initially, these were identified as activities that elute at high salt concentrations as the third peak of activity from DEAE-cellulose columns ('PDE-III' or 'low Km' cyclic AMP phosphodiesterase) and show a marked preference for cyclic AMP as a substrate. The best characterized inhibitor for this series is rolipram, such that this class is on occasion referred to as rolipram-sensitive cyclic AMP phosphodiesterase. It would appear that within this species are both soluble and membrane bound forms, displaying quite different subunit molecular weights. Further subdivision of this group has relied upon extensive purification protocols and improved separation methods that allow separation from the cyclic GMP stimulated phosphodiesterase, an activity that could serve to mask the presence of these isoenzymes (Reeves, *et al.*, 1987)

Cyclic AMP hydrolysis is insensitive to concentrations of cyclic GMP, distinguishing these from cyclic GMP inhibited phosphodiesterases which are also relatively cyclic AMP specific. Purification, however, must account for contamination and hence, for example, chromatography of liver supernatant on N^6-$H_2N(CH_2)$-cyclic AMP agarose separated the cyclic GMP stimulated phosphodiesterase from the 'low K_m' cyclic AMP phosphodiesterase activity which did not bind to this matrix. Further chromatography on Ultrogel AcA-34 separated the cyclic GMP inhibited phosphodiesterase from the cyclic GMP insensitive phosphodiesterase (Yamamoto, *et al.*, 1984). It would appear that within this species are both soluble and membrane bound forms, each displaying quite different subunit molecular weights.

One enzyme in this class identified in rat liver membranes is associated exclusively with the plasma membrane (Marchmont and Houslay, 1980) and has thus been termed the peripheral plasma membrane

phosphodiesterase. Interestingly, it can be activated by phosphorylation triggered by insulin (Marchmont and Houslay, 1980).

1.9.8. Class V

Cyclic GMP specific phosphodiesterases

Cyclic GMP binding cyclic GMP specific phosphodiesterases are a heterogenous family of enzymes consisting of several subgroups, including the widely distributed cyclic GMP stimulated cyclic nucleotide phosphodiesterase (Erneaux, *et al.*, 1981), the retinal cyclic GMP phosphodiesterases in both rods and cones (Gillespie and Beavo, 1988), a cyclic GMP specific phosphodiesterase from dictyostelium discoideum (Dicou and Brachet, 1980) and cyclic GMP specific phosphodiesterase in lung (Francis, *et al.*, 1980) and platelets (Hamet, *et al.*, 1984). Indeed, the cyclic GMP specific phosphodiesterases represent a major portion of phosphodiesterase activity in mammalian heart, liver, adipose tissue and adrenal gland.

These phosphodiesterases exhibit high specificity for cyclic GMP with, for example, bovine lung cyclic GMP specific phosphodiesterase hydrolysing cyclic AMP at a rate which is approximately 100 times slower than the hydrolytic rate for cyclic GMP (Francis, *et al.*, 1980). In addition to their high specificity for cyclic GMP over cyclic AMP, class V phosphodiesterases are related by several other common features. These include, noncatalytic cyclic GMP binding sites of relatively high affinity and unknown function, similar catalytic subunit size and association into dimers, micromolar K_m values for cyclic GMP and submicromolar K_i values for inhibition by dipyridamole, a relatively specific inhibitor of this class of enzymes.

1.9.9. Tissue distribution.

Examination of the tissue distribution of individual isoenzymes have revealed that there are marked regional differences in the distribution of phosphodiesterase isoenzymes, with some isoenzymes being widespread and others having only limited distribution. The type V_{B1} isoenzyme, for example, is only present in high concentration in the outer segments of retinal rod cells where it is involved in photoreceptor responses (Hurwitz, *et al.*, 1985). Conversely type III isoenzymes are found as physiologically important enzymes in heart, platelets, smooth muscle, adipocytes and liver (Hall, 1993). In addition, compartmentalization within tissues is viewed as a means to explain discrepancies amongst published results.

1.9.10. Inhibitors

Having used methylxanthines for decades to treat asthma, only in 1962 did Butcher & Sutherland discover their mechanism of action. Naturally this stimulated a great deal of enthusiasm as regards the possible therapeutic potential of such drugs. Unfortunately the ensuing research provided few therapeutically effective drugs. Recent advances however, have rekindled interest such that development of selective inhibitors remains an active area of research and indeed pharmaceutical companies have been rewarded with several drugs exhibiting selectivity for individual phosphodiesterases. Such drugs have been exploited as smooth muscle relaxants (especially blood vessels), cardiotonic agents, antithrombotic compounds and antidepressants. By far the most established are type III inhibitors, reflecting the pharmaceutical industries interest in identifying novel positive inotropic agents.

Since the phosphodiesterase isoenzyme(s) of physiological importance in controlling tissue cyclic nucleotide content vary in different

tissues, it follows that useful inhibitors must almost always be at least partially selective. If this is achieved however, the potential benefits of such selectivity are enormous.

1.9.11. Functional significance and pharmacological implications of multiple isoforms.

Given our current knowledge of phosphodiesterase isoenzymes an obvious question to ask would be what is the functional significance of there being so many isoenzymes responsible for cyclic nucleotide degradation? Evidence to date suggests that many of the isoenzymes are differentially expressed in selected cells and that they are crucial for function of that particular cell. Another possible reason for such diversity is to allow for differential regulation of individual isoenzymes by discrete signalling processes. In addition, individual forms may respond at different rates, allowing for short and long term effects in response to one signal.

1.9.12. Summary and conclusions.

Much more information is needed to rationalise the exact number of different isoenzymes in the supergene family. Only when this is accomplished and their characterization complete will we fully understand the regulation of cyclic nucleotide degradation in cells by these enzymes. Even more importantly, it remains to be established exactly which isoenzyme genes are translated and expressed in individual cells, a potentially important factor in drug tissue selectivity. The existence of a large number of phosphodiesterase isoenzymes and their differential expression does however create scope for precise and even cell specific regulation of intracellular cyclic nucleotide levels.

The wealth of information discussed above will hopefully highlight the fact that cyclic nucleotide phosphodiesterases provide one of the main mechanisms for integrating, at an intracellular level, the control of multiple signals that may regulate a particular cellular response. This important feature of cell signalling is now being appreciated and the contribution of phosphodiesterases should not be understated.

1.10. Influence of insulin on cyclic AMP metabolism

Insulin has been shown in various tissues and cell culture systems to influence intracellular cyclic AMP concentrations. This includes evidence for its modulation at the level of the receptor, G-protein, adenylate cyclase catalytic subunit and cyclic AMP phosphodiesterase.

1.10.1. Insulin's modulation of adenylate cyclase

Whilst insulin cannot modify unstimulated cyclic AMP levels in intact hepatocytes (Pilkis, 1970) it is well established that insulin can lower cyclic AMP levels which have been previously raised by another hormone. This effect has been suggested to be mediated by an inhibitory effect on adenylate cyclase (Heyworth and Houslay, 1983) and by an activation of cyclic AMP phosphodiesterase (Beavo, 1990). Insulin was shown to inhibit adenylyl cyclase (Heyworth and Houslay, 1983). The GTP dependent nature of this inhibitory effect, its toxin sensitivity and its abolition by glucagon desensitization suggest involvement of a G-protein (Heyworth, *et al.*, 1986; Houslay, 1990). This suggests that insulin exerts its action on adenylate cyclase via a G-protein. Insulin treatment of cells has also been reported to stimulate a high affinity GTPase in human platelets (Gawler and Houslay, 1987). Evidence such as this clearly suggests an interaction of the insulin receptor with the G-protein system. Finally, insulin has been shown to phosphorylate and attenuate functioning

of the β-adrenoceptor in smooth muscle cells (Hadcock, *et al.*, 1992) whilst insulin treatment of leukocytes acutely increases the number of cell surface β-adrenoceptors (Sager, *et al.*, 1990).

1.10.2. Insulin's control of phosphodiesterase activity

One potential mechanism for the anti-lipolytic actions of insulin in adipose tissue (Steinberg, *et al.*, 1975) and its anti-glycogenolytic actions in liver (Denton, *et al.*, 1981) is its ability to decrease cyclic AMP levels by stimulating the activity of a 'low Km' cyclic AMP and cyclic GMP hydrolysing particulate phosphodiesterase activity that was inhibited by cyclic GMP and cilostamide (Schmitz-Peiffer, *et al.*, 1992). Evidence for insulin regulation of phosphodiesterase activity was established in 1985 (Beebe, *et al.*, 1985). Here Beebe *et al.* showed that insulin could selectively antagonize the lipolytic glycogenolic actions of a variety of analogues of cyclic AMP, and this correlated with the ability of these analogues to serve as phosphodiesterase substrates, insulin antagonizing the action of those which were readily hydrolysed. In hepatocytes, for example, insulin can decrease cyclic AMP levels which are elevated by previous challenge with glucagon (Heyworth, *et al.*, 1983). Subsequently, others have also concluded that the anti-lipolytic effect of insulin in human adipocytes requires phosphodiesterase activation (Lonroth and Smith, 1986; Kather and Scheureer, 1987).

In hepatocytes three phosphodiesterase subtypes are known to be activated by insulin and whilst the molecular basis of this is uncertain, the activation of a 52kDa plasma membrane phosphodiesterase is thought to be achieved by tyrosyl phosphorylation (Pyne, *et al.*, 1989). Further investigation has revealed that insulin regulates at least two other phosphodiesterases in liver, both of which are membrane bound. The peripheral plasma membrane phosphodiesterase is activated by insulin

(Marchmont and Houslay, 1980) and belongs to the type IV cAMP specific, cGMP insensitive class of phosphodiesterase. Dense vesicle phosphodiesterase activity is also raised by insulin, this enzyme belonging to the type III cGMP inhibited cAMP phosphodiesterase class (Heyworth, *et al.*, 1983). A similar increased cyclic GMP inhibited cyclic AMP phosphodiesterase activity is also seen in platelets in response to insulin. This was due to insulin induced serine phosphorylation of the enzyme (Lopez-Aparicio, *et al.*, 1992). As expected, therefore, the insulin stimulated protein kinase involved did not absorb to anti-phosphotyrosine antibodies (Lopez-Aparicio, *et al.*, 1992).

1.11. The role of protein kinase C in the control of cyclic AMP metabolism.

1.11.1. Introduction

The interaction of protein kinase C with components of the cyclic AMP metabolizing system has been documented in many instances and current knowledge is summarized below. The level of importance this has in regulating endogenous intracellular cyclic AMP levels is still uncertain.

1.11.2. Interaction with inhibitory G-proteins

Phosphorylation of purified 'G_i' by protein kinase C has now been shown to occur *in vitro* (Katada, *et al.*, 1985) but only when in its holomeric, GDP-bound state (O'Brien, *et al.*, 1987). This suggests that phosphorylation may serve to stabilize the inactive, GDP-bound form of G_i. Indeed, work in hepatocytes (Bushfield, *et al.*, 1990) shows that the rate of G_i-2 phosphorylation closely matches the rate of loss of G_i function. Further supporting this theory is work showing that ADP-ribosylation of α-G_i, which can only occur on the GDP-bound holomeric

form, was significantly increased in cells pre-treated with phorbol ester for 15 minutes (Choi and Toscano, 1988).

Inactivation of G_i as a mechanism of phorbol ester mediated sensitization of adenylate cyclase was first proposed by Jakobs and co-workers (Katada, *et al.*, 1985). Such a mechanism is supported by work where pertussis toxin treatment of cells, to inactivate G_i, results in potentiation of the capacity of stimulatory hormones to activate adenylate cyclase, presumably via removal of a 'tonic' inhibitory input (Bushfield, *et al.*, 1990; Senogles, *et al.*, 1990). In all studies documented, the augmentation in hormonal stimulation of adenylate cyclase caused by phorbol esters was of similar magnitude to the potentiating effects of pertussis toxin (Abou-Samra, *et al.*, 1987). Perhaps more importantly the effects of phorbol esters and pertussis toxin were not additive, suggesting a similar target for action in each case, namely inactivation of G_i.

It must be stated that the ability of phorbol esters to elicit the phosphorylation of G_i is not a ubiquitous phenomenon. Speculation as to why this is so could be that a particular isoform of protein kinase C is required, that G_i-2 is present principally in a dissociated state and is therefore a poor substrate or that cell specific co-factors are needed for protein kinase C and G_i-2 to interact productively.

1.11.3. Interaction with the catalytic subunit of adenylate cyclase

Protein kinase C has been shown to phosphorylate adenylate cyclase, conferring a 'sensitization' on the enzyme, with enhanced GTP-, Na(Al)F-, PGE_1- and isoprenaline-stimulated activity observed (Jacobowitz, *et al.*, 1993). The extent of such an effect varied between subtypes of adenylate cyclase, with the effect on the type 2 enzyme being most pronounced (Jacobowitz, *et al.*, 1993). Treatment of purified

adipocyte plasma membranes with purified protein kinase C has also been shown to magnify adenylate cyclase activity (Nagshineh, *et al.*, 1986).

As discussed above, there is the possibility with such an observation that the 'sensitizing' effect of protein kinase C on adenylate cyclase in cells is due to withdrawl of a 'tonic' inhibitory input from G_i. Reconstitution of the purified enzyme with G_s seems the logical step to determine if this is so.

Once again, phorbol-ester mediated activation of adenylate cyclase is not a ubiquitous observation and may reflect differences in sensitivity of adenylate cyclase isoforms as well as the complement of protein kinase C isoforms in the cells studied. After treating NIH3T3 cells with phorbol ester, the fact that γ-PKC has a facilitatory and α-PKC an inhibitory effect on forskolin stimulated cAMP levels (Gusovsky and Gutkind, 1990) highlights again how the protein kinase C isoform complement in a given cell is of crucial importance.

1.11.4. Interaction with cyclic AMP phosphodiesterases

Treatment of hepatocytes with phorbol esters is known to potentiate glucagon stimulated increases in cyclic AMP. This effect is abolished, and in fact reversed, in the presence of IBMX (Irvine, *et al.*, 1986). This sensitizing action seems to be due to protein kinase C activation and therefore possibly phosphorylation and inhibition of cyclic AMP phosphodiesterase activity. Phorbol ester induced potentiation of hormone stimulated increases in intracellular cyclic AMP concentrations due to phosphodiesterase inhibition has since also been shown in anterior pituitary cells (Abou-Samra, *et al.*, 1987).

Despite the abtruse nature of the above experiments they clearly demonstrate the profound effects that activation of protein kinase C has on intracellular cyclic AMP concentrations.

Figure 1.1. An introduction to signal transduction

Depicted in this diagram are some of the major cellular signal transduction systems. In addition, some of the agonists which act via these systems are also listed.

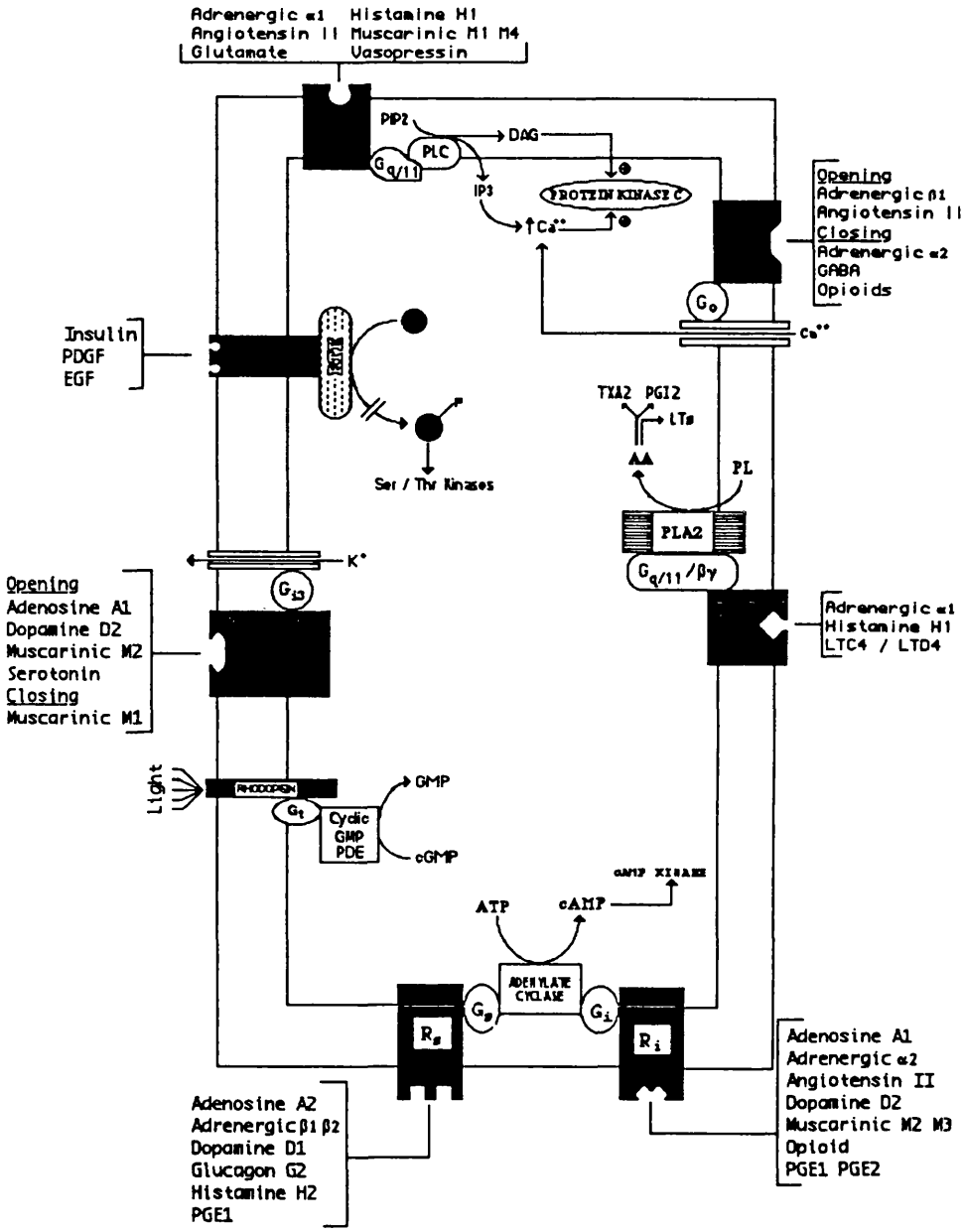

Adrenergic α1 Histamine H1
Angiotensin II Muscarinic M1 M4
Glutamate Vasopressin
PIP2
DAG
PLC
$G_{q/11}$
IP3
PROTEIN KINASE C
$\uparrow Ca^{++}$
Opening
Adrenergic β1
Angiotensin II
Closing
Adrenergic α2
GABA
Opioids
G_o
Ca^{++}
Insulin
PDGF
EGF
TXA2 PGI2
LTs
AA
PL
PLA2
$G_{q/11}/\beta\gamma$
Ser / Thr Kinases
K^+
G_{i3}
Opening
Adenosine A1
Dopamine D2
Muscarinic M2
Serotonin
Closing
Muscarinic M1
Adrenergic α1
Histamine H1
LTC4 / LTD4
Light
RHODOPSIN
G_t
Cyclic GMP PDE
GMP
cGMP
cAMP XIBARE
ATP
cAMP
ADENYLATE CYCLASE
G_s
G_i
R_s
R_i
Adenosine A2
Adrenergic β1 β2
Dopamine D1
Glucagon G2
Histamine H2
PGE1
Adenosine A1
Adrenergic α2
Angiotensin II
Dopamine D2
Muscarinic M2 M3
Opioid
PGE1 PGE2

Figure 1.2. Structure of the insulin receptor

Shown in this figure is a diagrammatic representation of insulin receptor structure.

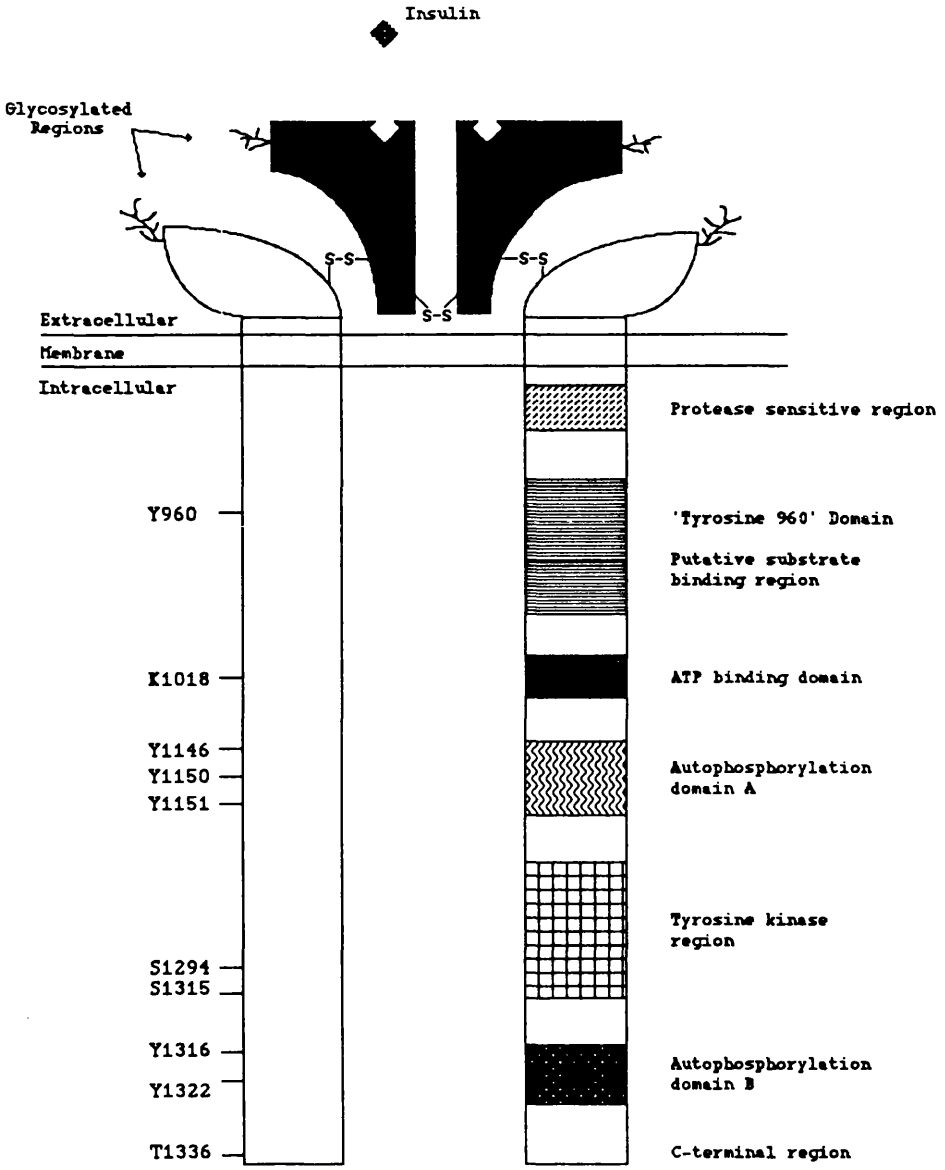

Insulin
Glycosylated Regions
S-S
S-S
S-S
Extracellular
Membrane
Intracellular
Protease sensitive region
Y960
'Tyrosine 960' Domain
Putative substrate binding region
K1018
ATP binding domain
Y1146
Y1150
Y1151
Autophosphorylation domain A
Tyrosine kinase region
S1294
S1315
Y1316
Y1322
Autophosphorylation domain B
T1336
C-terminal region

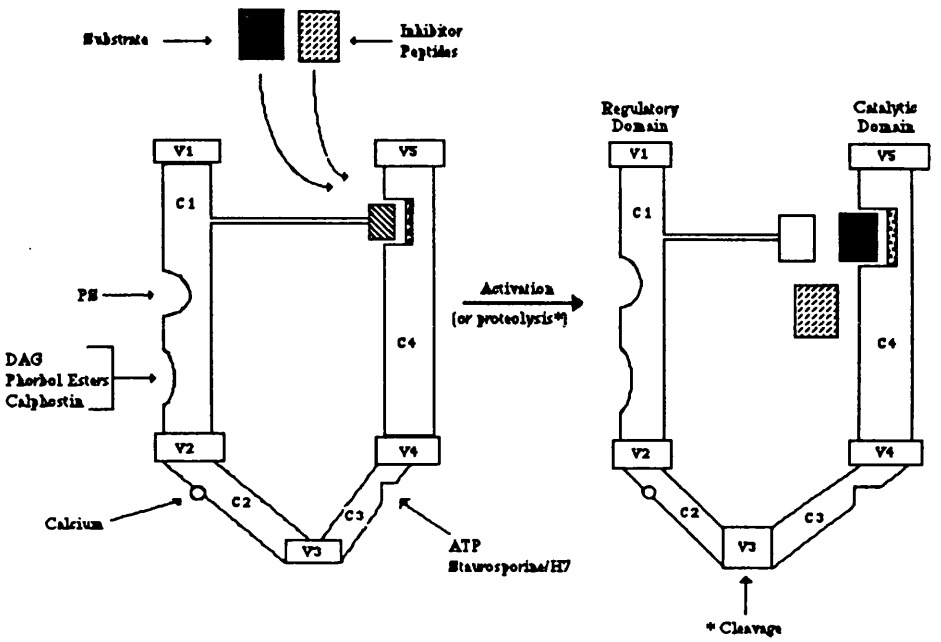
Table 1.1. The protein kinase C family

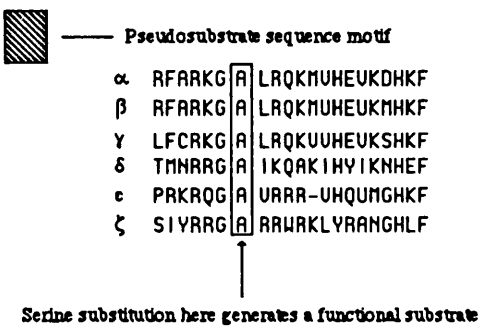
Characteristics of subspecies within the protein kinase C family are detailed here. Calculated molecular masses are from the cDNA sequence, apparent molecular masses are estimated from SDS-PAGE. Information taken from the following references, (Kikkawa, *et al.*, 1989; Hug and Sarre, 1993; Nishizuka, 1988).

Subspecies	Calculated Molecular Mass (kDa)	Apparent Molecular Mass (kDa)	Amino Acid Residues	Activators	Tissue Expression
α	76 799	80–81	672	Ca^{2+}, DAG, PS, AA	Universal
β_I	76 790	79-80	671	Ca^{2+}, DAG, PS	Some tissues
β_{II}	76 933	80	673	Ca^{2+}, DAG, PS	Many tissues
γ	78 366	77–84	697	Ca^{2+}, DAG, PS, AA	Central nervous system only
δ	77 517	74-79	673	DAG, PS	Fairly ubiquitous
ε	83 474	89-96	737	DAG, PS	Brain and others
η (L)	77 972	78-82	-	?	Lung, skin, heart
θ	81 571	79	-	?	Prominent in skeletal muscle
ζ	67 740	78-80	592	PS	Many tissues
λ	67 200	-	-	?	Ovary, testis and others

Figure 1.3. Structure and function of the pseudosubstrate region of protein kinase C

The proposed mechanism for autoinhibition of protein kinase C by the pseudosubstrate region is highlighted. Also listed are the amino acid sequences in the pseudosubstrate region for various protein kinase C isoforms.


Substrate
Inhibitor Peptides
V1
V5
C1
C4
PS
DAG
Phorbol Esters
Calphostin
V2
V4
C2
C3
V3
Calcium
ATP
Staurosporine/H7
Activation (or proteolysis*)
Regulatory Domain
Catalytic Domain
* Cleavage


Pseudosubstrate sequence motif
α RFARKGALRQKNVHEVKDHKF
β RFARKGALRQKNVHEVKNHKF
γ LFCRKGALRQKVVHEVKSHKF
δ TMNRRGAIKQAKIHYIKNHEF
ε PRKRQGAVRRR-VHQVNGHKF
ζ SIYRRGARRWRKLYRANGHLF
Serine substitution here generates a functional substrate

Chapter 2

Methods and materials

2.1. Methods

2.1.1. Assay of intracellular cyclic AMP concentration.

2.1.1.1. Introduction.

The receptor protein binding displacement assay was used to determine intracellular cyclic AMP concentration in cells. It is based on competition for protein binding sites between radiolabelled cyclic AMP and the unlabelled cyclic AMP to be quantified. This can be considered as a radioimmunoassay like system where antibody is replaced by binding protein. Use of a naturally occurring binding protein preparation (usually a crude preparation of the regulatory subunit of cyclic AMP dependent protein kinase) which interacts with cyclic AMP with high affinity results in a comparatively simple, but highly sensitive and specific assay. The method used here is essentially a modification of those developed by Gilman (Gilman, 1970) and Brown *et al.* (Brown, *et al.*, 1972).

2.1.1.2. Preparation of cyclic AMP binding protein.

This procedure was carried out essentially as described by Brown *et al.* (Brown, *et al.*, 1972). Briefly, bovine adrenal glands (approximately 30) were obtained and transported to the laboratory on ice. After removal of excessive fat, the glands were hemisected and the medulla removed. Cortical tissue was then scraped from the gland capsule, pooled and transferred into a pre-cooled Waring blender along with 1.5 volumes of ice cold homogenization buffer (0.25M sucrose, 25mM KCl, 5mM $MgCl_2$ and 50mM Tris HCl, pH 7.4). After homogenization at maximum speed the tissue was transferred to centrifuge tubes on ice and centrifuged at 2000g for five minutes at 4°C. The supernatant was decanted then re-centrifuged at 6000g for 15 minutes at 4°C and the final supernatant fraction pooled, aliquoted as a homogenous mixture and stored at -20°C until use.

2.1.1.3. Sample preparation.

Chinese hamster ovary cells were grown to confluence in 6-well plates under conditions described in Section 2.1.15, at which stage they were used for experimental purposes and numbered approximately 1 million cells per well. Wells were prepared such that they contained a final volume of 0.5ml Ham's F12 medium upon addition of any ligands at the desired concentration and for the desired time. Note that ligands were prepared in medium and added in a volume not exceeding 10% of the total incubation volume.

After stimulation of the cells, as detailed in legends for individual experiments in Chapter 5, medium was aspirated and cyclic AMP extracted in 2% (w/v) ice-cold perchloric acid (250µl per well). After an incubation on ice for between 15-30 minutes, each well was thoroughly scraped to disturb the cell monolayer. A high degree of efficiency at this stage was obtained and ensured by monitoring cell number under a microscope. The contents of each well were then centrifuged at 13 000g for two minutes at 4°C. The supernatant fraction was neutralized with 0.5M triethanolamine in 2M KOH using universal indicator. The precipitate at this stage was pelleted by centrifuging as above and the supernatant fraction used as sample for the binding protein assay.

2.1.1.4. Assay procedure for intracellular cAMP determination

The incubation buffer used in the assay was 50mM Tris HCl, pH 7.4, containing 4mM EDTA. Using this, various dilutions (0-320 pmols/ml) of unlabelled cyclic AMP were prepared giving corresponding values in the assay of 0.06, 0.12, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0 and 16.0 pmol/50µl. These were used to prepare a standard curve for unknown cyclic AMP determination by incubating with a fixed concentration of

labelled cyclic AMP and binding protein, also used for unknown samples as shown below.

[5',8-^{3}H]-cAMP was diluted in assay buffer to give approximately 500 000 cpm/ml. Binding protein was diluted 1:30 in assay buffer for use. Samples were then set up as shown below:

	Sample	Buffer	^{3}H-cAMP	Bind. Protein
Background	-	200µl	100µl	-
Total bound	-	100µl	100µl	100µl
Standards	50µl	50µl	100µl	100µl
Unknowns	5-50µl	95-50µl	100µl	100µl

The incubation was commenced by the addition of binding protein, hence this was always added last. Samples were mixed then allowed to reach equilibrium by incubation at 4°C for two hours. At this time the reaction was terminated by adding 250µl of a well mixed suspension of 2% (w/v) Norit-GSX charcoal and 1% (w/v) BSA in ice-cold assay buffer. Tubes were rapidly vortexed then centrifuged for 4 min at 13 000g and 4°C to sediment the charcoal containing cyclic AMP which had not bound to binding protein during the incubation. 0.3ml of each supernatant was taken for counting, incorporating a purpose-designed curve fitting programme which allowed quantification of unknown cyclic AMP values.

2.1.2. Phosphodiesterase assay

This assay is essentially based on that originally described by Thomson and Appleman (Thomson and Appleman, 1971).

2.1.2.1. Preparation of reagents

- 3', 5' cyclic AMP: Prepared at a concentration of 10mM in 20mM Tris HCl pH 7.4 containing 5mM $MgCl_2$ and frozen as stock. A 1μM solution was freshly prepared on the day of each assay. Note, Mg^{2+} ions are required as an essential co-factor for phosphodiesterase activity.

- 3', 5' [8-^{3}H] cyclic AMP: Added to give approximately 100 000 d.p.m. per tube.

- *Hannah ophiophagus* snake venom: Stock solution of 10 mg/ml prepared in distilled water and frozen. Diluted to 1 mg/ml using distilled water for use in assay.

- Dowex 1-chloride: 100g of Dowex 1-chloride resin was washed in 1M NaOH for 15 minutes after which it was extensively washed with deionized water until the pH of the eluate fell to 7.0. The resin at this stage was then washed further in 1M HCl for 15 minutes followed by yet more extensive washing until the pH of the eluate was between 4.0 and 5.0. For use in the assay 2 parts of Dowex slurry (Dowex:water 1:2) was mixed with 1 part ethanol. Ethanol is used to prevent non-specific binding of labelled adenosine to Dowex and to inhibit adenosine deaminase (often present in samples) converting adenosine to inosine, which does bind to Dowex.

- Samples were all prepared from CHO cell clones. The following procedure was always followed: Cells were split upon reaching confluence into the desired number of Corning 75cm cell culture flasks at a dilution of 1:6. Two days later cells were ~80% confluent and were fed again with fresh medium in the evening. The following morning cells were treated with ligand (same evening in the case of TPA down-regulation) as indicated, if at all. Subsequently, medium was aspirated and ice cold homogenization buffer (10mM Tris HCl pH 7.5, 0.1mM EDTA and protease inhibitor cocktail (2.5mM benzamidine, 0.2mM PMSF, 1μg/ml

antipain, 1μg/ml leupeptin and 1μg/ml pepstatin A)) was added. Cells were scraped into this medium and pelleted by centrifugation at 13 000g (4°C). At this stage pellets were frozen at -80°C. On day of assay pellets were thawed and resuspended in homogenization buffer to give required protein concentration (see Section 2.1.2.2). Disruption of cells was completed by homogenization with 15 strokes in a glass ground hand driven tissue homogenizer.

2.1.2.2. Assay procedure for phosphodiesterase activity determination

At 4°C, 25μl of enzyme sample (containing ~30μg of protein) was added to 25μl of 20mM Tris HCl pH 7.4 containing 5mM $MgCl_2$, which contained any inhibitory drugs to be used at four times the desired final concentration. Blanks, containing 50μl of Tris/Mg^{2+} buffer only, were incorporated in every assay. To this, 50μl of ^{3}H-cyclic AMP was added. In all results shown a 'cold' cyclic AMP concentration of 1μM was used. Tubes were mixed then incubated at 30°C for 10 minutes. Samples were boiled immediately for two minutes and allowed to cool to 4°C again. Boiling terminates the initial reaction by completely inactivating phosphodiesterase activity. 25μg (25μl of 1mg/ml solution) of snake venom was added, and tubes incubated again at 30°C for 10 minutes before addition of 400μl Dowex. Note, Dowex was kept well stirred during use to ensure a homogenous suspension. Tubes were mixed well and left to stand for 15 minutes then vortexed again before sedimenting the Dowex resin by centrifugation at 13 000g using a bench-top microcentrifuge. A 150μl aliquot of the resulting supernatant was added to 3ml of scintillation fluid for counting.

2.1.2.3. Calculation of results.

Correct c.p.m. per sample was calculated by subtracting the blank value in each case. This is necessary as it is generally found that 2-5% of tritiated cyclic AMP does not bind to the Dowex resin. Determining the protein content of each sample allowed results to be expressed relative to protein content of each sample. Finally, specific activity in each case was calculated as pmol/min/mg protein.

2.1.3. Purification of human insulin receptors

2.1.3.1. Preparation of human placental membranes

To limit proteolysis, all experimental manipulations were performed at 4°C and all buffers contained 0.2mM PMSF and 2.5mM benzamidine. Normal placentae were obtained immediately after delivery and kept on ice. Each placenta was trimmed of amnion and chorion, cut into small pieces, washed with 1 litre of ice-cold homogenization buffer (50mM Hepes pH 7.6, 0.25M sucrose) and then resuspended to approximately 750ml using this buffer. Homogenisation was then performed for 1.5 minutes (30 sec low speed, 1 min high speed) using an Ato-mix homogeniser (MSE). The homogenate was then filtered through one layer of nylon netting to remove most of the fibrous tissue before further homogenisation for 1.5 mins using a polytron homogeniser at setting 7. This homogenate was centrifuged at 600g for 10 minutes and the resulting supernatant fraction centrifuged at 10 000g for 20 minutes. Whilst stirring on ice the supernatant from this step was adjusted to 0.1M NaCl and 0.2mM $MgSO_4$ and centrifuged at 50 000g for 1 hour to pellet the membranes, which were then washed twice by resuspension (~300ml) in 50mM Hepes pH 7.6 and centrifuged as before. Finally, the membranes were resuspended to approximately 20mg of protein per ml in 50mM

Hepes pH 7.6, 1μg/ml pepstatin A, 1μg/ml leupeptin, 1μg/ml antipain and were either solubilized immediately or flash-frozen in liquid N_2 until use.

2.1.3.2. Solubilization of insulin receptors from human placental membranes

The resuspended placental membranes were solubilized using the non-ionic detergent Triton X-100. Membranes were stirred for 1 hour at 4°C with 1% (w/v) Triton X-100 and then centrifuged at 150 000g for 1 hour at 4°C. The supernatant, containing approximately 95% of the insulin binding activity, termed crude placental extract, was flash-frozen as small aliquots (normally 30μl) in liquid N_2 and stored at -70°C until use.

2.1.3.3. Immunopurification of insulin receptor from crude placental extract

Having previously determined the ratio of crude placental extract to immunoadsorbent that was sufficient to barely saturate the insulin receptor binding sites on the immunoadsorbent, 0.4mg of cellulose/0.1mg of antibody were used per ml of crude placental extract. (O'Brien, *et al.*, 1986) All operations were performed at 4°C. Immunoadsorbent was pre-washed three times with 50mM Hepes pH 7.6, 1M NaCl, 10% (v/v) glycerol and 0.1% (w/v) Triton X-100, using 1ml of buffer per mg of cellulose in immunoadsorbent. A further three washes (again 1ml/mg cellulose) with 50mM sodium acetate pH 5, 1M NaCl, 10% (v/v) glycerol and 0.1% (w/v) Triton X-100 were followed by a final two washes with 25mM Hepes pH 7.6, 10% (v/v) glycerol and 0.1 % (w/v) Triton X-100. Each step required centrifugation at 2500g for 3 minutes to pellet the immunoadsorbent before resuspension in the subsequent buffer. The immunoadsorbent was then resuspended in crude placental extract (2ml and 10ml, respectively, were used) and rotated end-over-end for 1 hour.

The immunoadsorbent was then washed six times with 50mM Hepes pH 7.6, 1M NaCl, 10% (v/v) glycerol and 0.1% (w/v) Triton X-100. The immunoadsorbent was then resuspended in 750 µl of this buffer including 0.2mM PMSF, 2.5mM benzamidine, 1µg/ml pepstatin A, 1µg/ml leupeptin and 1µg/ml antipain. Protein concentrations were assayed directly by Lowry assay (Section 2.1.14.2).

2.1.3.4. Preparation of immunoadsorbents

Anti-insulin receptor monoclonal antibodies were partially purified from ascites fluid by precipitation with 40% $(NH4)_2SO4$ and then coupled to aminocellulose by diazotisation (Hales and Woodhead, 1980). The resulting immunoadsorbents, usually containing 200-300µg protein per mg of cellulose, were resuspended to 5mg/ml cellulose. For use in the studies presented here, immunoadsorbents were kindly provided by Dr M. Saville.

2.1.4. ^{125}I - insulin binding to solubilized insulin receptors

The insulin binding activity of the Triton X-100 extracts and purified receptors was measured by incubating the receptor preparations for 16-20 hours at 4°C with [^{125}I]-insulin (50pM) in 75 mM Tris HCl pH 7.8, 30mM NaCl, 0.5mM EDTA, 0.1mM PMSF, 10mM glucose, 0.1% (w/v) dialysed BSA, 0.05% (w/v) Triton X-100 in a total volume of 200µl. Receptor bound radioactivity was separated from free insulin by the polyethylene glycol precipitation method of Cuatrecasas (Cuatrecasas, 1972), which selectively precipitates the high molecular weight receptor - [^{125}I]-insulin complexes. Ice-cold carrier human gamma globulin (0.625mg in 500µl of 0.05M sodium phosphate buffer pH 7.4) and polyethylene glycol 6000 (500µl, 25% (w/v) in distilled water) were added with thorough mixing. After 10-15 minutes on ice the samples were centrifuged at 1700g for 30 minutes at 4°C and the pellets were washed

once with 9% (w/v) polyethylene glycol containing 0.05% (w/v) Triton X-100. Nonspecific binding was determined by addition of buffer only to the incubations. This gave similar values to binding in the presence of sample and 5μM insulin (less than 7% of total precipitated counts). Specific binding was calculated as the difference between total and non-specific binding.

2.1.4.1. Calculation of insulin binding activity

Human placental membranes were prepared and solubilized as described above. A sample of crude placental extract was used to construct a two-fold dilution series to which specific [^{125}I]-insulin binding was assayed, again as described above. Total insulin binding activity (expressed as arbitrary units) was calculated as the product of the total placental extract volume (in this case 42ml) and the reciprocal of the sample dilution required to bind 5% of the counts added in the assay (in this case 185).

2.1.5. Kinase activity of purified human insulin receptor

Partially purified placental insulin receptors (10μl of a 10 fold dilution of preparation described in Section 2.1.3 (equivalent to 21ng per incubation)) were preincubated for 30 minutes at 4°C in a final volume of 30μl containing 50mM Hepes pH 7.6, 0.1% (w/v) Triton X-100, 0.2mM sodium vanadate, 2mM dithiothreitol and 13.3nM insulin unless otherwise stated. In experiments where the receptor was pre-activated, this was achieved by incubating the receptor at this stage with 10μl 100mM Hepes pH 7.6, 20mM $MgCl_2$, 8mM $MnCl_2$ and 1mM 'cold' ATP for 15 minutes at 23°C. The assay reaction was initiated by the addition of 10μl of 100mM Hepes pH 7.6, 20mM $MgCl_2$, 8mM $MnCl_2$ and 1mM [γ-^{32}P]-ATP (0.5-2μCi), giving a final ATP concentration of 250μM. After incubation

at 23°C for the desired time the reaction was terminated by addition of 20µl of 0.19M Tris HCl pH 6.8, 6% (w/v) SDS, 30% (v/v) glycerol, 15mM EDTA, 300mM DTT and 0.02% (w/v) bromophenol blue with immediate boiling for 5 minutes. These samples were subsequently analysed by electrophoresis in 7.5% SDS-PAGE gels which were then stained, dried and the β-subunit located by autoradiography. Results were quantified by excision of a gel chip corresponding to the desired band and determination of ^{32}P by Cerenkov counting as well as by scanning densitometry.

2.1.6. Phosphorylation of the purified inhibitory guanine nucleotide binding protein Gi

This method, based on that of O'Brien *et al.* (O'Brien, *et al.*, 1987), consists of several pre-incubation stages for various reagents which are then added together to initiate the phosphorylation reaction. Purified G-protein used in these experiments, prepared by a series of chromatographic steps as described previously (Milligan and Klee, 1985) was a gift from Dr E. Tang. Pre-incubation mixture 1 was a total volume of 20µl and contained purified receptor (20ng/assay), together with 24nM $MgCl_2$, 4mM $MnCl_2$, 0.2mM $NaVO_3$ and 2mM dithiothreitol in the presence or absence of 10nM insulin and was incubated for 15 minutes at 23°C. Pre-incubation mixture 2 was 10µl of the purified G-protein plus 5mM $MgCl_2$ and was incubated for 10 minutes at 32°C. Both of these mixtures were mixed and incubated for a further 10 minutes at 23°C. Phosphorylation was commenced by adding 10µl of 0.4mM [γ-^{32}P]ATP (0.5-2µCi) which was then incubated for various time periods, up to 60 minutes, at 23°C. Reactions were stopped and samples analysed as in Section 2.1.5.

2.1.7. Phosphorylation of the peptide IRβ by the immobilised insulin receptor

This procedure is based on that published by Shoelsen *et al.* (Shoelsen, *et al.*, 1988). Partially purified placental insulin receptors (5µl of a 10 fold dilution of preparation described in Section 2.1.3 (equivalent to 11ng per incubation)) were pre-incubated for 30 minutes at 4°C in a final volume of 10µl containing 50mM Hepes pH 7.6, 0.1% (w/v) Triton X-100 and 13.3nM insulin unless otherwise stated. In experiments where the receptor was pre-activated, this was achieved by incubating the receptor at this stage with 10µl 100mM Hepes pH 7.6, 10mM $MnCl_2$ and 25µM 'cold' ATP for 10 minutes at 23°C. Otherwise the same volume of this buffer lacking ATP was added instead. The peptide IRβ (TRDIYETDYYRK) was used at a final concentration in the assay of 0.33mM. The reaction was initiated by the addittion of 10µl of the peptide in 100mM Hepes pH 7.6, containing 25µM [γ-^{32}P]-ATP (0.5-2µCi per assay). The reaction was stopped by adding 60µl of 5% (w/v) TCA, 1% (w/v) BSA, incubating for 30 minutes at 4°C centrifuging at 13000g for 5 minutes and applying 50µl of the resulting supernatant to 2x2cm squares of Whatman P81 phosphocellulose paper which were washed four times (15 minutes each) in 75mM phosphoric acid, rinsed in acetone, then dried and added to 3ml scintillant. Phosphate incorporation was determined by Cerenkov counting.

2.1.8. SDS-PAGE electrophoresis and immunoblotting of proteins

Immunoblotting combines the resolution of gel electrophoresis with the specificity of immunochemical detection. It can be summarized in six major steps;

a. Preparation of the antigen sample.

Samples, normally containing 100μg total protein, were mixed with an equal volume of x2 sample buffer and boiled for three minutes prior to loading onto SDS-PAGE gels.

b. Resolution of the sample by gel electrophoresis.

This procedure was carried out essentially as decribed by Laemmli (Laemmli, 1970). Since protein transfer will be better for low percentages of acrylamide and cross-linker as well as for thin gels, the lowest percentage of acrylamide giving the desired resolution and gels of ~0.4 mm thickness should ideally be used. Resolving gels and subsequently stacking gels were cast, whereupon samples were loaded and gels run either at 60mA per gel for 2.5 hours or 6mA per gel overnight. At this stage gels were either stained, de-stained and dried for analysis or used for blotting as described below.

c. Transfer of the separated polypeptides to a membrane support.

Nitrocellulose is the most commonly used membrane. Electrophoretic elution was the method by which protein transfer was carried out. Each blot required one sheet of nitrocellulose paper and two sheets of absorbent filter paper cut to the size of the gel. As shown in Figure 2.1, the immunoblot apparatus was set up tightly in the following order; gel, membrane, filter paper and support pad sandwich. This was thoroughly soaked in blotting buffer, care being taken to exclude air bubbles. This sandwich was immersed in the transfer tank with the membrane closest to the positive electrode. Protein was transferred for two hours at 1mA with cooling.

Staining with ponceau S was often used at this stage to visualize proteins transferred onto nitrocellulose. Ponceau S was applied as a 0.2% (w/v) solution in 0.3% (w/v) trichloroacetic acid. Using PBS, blotting buffer was washed from nitrocellulose which was then shaken in ponceau

S solution for approximately 1 minute. Nitrocellulose was washed briefly with PBS and proteins visualized. Ponceau S solution was washed off with blotting buffer followed by PBS and then the membrane was processed as normal.

d. Blocking nonspecific binding sites on the membrane.

It is essential at this stage to prevent nonspecific binding of immunological reagents to the membrane, hence the reason for blocking with protein detergent solution. In this case non-fat dried milk and donkey serum were chosen to provide clean backgrounds. Therefore, the membrane, rinsed several times with PBS, was added to blocking solution and incubated at room temperature for two hours with agitation.

e. Addition of antibody.

Nitrocellulose paper was removed from the blocking solution and rinsed three times for 15 mins with PBS / 0.05% (v/v) NP40. The first antibody solution was added to the nitrocellulose and incubated overnight at room temperature with agitation.

f. Secondary antibody detection system.

Nitrocellulose was removed from the first antibody solution and washed three times for 15 minutes in PBS / 0.05% (v/v) NP40. The second antibody solution was then added and incubated at room temperature, with shaking, for two hours.

g. Enhanced chemiluminescence detection system.

This system is summarized in Figure 2.2. In essence this is a light emitting non-radioactive method for detection of immobilized specific antigens, conjugated directly or indirectly with horseradish peroxidase-labelled antibodies. After treating blots, results were visualized by a short exposure to blue-light sensitive autoradiography film.

2.1.8.1. Buffers associated with SDS-PAGE electrophoresis and Western blotting.

- x2 sample buffer:

0.5M Tris HCl pH 6.8	1.25ml
Glycerol	1.25ml
10% (w/v) SDS	2.00ml
β-mercaptoethanol	0.25ml (added on day of use)
Distilled H_2O	0.25ml
Bromophenol blue	0.2% (w/v)

- Resolving gel mixtures:	8%	10%
Distilled H_2O	11.6ml	9.9ml
30% (w/v)Acrylamide / 0.8% (w/v) Bis Acrylamide	6.7ml	8.3ml
1.5M Tris HCl pH 8.8	6.3ml	6.3ml
10% (w/v) SDS	0.25ml	0.25ml
10% (w/v) APS (prepared fresh)	0.25ml	0.25ml
TEMED	0.015ml	0.01ml

- Stacking gel mixtures:

Distilled H_2O	3.4ml
30% (w/v) Acrylamide / 0.8% (w/v) Bis Acrylamide	0.83ml
1M Tris HCl pH 6.8	0.63ml
10% (w/v) SDS	0.05ml
10% (w/v) APS	0.05ml
TEMED	0.005ml

- Running buffer:

Glycine	72g per 5 litres distilled H_2O.	
Tris	15g	"
SDS	5g	"

- Blotting buffer:

As for running buffer except using only 4 litres distilled H_2O with 1 litre methanol.

- Phosphate buffered saline (PBS) (10x):

NaCl	160g per 2 litres distilled H_2O	
KCl	4g	"
NaH_2PO_4	23g	"
KH_2PO_4	4g	"

- Blocking solution:

2% (v/v) Donkey serum

4% (w/v) Dried milk (in PBS)

0.05% (v/v)NP-40

- First antibody solution:

1% (w/v) Dried milk (including thimerosal if to be stored) in PBS containing desired antibody at the required dilution, here1:100 unless otherwise stated.

- Second antibody solution:

1% (w/v) Dried milk in PBS/0.05% (v/v) NP-40 containing 100µl of HRP-linked anti-rabbit second antibody per 100ml of solution.

2.1.9. Peptide conjugation and immuniziation of rabbits

Since many short peptide sequences themselves are poor immunogens they are often coupled to a suitable carrier and in this case keyhole limpet hemocyanin (KLH) was chosen, using glutaraldehyde as the coupling reagent. 10mg KLH and 3mg of peptide were dissolved in 1ml of 0.1M phosphate buffer pH 7.0 (0.1M NaH_2PO_4 added to 0.1M Na_2HPO_4 until desired pH was reached). To this was added 0.5ml of 21mM glutaraldehyde, dropwise and over as long a time period as possible (eg. 12.5µl every 15 minutes over 10 hours). This remained at room temperature overnight and was mixed with Freund's complete adjuvant for immunization the next day. Booster immunization was performed with material prepared in a similar fashion but with 5mg KLH and 1.5mg peptide using incomplete Freund's adjuvant. The entire 1.5ml provides enough material for immunizing at least three rabbits. A mixture of immunogen (± PBS to appropriate dilution) and adjuvant were well mixed to form a milky emulsion before injection.

Recommended dosage is between 50 and 1000 µg of immunogen per rabbit. Injection of large volumes is not recommended and injection of smaller volumes (~350µl) at multiple (3) sites is common practice. With this in mind 100µl of immunogen (200µg peptide) was mixed with 400µl PBS and 500µl adjuvant before injection. Skin was pinched between thumb and forefinger, pulled away from body and the needle (25-gauge) inserted into the space created. Care was taken to ensure the needle was not inserted into muscle or body wall. The desired amount was injected then the needle slowly withdrawn and the wound rubbed gently to stop any of the inoculum from escaping.

A test bleed was taken at 7 - 10 days after initially injecting the animal. This involved collecting a small volume of blood (~5ml) from the ear vein in rabbits, a suitable site due to easy accessibility and low number

of nerve endings. At this stage, and whenever other bleeds were taken, collected blood was allowed to clot for 30 - 60 minutes at 37°C then left overnight at 4°C to allow it to contract. Serum was then removed from the clot and any remaining insoluble material removed by centrifugation at 10 000g for 10 minutes at 4°C. After normally several boosts, again ~350ml at three sites, if useful antibodies were produced a final bleed was carried out. Blood collected was treated as before then aliquoted into small volumes, snap frozen in liquid N_2 and stored at -80°C until use.

2.1.10. Preparation of isolated hepatocytes

Preparation of isolated hepatocytes was performed essentially as described by Berry and Friend (Berry and Friend, 1969). Rats were anaesthetised by one intra-peritoneal injection of 0.4ml of a 60mg/ml solution of Sagatal (sodium pentobarbitone solution containing 4mg/ml heparin). Upon loss of the flexor and corneal responses, the abdominal cavity was opened. The inferior vena cava and hepatic portal vein were ligated and the vena cava then cannulated with a 16 gauge needle containing a solution of heparin (10mg/ml). The hepatic portal vein was also then cannulated with a 19 gauge needle and the ligatures tightened to secure the cannulae. The liver was perfused in the physiological direction with 80ml of Krebs buffer (25mM $NaHCO_3$, 1.2mM $MgSO_4$, 1.2mM KH_2PO_4, 5mM KCl and 120mM NaCl) pH 7.4 containing 1mg/ml EDTA at a constant temperature of 37°C and a flow rate of 30ml/min using a Watson - Marlow peristaltic pump. The EDTA containing buffer was washed out with 50ml of Ca^{2+} free Krebs buffer containing 20mM glucose. 60mg of collagenase was added to 100ml of this buffer and circulated for 30 minutes. After approximately 20 minutes it became apparent that the liver structure had become disrupted. At this stage cannulae were removed and the liver transferred to a plastic beaker

containing 35ml of Ca^{2+} free Krebs buffer and gently disrupted. The resulting cell suspension was gently filtered through nylon mesh with a pore size of 150μM and washed with a further 20ml of buffer. The suspension was then centrifuged at 100g for 2.5 minutes. The pellet was then washed several times by resuspending in Krebs buffer containing 2.5mM $CaCl_2$ and centrifuging as before.

2.1.11. Partial purification of protein kinase C using DE52 ion-exchange chromatography.

2.1.11.1. Preparation of DE52 column

DE52 is an anion exchange cellulose obtained in pre-swollen form. To prepare, this was stirred in 0.5M Tris HCl pH 7.5 buffer (20ml of buffer for every dry gram of cellulose). Whilst stirring, pH was adjusted to 7.5. Once pre-equilibration was complete the slurry was allowed to settle and the supernatant fraction decanted. The slurry was redispersed in buffer then allowed to settle, again the supernatant being decanted but this time leaving 20% of the wet settled volume on top of the resin. At this stage the slurry was used for packing columns.

This was done by pouring the stirred slurry into the chosen column as swiftly as possible to prevent convection currents in the slurry becoming established. Eluent from the column was allowed to run to waste then buffer run through the column until equilibration was complete (pH of eluate same as that of initial buffer) and the column bed height was constant. The sample, diluted appropriately in starting buffer, was then loaded onto the column at a controlled flow rate.

2.1.11.2. Partial purification of protein kinase C

The liver of an anaesthetised rat was perfused with ice-cold PBS containing 2mM EDTA and 2mM EGTA until all blood had been cleared

and the liver was cold to touch. The liver was then immediately excised, weighed and placed in approximately 40ml ice-cold homogenization buffer where it was cut into fine pieces. This was homogenised with 15 strokes of a motorized homogeniser. After sitting on ice for 20 minutes this homogenate was spun at 48000 rpm (4°C) using a Ti-50 rotor. The supernatant from this spin was made up to 200ml with column buffer and loaded onto a DE52 column which was then washed with column buffer for approximately 1-2 hours until protein concentration in the eluate fell sufficiently. At this stage protein kinase C was first eluted and collected in individual fractions (~3ml) by applying buffer A (80mM NaCl) until protein cocentrations of the eluate showed that peak 1 had passed. Buffer B (250mM NaCl) was then applied to elute the second peak of protein kinase C activity. All fractions were then tested for protein kinase C activity.

Homogenization buffer:

20mM Tris HCl pH 7.5

10mM EGTA

2mM EDTA

0.5% (v/v) Triton X-100

50mM β-mercaptoethanol

10mM Sodium Vanadate

10mM Sodium Fluoride

10mM β-glycero phosphate

0.1mg/ml Trypsin inhibitor

Protease inhibitor cocktail (see Section 2.1.2.1)

Column buffer:

20mM Tris HCl pH 7.5

50mM β-mercaptoethanol

0.1mg/ml Trypsin inhibitor

Protease inhibitor cocktail (see Section 2.1.2.1)

Buffer A:

(Prepared in column buffer)

80mM NaCl

2mM EDTA

Buffer B:

(Prepared in column buffer)

250mM NaCl

2mM EDTA

2.1.12. Protein kinase C activity assay

Assay mixes for these assays were composed to represent three important conditions, namely including A) phosphatidylserine, diacylglycerol and calcium, B) phosphatidylserine, diacylglycerol, and EGTA and C) EGTA only. These were composed as follows:

	A	B	C
Tris / HCl pH 7.5	20mM	20mM	20mM
$MgNO_3$	20mM	20mM	20mM
Histone H1 (III-S)	1mg/ml	1mg/ml	1mg/ml
ATP	40μM	40μM	40μM
$CaCl_2$	2mM	--------	--------
EGTA	--------	2mM	2mM

(prepared in distilled water)

The above were mixed with an equivalent volume of PS/DAG mixture (A + B) or an equivalent volume of 20mM Tris HCl pH 7.5 (C) to complete the final assay mixture. To this was added γ-[^{32}P] ATP at 5μCi/ml.

Phosphatidylserine (0.1mg/ml) and diacylglycerol (6μg/ml) were prepared by mixing well then drying under nitrogen, with final resuspension in 20mM Tris HCl pH 7.5. Sonication using a Jencons ultrasonic probe processor for 3 x 30 seconds at 80 Watts with 30 second intervals was then performed.

The assay required incubating 100μl of final assay mixture, including label, with 100μl of column buffer containing the fraction sample at the appropriate dilution (approx 1:10) for 15 minutes at 37°C. After this time the reaction was stopped by adding 30μl of ice-cold 100% (w/v) TCA and the samples placed on ice. Using custom built 96 well vacuum filter apparatus and Whatman GF/C glass microfibre filter paper, samples were loaded into wells which were washed several times with 10% (w/v) TCA and then with methanol containing bromophenol blue to fix samples and allow them to be visualized. Activity in each fraction sample was now represented by a specific spot on the GF/C paper which was cut out and counted for radioactivity.

2.1.13. Streptozotocin injections

Male Sprague-Dawley rats (200-270g) were used. Diabetes was induced as previously described (Gawler, *et al.*, 1987) using one intraperitoneal injection of streptozotocin (80mg per kg body weight, 0.3ml per animal) in sterile 0.1M citrate buffer pH 4.5 (0.1M sodium citrate, and add 0.1M citric acid to desired pH). Urine glucose was monitored (Diabur-Test 5000 kit) and animals used when diabetic

(normally several days after injection). Animals were only confirmed as diabetic if elevated glucose was detected in the urine and if the blood glucose concentration was >12mM, as assessed using Dextrostix.

2.1.14. Protein assays

2.1.14.1 Bradford method

Protein content of samples was determined using the Bio-Rad protein assay kit based on the method of Bradford (Bradford, 1976). In all assays BSA was used as the standard. Standard solutions were prepared ranging from 0 - 20μg protein and treated similarly to unknown samples (normally 5 - 50μl, containing 0.5 - 20μg protein or equivalent of standard curve) in being diluted to 0.8ml with distilled H_2O. To this was added 0.2ml Bio-Rad dye reagent followed by thorough mixing. Absorbance at 595nM of all samples was read, allowing determination of unknown protein concentrations from the standard curve.

2.1.14.2 Lowry method

Protein determination was also carried out by a method based on that of Lowry *et al.* (Lowry, *et al.*, 1951). The following stock solutions are required:

1. Copper tartrate carbonate (10% (w/v) $Na_2C O_3$, 0.1% (w/v) $CuSO_4.5H_2O$, 0.2% (w/v) sodium potassium tartrate)
2. 0.8M NaOH
3. 10% (w/v) SDS

Immediately before use, one part of each of these solutions was mixed along with one part H_2O to give reagent 'A', which was gently warmed to dissolve any precipitate. Standard solutions of BSA were prepared in duplicate containing 0-100μg of protein per ml and unknown protein solutions prepared in triplicate. All samples were made up to 1ml

with H_2O and then 1ml reagent 'A' added to each sample. After 10 minutes 0.5ml reagent 'B' was added (Folin and Ciocalteau's reagent diluted 1:6 with distilled H_2O), mixed and colour development allowed to occur for 30 minutes before absorbance at 750nM was measured.

2.1.15. Cell Culture

2.1.15.1. Growth medium for CHO cells

All cells required the following growth medium:

500 ml	Ham's F12 medium
55 ml	Foetal calf serum (heat-inactivated)
5.5 ml	Penicillin / Streptomycin solution

In addition the following were added to the above medium as selection markers:

400 µg/ml	G418 (for cells transfected with insulin receptor)
150 µg/ml	Hygromycin (for cells transfected with PKC)

2.1.15.2. Passage of cells

Media was removed from 75cm cell culture flasks containing confluent cells with a sterile pipette. Approximately 3ml of trypsin solution was added to each flask and incubated for a few minutes. When the cells began to 'round-up', 3ml of growth medium was added to stop the action of trypsin. Cells not yet dislodged from the plate were displaced by gentle tapping of the plate and trituration of the media. This media was then transferred to a sterile centrifuge tube and spun for 3 minutes at ~1000g. The media/trypsin solution was then aspirated and the pellet resuspended in fresh medium. The desired amount of these cells were then seeded in new 75cm flasks in a final volume of ~10ml.

2.1.15.3. Freezing cells

When freezing cells, the cell pellet after centrifugation (Section 2.1.15.2) was this time resuspended in freezing medium (1.0-1.5ml per flask of confluent cells). Freezing medium consisted of 10% (v/v) DMSO, 25% (v/v) foetal calf serum and 65% (v/v) normal growth medium. After aliquoting into cryotubes, cells were frozen slowly overnight in a -80°C freezer then transferred to liquid nitrogen the following day and stored there until required.

2.2. Materials

2.2.1. Chemicals and General Reagents

Sigma Chemical Co,	Antipain
Poole,	ATP (disodium salt)
Dorset, U.K.	Benzamidine
	BSA
	Bromophenol blue
	Calmodulin
	Cyclic AMP (disodium salt)
	Cyclic GMP
	Charcoal (Norit A)
	DAG
	Dowex 1-chloride
	Glucagon
	Glutaraldehyde
	Histone-H1 (III-S)
	Human gamma globulin
	IBMX
	KLH
	Leupeptin

	Nonidet-P40
	Pepstatin
	PGE_1
	PMSF
	Ponceau S
	Snake venom (*Hannah ophiophagus*)
	Streptozotocin
	TEMED
	Thimerosal
	TPA
	Triton X-100
	Trypsin inhibitor
Amersham,	ECL kits
Bucks, U.K.	Hyperpaper
	Molecular weight markers
	Nitrocellulose
Fisons,	Hepes
Loughborough,	TCA
England:	
Boehringer,	Dithiothreitol
East Sussex, U.K.	Tris
	Diabur test-5000 kits
	Hygromycin
	TEA
BDH, Poole,	β-mercaptoethanool

Dorset, U.K.	Universal indicator DMSO
Kodak Ltd, Manchester, U.K.	X-O Mat-S film
Antibody Production Unit, Law Hospital, Lanarkshire, Scotland:	HRP-linked sheep anti-rabbit IgG Donkey serum
Ames, Slough, U.K.	Dextrostix
FSA Lab Supplies, Loughborough, U.K.	Folin & Coicalteau's reagent
Schering Ltd, Berlin, Germany.	Rolipram
Pfizer, Sandwich, U.K.	Cilostamide
Bio-Rad Laboratories Ltd, Herts, U.K.	Bradford reagent
Lipid Products, London, U.K.	Phosphatidylserine

Novo Laboratories Ltd, Basingstoke, U.K.	Insulin (porcine)
SmithKline Beecham, Epsom, U.K.	IRβ peptide
DIFCO Laboratories, Detroit, U.S.A.	Freund's complete adjuvant Freund's incomplete adjuvant
Hannah Institute Ayr, Scotland.	Peptides for antibody production
Packard, Groningen, N.L.	Scintillation fluid
Rhone Merieux, France.	Sagatal
Whatman, Maidstone, Kent, U.K.	P81 paper GF/C paper DE52 ion-exchange resin
Worthington Biochemical Corporation, New Jersey, U.S.A.	Collagenase

All other chemicals were of analar grade or were of the highest grade available commercially.

2.2.2. Radiochemicals

Amersham,	[8-^{3}H] - Adenosine 3', 5'-cyclic monophosphate
Bucks, U.K.	[5',8-^{3}H] - Adenosine 3', 5'-cyclic monophosphate
	[^{125}I] - Insulin
	[γ ^{32}P] - Adenosine 5'-triphosphate

2.2.3. Animals

Male Sprague Dawley rats (220-250g) were used as the source of tissue for partial purification of protein kinase C. Antibodies were raised in New Zealand White rabbits purchased at 3 months old from MRC accredited sources.

2.2.4. Cell Culture Materials

All materials required for cell culture, unless otherwise listed, were purchased from Gibco. BRL, Paisley, Scotland. Transfected CHO cells were a gift from Dr. J. Tavare, Bristol University.

2.2.5. Miscellaneous

Bovine adrenal glands were obtained (Duke St abattoir, Glasgow) immediately after slaughter, transported at 4^{o}C and processed without delay. Human placenta were obtained (Rottenrow maternity hospital, Glasgow) as soon as possible after birth and again transported at 4^{o}C then processed without delay.

Figure 2.1. Diagram of immunoblotting apparatus

This digram highlights the composition of the gel/membrane sandwich and the orientation of these in relation to the electrodes of the immunoblotting tank.

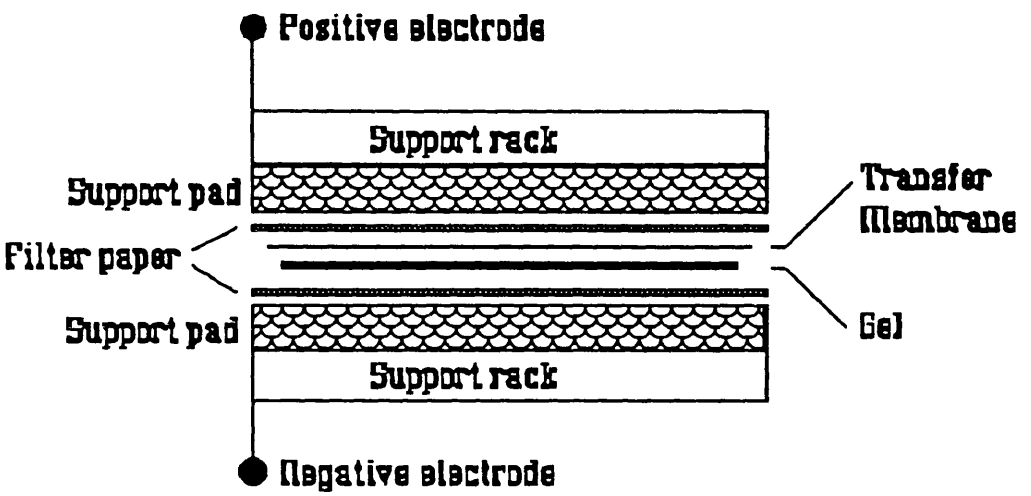
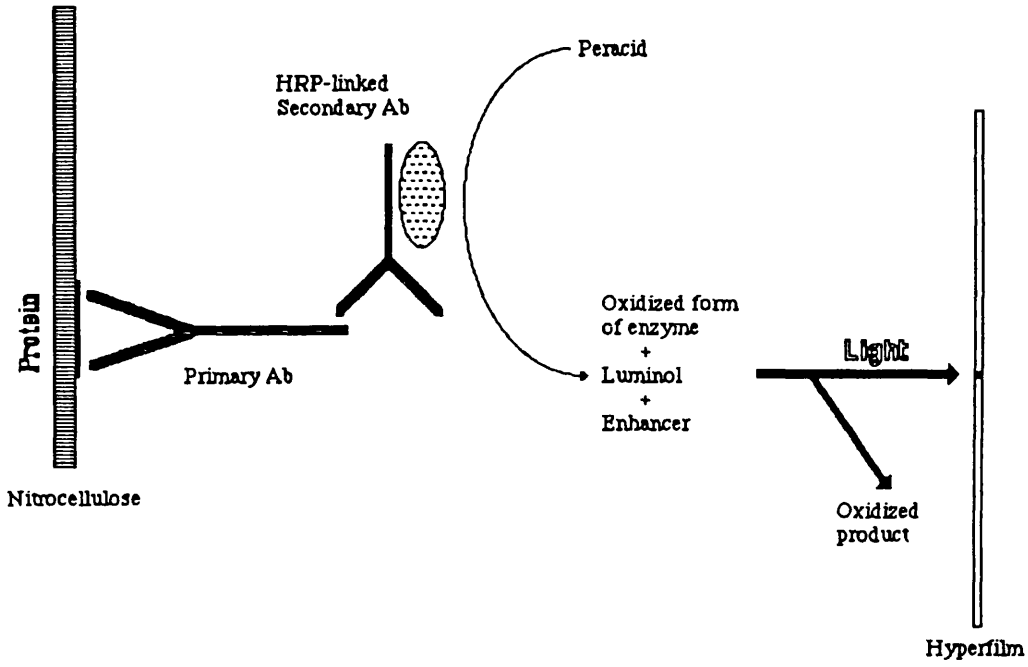

Positive electrode
Support rack
Support pad
Transfer Membrane
Filter paper
Gel
Support pad
Support rack
Negative electrode

Figure 2.2. ECL method of protein detection

This diagram highlights the basis of the enhanced chemi-luminescence method of detecting immunoreactive proteins, as was used here.


Peracid
HRP-linked
Secondary Ab
Protein
Primary Ab
Oxidized form
of enzyme
+
Luminol
+
Enhancer
Light
Oxidized
product
Nitrocellulose
Hyperfilm

Chapter 3

Production of protein kinase C isoform specific antibodies

3.1. Introduction

3.1.1. The immune response

The immune system functions principally to protect animals from infectious organisms and from their toxic products. The system can be subdivided into two broad categories, adaptive and non-adaptive immunity. Non-adaptive immunity is mediated by cells which respond in a non-specific manner to foreign molecules and encompasses phagocytosis by macrophages, secretion of lysozyme and cell lysis by natural killer cells. Adaptive immunity is mediated by lymphocytes, cells which synthesize cell-surface receptors or secrete proteins that bind specifically to foreign molecules. These proteins are called antibodies and it is this system which can be exploited by introducing a specific immunogen into a host in order to induce formation of the corresponding specific antibody.

Lymphocytes can be grouped into three basic types - B cells, cytotoxic T cells and helper T cells. All three carry cell-surface receptors that can bind antigens. Note that one cell is capable of recognizing only one particular antigen as all of the antigen receptors on a single cell are identical. B cells secrete antibodies and carry a modified form of the same antibody on their surface, where it acts as a receptor for antigens. Cytotoxic T cells lyse foreign or infected cells upon binding to B cells via their surface antigen receptor, known as the T-cell receptor. Helper T cells play a key regulatory role in controlling the response of B cells and cytotoxic T cells.

Adaptive immunity can be further subdivided into two categories, humoral and cell mediated immunity. Cell mediated responses are typified by binding of cytotoxic T lymphocytes to foreign or infected cells and subsequent lysis of these cells. The humoral response results in the generation of circulating antibodies that bind to foreign antigens. It is

mediated by B cells in conjunction with helper T cells and forms the basis of the techniques employed here.

When an animal first encounters an antigen the primary immune response is slow and weak. On second exposure to the same antigen a stronger and more rapid response ensues. This capacity to mount a strong and specific secondary response is termed immunological memory and is again exploited here.

3.1.2. Antibody molecules

Antibodies are a large family of glycoproteins that share key structural and functional features. Structurally, antibodies are often visualized as having a 'Y' shape. Each Y shaped antibody is composed of four polypeptides, two identical copies of both a heavy and a light chain. Antibodies are classified into five classes, IgG, IgM, IgA, IgE and IgD, on the basis of the number of Y-like units and the type of heavy chain polypeptide they contain. IgG's contain one Y unit and are the most abundant in serum.

Antibody-antigen binding is entirely dependent on noncovalent interactions and exists in equilibrium with dissociated components. These interactions include hydrogen bonds, van der Waals forces, coulombic interactions and hydrophobic backbones. Polyclonal sera tend to contain complex mixtures of antibodies of differing affinities.

3.1.3. Production of antibodies

Plasma cells are the main sites of antibody production. The cells live for three to four days and allocate approximately 40% of their total protein synthesis to antibody production. Antibody production is maintained by regulating the differentiation of B cells into plasma cells and memory cells.

When producing antisera to a specific antigen, choosing the site of injection for the immunogen is very important. Ideal sites should have high numbers of antigen presenting cells and low rates of antigen degradation. To aid in this respect, immunogens are often mixed with adjuvants which protect the antigen from rapid dispersal by trapping it in a local deposit. Adjuvants may also contain substances which aggravate the host animal's immune response (see Section 3.1.5.).

Upon phagocytosis, antigen presenting cells degrade the antigen and display fragments of it on the cell surface, bound to an MHC (major histocompatibility complex) class II protein. If antigen fragments do not bind to MHC class II proteins of a given animal the antigen is masked from the immune system and no immune response occurs.

Not all molecules researchers wish to raise an antibody to, such as short peptide sequences in this case, are good immunogens due to T cell tolerance or lack of the appropriate class II proteins. This is most readily overcome by coupling such small molecules, or haptens, to soluble carrier proteins using a bifunctional coupling reagent. The carrier provides binding sites for MHC class II - T-cell binding sites, while the hapten provides an epitope for binding to the antibodies on the B cell surface. Both of these binding steps are essential in optimizing the immune response.

3.1.4. Coupling of the peptide

Most coupling methods rely on free amino groups (terminal or lysine side chains), sulphydryl groups (cysteine side chains), phenolic groups (on tyrosine residues) or carboxylic acid groups (terminal or on aspartate and glutamate). Coupling should be designed to link the peptide through a terminal amino acid.

Proteins commonly used as carriers include keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA). KLH is often preferred as BSA is often a good immunogen in its own right.

Glutaraldehyde is also often used as a coupling reagent. A single-step coupling method involves mixing the peptide and carrier at the appropriate ratio with a limiting amount of glutaraldehyde to minimize overcoupling.

3.1.5. Adjuvants.

These are non-specific stimulators of the immune response and their judicious use is essential to induce a strong antibody response. Complete adjuvants have two active components. Firstly, mineral oils are present to form a deposit preventing rapid catabolism of the immunogen. Secondly, a substance that will nonspecifically stimulate the immune response is used. This normally relies upon heat killed bacteria, such as *mycobacterium tuberculosis*, as is present in Freund's adjuvant. Adjuvant lacking the latter component is termed incomplete adjuvant.

3.1.6. Injections.

Subcutaneous injections are widely used in rabbit immunizations as the injected inoculum will drain quickly into the local lymphatic system and become concentrated in the lymph nodes closest to the injected sites. The recommended dosage is between 50 and 1000 μg of immunogen per rabbit.

Injection of large volumes is not recommended and injection of smaller volumes (~350μl) at multiple sites is common practice. The skin should be pinched between thumb and forefinger, pulled away from body and a needle (25-gauge) inserted into the space that has been created. Care should be taken to ensure that the needle is not inserted into muscle or

body wall. The desired amount is then injected, the needle slowly withdrawn and the wound then gently rubbed to stop any of the inoculum from escaping.

3.1.7. Sampling serum

Test bleeds are often taken before immunizations begin for control purposes. After the initial injection, antibodies to the immunogen appear in the serum at approximately seven days, reaching maximal levels at ten days. Test bleeds can be taken at this stage. This normally involved collecting a small volume of blood (~5ml) from the ear vein in rabbits, a suitable site due to its easy accessibility and low number of nerve endings. Subsequent boosts (normally approximately four weeks apart, although longer if desired as animal remains primed up to one year) are given until a high titre to the antigen of interest is obtained. At this time the rabbit is bled. Collected blood should be allowed to clot for 30 - 60 minutes at 37°C then left overnight at 4°C to allow it to contract. Serum should then be removed from the clot and any remaining insoluble material removed by centrifugation at ~10 000g for 10 minutes at 4°C.

One practical advantage of antibodies having compact and stable protein domains is that they are resistant to a broad range of mildly denaturing conditions. This makes storage for several years below -20°C possible. The only real problem associated with storing antibodies is contamination of these solutions with bacteria or fungi, which is best prevented by addition of sodium azide at a final concentration of 0.02% (w/v).

3.2. Peptide sequences used to raise antibodies to individual protein kinase C isoforms

The peptide sequences displayed below were synthesized with the express intention of raising isoform specific PKC antibodies. Each one represents a distinct peptide sequence from within the amino acid structure of that particular isoform. Before synthesis each peptide sequence was checked for cross-reactivity with other PKC-isoform sequences to ensure specificity of the resultant antibodies.

PKC-α

Peptide used: (C) QFVHP ILQSA V

Amino acids 662-672

Gln-Phe-Val-His-Pro-Ile-Leu-Gln-Ser-Ala-Val

PKC-β_I / β_{II}

Peptide used: (C) IQAHI EREVL I

Amino acids 623-633

Ile-Gln-Ala-His-Ile-Glu-Arg-Glu-Val-Leu-Ile

PKC-ε

Peptide used: (C) NQEEF KGFSY FGEDL M

Amino acids 721-736

Asn-Gln-Glu-Glu-Phe-Lys-Gly-Phe-Ser-Tyr-Phe-Gly-Glu-Asp-Leu-Met

PKC-γ

Peptide used: (C) NYPLE LYERV RTG

Amino acids 306-318

Asn-Tyr-Pro-Leu-Glu-Leu-Tyr-Glu-Arg-Val-Arg-Thr-Gly

PKC-ζ

Peptide used: (C) GFEYI NPLLL SAEES V

Amino acids 618-633

Gly-Phe-Glu-Tyr-Ile-Asn-Pro-Leu-Leu-Leu-Ser-Ala-Glu-Glu-Ser-Val

PKC-δ

Peptide used: (C) SFSDK NLIDS MDQTA

Amino acids 643-657

Ser-Phe-Ser-Asp-Lys-Asn-Leu-Ile-Asp-Ser-Met-Asp-Gln-Thr-Ala

All six antibodies raised exhibited immunoreactivity against purified protein kinase C from bovine brain and were used in subsequent experiments. For example, Figures 3.1-3.4 show immunoblots where samples of extracts from CHO cells transfected with individual PKC-isoforms were analysed by SDS-PAGE electrophoresis and immunoblotting (as described in Section 2.1.8) using antisera to the α, β, γ and ε isoforms of protein kinase C. The results obtained clearly highlight specificity of the raised antibodies for each individual protein kinase C isoform.

The CHO cells used in these blots were transfected with PKC-α (CHO-α), PKC-β (CHO-β), PKC-γ (CHO-γ) and PKC-ε (CHO-ε) as well as control cells (CHO-T).

Figure 3.1. Expression of PKC-α in CHO cell clones

Extracts from Chinese hamster ovary (CHO) cells transfected such that they stably overexpressed an individual protein kinase C isoform were used to assess the specificity of the antibody raised against PKC-α peptide. Five cell clones were tested with the anti-PKC-α antibody (used at a dilution of 1 in 100); namely cells overexpressing PKC-α (α), PKC-β_I (β), PKC-γ (γ), PKC-ε (ε) and a control cell clone not overexpressing any protein kinase C isoform (T). Immunoreactivity was detected at ~80kDa.

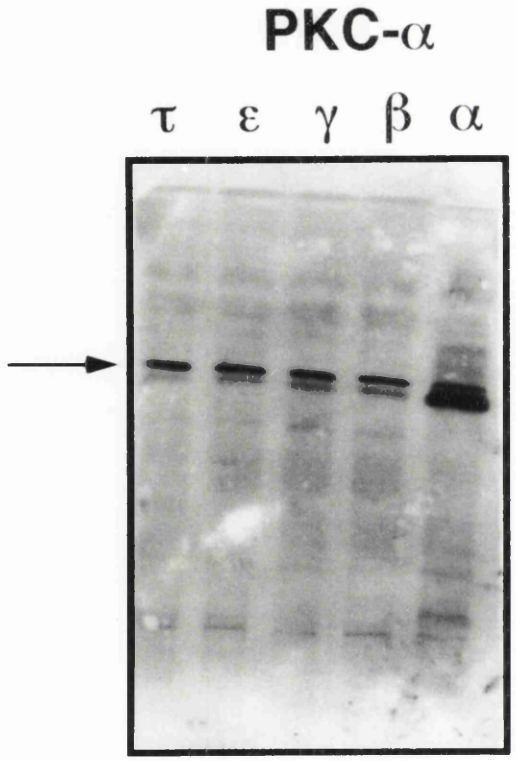

PKC-α
τ ε γ β α

Figure 3.2. Expression of PKC-β in CHO cell clones

Extracts from Chinese hamster ovary (CHO) cells transfected such that they stably overexpressed an individual protein kinase C isoform were used to assess the specificity of the antibody raised against PKC-β peptide. Five cell clones were tested with the anti-PKC-β antibody (used at a dilution of 1 in 100); namely cells overexpressing PKC-α (α), PKC-β_I (β), PKC-γ (γ), PKC-ε (ε) and a control cell clone not overexpressing any protein kinase C isoform (T). Immunoreactivity was detected at ~80kDa.

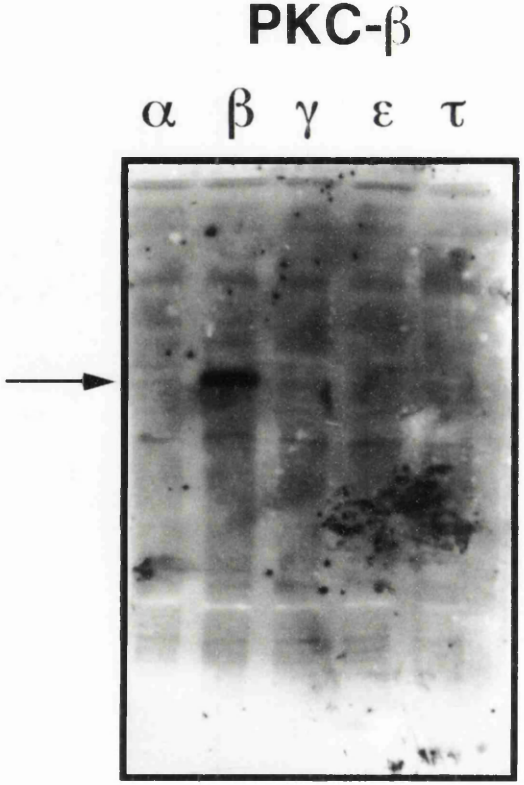

PKC-β
α β γ ε τ

Figure 3.3. Expression of PKC-γ in CHO cell clones

Extracts from Chinese hamster ovary (CHO) cells transfected such that they stably overexpressed an individual protein kinase C isoform were used to assess the specificity of the antibody raised against PKC-γ peptide. Five cell clones were tested with the anti-PKC-γ antibody (used at a dilution of 1 in 100); namely cells overexpressing PKC-α (α), PKC-β_I (β), PKC-γ (γ), PKC-ε (ε) and a control cell clone not overexpressing any protein kinase C isoform (T). Immunoreactivity was detected at ~78kDa.

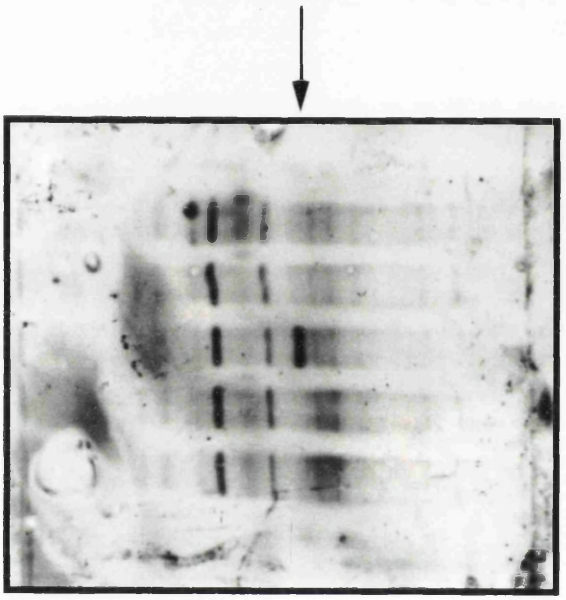
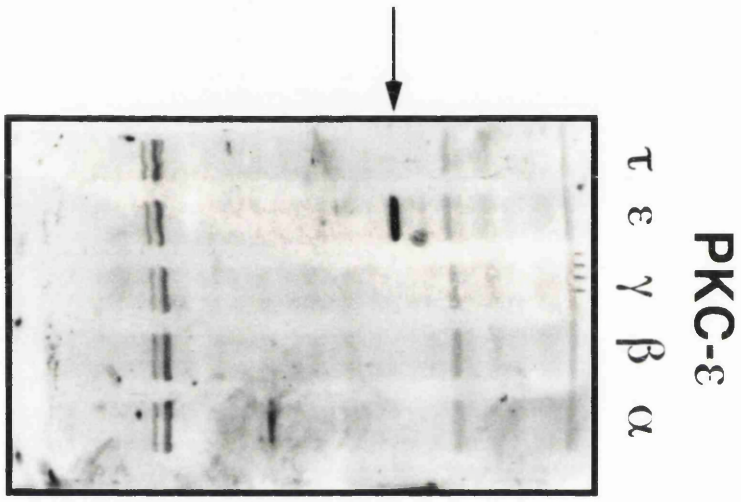

PKC-γ
τ ε γ β α

Figure 3.4. Expression of PKC-ε in CHO cell clones

Extracts from Chinese hamster ovary (CHO) cells transfected such that they stably overexpressed an individual protein kinase C isoform were used to assess the specificity of the antibody raised against PKC-ε peptide. Five cell clones were tested with the anti-PKC-ε antibody (used at a dilution of 1 in 100); namely cells overexpressing PKC-α (α), PKC-β_I (β), PKC-γ (γ), PKC-ε (ε) and a control cell clone not overexpressing any protein kinase C isoform (T). Immunoreactivity was detected at ~85kDa.


PKC-ε
τ ε γ β α

Chapter 4

Protein kinase C activity and expression in normal rat liver and liver of streptozotocin-induced diabetic rats

4.1. Introduction

It has been suggested by a number of investigators that changes in either the expression or activity of protein kinase C might give rise to insulin-resistant states such as are seen in diabetes and obesity (Grunberger, 1991). One suggestion (Turinsky, *et al.*, 1990) proposed that insulin resistance occurred as a consequence of a persistent increase in diacylglycerol levels, leading to increased protein kinase C activity and ultimately phosphorylation and inhibition of insulin receptor function (Kadowaki, *et al.*, 1984; Shmueli, *et al.*, 1993). Protein kinase C has also been implicated as part of the insulin stimulated signal transduction pathway (Considine and Caro, 1993) and in this respect changes in activity and/or expression of a specific isoform may also affect insulin signalling processes.

To date, however, observations describing alterations in protein kinase C activity, expression and distribution in diabetic states has not identified any consistent pattern. This is evident from the data summarised in Table 4.1. Results appear to vary both between the various model systems and the tissue examined. For example, using the streptozotocin-induced diabetic rat both an overall decrease (Kim, *et al.*, 1991), as well as no overall change (Borghini, *et al.*, 1994) in protein kinase C activity in sciatic nerve has been observed in the diabetic state. In addition, an overall increase has been detected in heart (Tanaka, *et al.*, 1991). Interestingly, this increased protein kinase C activity seen in diabetic tissue can often be ameliorated by calcium channel blockers such as verapamil (Tanaka, *et al.*, 1991), suggesting that calcium dependent protein kinase Cs are responsible for the increased activity in this case.

Evidence that increased protein kinase C activity (associated with serine/threonine phosphorylation of the insulin receptor) was responsible for decreased receptor tyrosine kinase activity has been shown in many

studies (Bollag, *et al.*, 1986; Takayama, *et al.*, 1984). In streptozotocin diabetic rats, whilst a reduced insulin receptor kinase activity was found in one study, this was not paralleled by any increase in protein kinase C activity (Karasik, *et al.*, 1990). There is considerable interest in the possibility that individual protein kinase C isoforms may have distinct functional roles, including regulation of insulin signalling. Their contribution might explain some of the anomalous results described in Table 4.1. In an attempt to further understand the role of protein kinase C, the enzyme was partly purified from the liver of normal and streptozotocin-induced diabetic rats and its activity assessed. Previous work in this laboratory has established that selectively altered expression of individual isoforms occurs upon streptozotocin-induced diabetes in rat hepatocytes (Tang, *et al.*, 1993). Therefore, protein kinase C isoform specific antisera were also used to investigate isoform expression and relate this to activity in partially purified fractions.

4.2. Results

4.2.1. Protein kinase C activity in normal and diabetic states

Protein kinase C activity was resolved into two distinct peaks after DE52 ion-exchange chromatography as described in Section 2.1.11. Typical results of assays to determine protein kinase C activity in fractions collected after chromatography are shown in Figures 4.1 and 4.2, with the results being summarized in Table 4.2. These data show that, using this system, a significant overall decrease in the protein kinase C activity of peak 1 occurred upon induction of diabetes. No significant difference in the activity of peak 2 occurred with diabetes induction. The overall level of activity in peak 2 was much less than that observed in peak 1.

By assaying fractions under different conditions it was possible to break down the protein kinase C activity into that which was attributable

to both the Ca^{2+}-dependent and Ca^{2+}-independent isoforms. Calcium dependent activity can be calculated as the activity expressed in the presence of PS/DAG/Ca^{2+} minus that activity expressed in the presence of PS/DAG/EGTA. Similarly, subtracting the activity expressed in the presence of EGTA alone from that activity expressed in the presence of PS/DAG/EGTA gave a measure of PS/DAG dependent protein kinase C activity. These results are summarized in Table 4.2. They indicate that a significantly greater amount of Ca^{2+}-dependent protein kinase C activity was found in peak 1 from normal rat liver as opposed to diabetic rat liver. The amount of Ca^{2+}-dependent protein kinase C activity in peak 2 appeared, however, not to be significantly altered upon induction of diabetes. PS/DAG-dependent activity in peak 1 was, again, significantly reduced in diabetic tissue, while little PS/DAG-dependent activity was apparent in peak 2.

4.2.2. Immunoblotting of fractions with isoform specific antisera.

Western blot analysis with isoform specific protein kinase C antisera was performed in order to determine the expression of individual isoforms in the range of fractions which formed the two observed peaks of activity. These are shown in Figures 4.3 - 4.6. PKC-α was present in peak 1 from both normal and diabetic animals (Figures 4.3 and 4.4). Interestingly, however, in samples from normal animals two distinct immunoreactive bands (~79 and ~83kDa) were observed whilst in samples from diabetic animals only one band (~80kDa) was seen. No PKC-α was observed in peak 2 from either normal or diabetic animals.

PKC-β expression was examined using an antiserum which could recognize both PKC-β_I/β_{II} forms. Previously (Tang, *et al.*, 1993) only PKC-β_{II} had been shown to be expressed in liver. As shown in Figure 4.5 and 4.6, a single band of immunoreactivity (~80kDa) was observed in

peak 1. Also, to a lesser degree, a similar band was also present in peak 2 from normal rats. The pattern in diabetic tissue appeared to be similar.

PKC-ε expression is shown in Figures 4.7 and 4.8. In normal animals one band of immunoreactivity at ~78kDa was observed in peak 1, whilst a doublet occurring at a higher apparent molecular weight (~81kDa) was detected in peak 2. In diabetic tissue one band at ~80kDa was detected in peak 1 and a single band, again at a higher apparent molecular weight (~85kDa), was detected in peak 2.

No PKC-ζ expression was found (Figures 4.9 and 4.10) in peak 1 from either normal or diabetic tissue. However, a prominent band at ~81-84kDa was observed in peak 2 of normal and diabetic samples. PKC-ζ was expressed at lower levels in diabetic samples.

PKC-γ was found not to be expressed in rat liver as no immunoreactive species consistent with the presence of the PKC-γ isoform were observed in these studies.

4.3. Discussion

Previous studies have suggested that streptozotocin-induced diabetes in rats causes alterations in protein kinase C activity (Kim, *et al.*, 1991; Inoguchi, *et al.*, 1992) and/or expression (Tang, *et al.*, 1993). Changes in expression may selectively involve individual isoforms (Tang, *et al.*, 1993), a fact which may at least partly explain the conflicting range of data reported in the literature concerning changes in activity (Wali, *et al.*, 1990; Tanaka, *et al.*, 1991; Borghini, *et al.*, 1994). In order to assess whether changes in activity ensued as a result of the induction of diabetes, a protein kinase C activity assay was utilized, together with immunoblotting techniques.

The model of streptozotocin-induced diabetes reflects a type-I hypoinsulinaemic diabetic state. However, it also exhibits characteristics

of insulin resistance seen in NIDDM. In this model, destruction of pancreatic β-cells leads to reduced insulin secretion and, therefore, hypoinsulinaemia. If insulin activation of protein kinase C occurs as an endogenous phenomenon then a reduction in protein kinase C activity as a direct result of hypoinsulinaemia might be expected. This in turn might explain the increased expression (up-regulation) of the enzyme which tends to be seen in this model (Tang, *et al.*, 1993).

Upon partial purification of protein kinase C from rat liver using DE52 ion-exchange chromatography two peaks of activity were observed. The first to elute contained a significantly greater proportion of protein kinase C activity than the latter. Immunoblotting analysis showed that the α and ζ isoforms were only present in one peak, whilst the ε and β_{II} isoforms were found in both peaks. Individual isoforms might be expected to have different charges, and this phenomenon forms the basis of separation using ion-exchange chromatography. In addition, phosphorylation or dephosphorylation of protein kinase C species can be expected to result in an alteration of their charge and hence their chromatographic properties on ion-exchange resin. Another possible reason for elution at different salt concentrations could be specific binding of isoforms to cellular organelles.

Since the streptozotocin-diabetic rat is hypoinsulinaemic, it is expected that the results observed are most closely representative of the pathogenesis of insulin dependent (type I) diabetes. Results presented here show that protein kinase C activity observed in peak 1 from streptozotocin induced diabetic rat liver tissue was significantly decreased from that seen in normal rat liver. This suggests that insulin might activate protein kinase C to some extent under normal physiological conditions.

The assay conditions used allow resolution of protein kinase C activity into that which is Ca^{2+}-dependent and Ca^{2+}-independent. From

results presented here, it is apparent that induction of diabetes caused a significant reduction in the proportion of both Ca^{2+}-dependent and PS/DAG-dependent protein kinase C activity occurring in peak 1.

Immunoblotting studies allowed characterization of the protein kinase C isoform content of both peaks. This was then correlated with the proportion of Ca^{2+}-dependent and Ca^{2+}-independent activity present in each. Based simply on which isoforms were detected, it was concluded that in both normal and diabetic samples peak 1 was composed of the α, β_{II}, and ε isoforms. Peak 2 was composed of the β_{II}, ε and ζ isoforms, with the amount of PKC-β_{II} and PKC-ε expressed in peak 2 being less than in peak 1. Results using rat hepatocytes have suggested that overall expression of the α, β_{II} and ε isoforms is increased in streptozotocin induced diabetic animals (Tang, *et al.*, 1993). It is therefore possible that this increased expression might be a direct compensatory response for the decreased activity of protein kinase C in this model of diabetes.

Therefore, relating immunoblotting and activity assay results, it would appear that the reduced Ca^{2+}-dependent protein kinase C activity observed in diabetic animals might be directly due to altered PKC-α or PKC-β_{II} activity as neither of the other Ca^{2+}-dependent isoforms (PKC-β_{I} or PKC-γ) are expressed in liver. Similarly, the only Ca^{2+}-independent isoform detected in peak 1 in these studies was PKC-ε, suggesting that altered activity of this isoform underlies the observed decrease in Ca^{2+}-independent activity. Nevertheless, changes in other isoforms which we do not have antisera for may also occur.

It is worth highlighting the pattern of PKC-α expression which changed from two distinct bands in normal samples to one in diabetic samples. Others have also observed multiple forms of PKC-α detected in this tissue, one explanation mooted was that post-translational modifications may alter the apparent molecular size upon SDS-PAGE

(Pears, *et al.*, 1992). This group also provided evidence to suggest that phosphorylation of PKC-α can account for the observed mobility shift observed upon SDS-PAGE (Pears, *et al.*, 1992). They further suggest that phosphorylation on at least one site is required for PKC-α to be active as treatment with PPase-1 induces a time dependent loss of activity, although this particular phosphorylation did not lead to an apparent mobility shift. Subsequent autophosphorylation of the enzyme did however result in altered mobility. It is therefore worth bearing in mind that phosphorylation or dephosphorylation could account for the small mobility shifts of proteins observed upon SDS-PAGE, as well as changes in the activity of the enzyme. In the case of PKC-α expression observed here, it is likely that the switch from two bands to one upon induction of diabetes results in a reduction in activity, as the contribution of the 'upper' band seen in normal samples is lost. This theory is the most likely explanation of the changes in total protein kinase C activity observed between normal and diabetic tissue.

Activity in peak 2 was very much less than that in peak 1 in both normal and diabetic samples. Very little Ca^{2+}-dependent activity was observed and immunoblotting studies confirmed that the detected activity was attributable to a small amount of PKC-β_{II} isoform. No PKC-α was detected in this peak. Furthermore, little or no PS/DAG-dependent activity was observed in peak 2, despite the presence of PKC-ε and PKC-ζ isoforms. An explanation for this may be that as the PKC-ε detected in peak 2 is of a higher molecular weight than that in peak 1 it may represent a phosphorylated form of the enzyme where this modification has resulted in inactivation. Lack of Ca^{2+}- or PS/DAG-dependent activity suggests that total activity detected in peak 2 may be due to the presence of a constitutively active form of protein kinase C, perhaps as represented by the species of ~50kDa detected by PKC-ε and PKC-ζ antibodies.

In summary, results presented in this chapter highlight a decrease in total protein kinase C activity from normal levels in liver of rats made diabetic by streptozotocin treatment. This difference is accounted for both by altered Ca^{2+}-dependent and Ca^{2+}-independent protein kinase C activity. These studies suggest that the α, β_{II} and ε isoforms of protein kinase C might be responsible for the observed changes in activity, and the increased expression of these isoforms observed in streptozotocin induced diabetic rat hepatocytes may be the direct result of a reduction in their activity.

Histone kinase activity detected in peak 2 did not exhibit characteristics to suggest that it was entirely due to protein kinase C. This dubiety is somewhat resolved by the fact that protein kinase C expression is detected in peak 2.

Table 4.1. Summary of published data concerning changes in protein kinase C activity or expression in diabetic states

Listed in this table is an abbreviated summary of results published in recent years studying changes in protein kinase C activity or expression in diabetic models. A brief resume of the results in each case is presented, together with the diabetic model studied, the substrate employed in the assay (where applicable) and the tissue used in each case.

References for Table 4.1.

1. (Xiang and McNeill, 1992)
2. (Tanaka, *et al.*, 1991)
3. (Inoguchi, *et al.*, 1992)
4. (Kim, *et al.*, 1991)
5. (Borghini, *et al.*, 1994)
6. (Hoffman, *et al.*, 1991)
7. (Tang, *et al.*, 1993)
8. (Wali, *et al.*, 1990)
9. (Craven and DeRubertis, 1989)
10. (Garcia-Paramio, *et al.*, 1993)
11. (Williams and Schrier, 1992)
12. (Nagy, *et al.*, 1991)
13. (Farese, *et al.*, 1992b)
14. (Egan, *et al.*, 1990)

Model	Tissue	Substrate	Cytosolic	Particulate	Ref
Streptozotocin	Heart	Histone	Decreased activity	Increased activity	1
Streptozotocin	Heart	Histone	94% Increased activity	41% Increased activity	2
			Both inhibited by verapamil		
Streptozotocin	Heart	Peptide (RKRTLRRL)	Unchanged activity	21% Increased activity	3
Streptozotocin	Sciatic nerve	Histone	Decreased activity	Unchanged activity	4
Streptozotocin	Sciatic nerve	Histone	No altered overall activity No altered expression or distribution of isoforms		5
Streptozotocin	Soleus muscle	Histone	Increased activity	Decreased activity	6
Streptozotocin	Adipocytes	Histone	Decreased activity	Increased activity	6
Streptozotocin	Hepatocytes	n/a	Increased α and β_{II} expression	Increased α, β_{II} and ε expression	7
			Reversed by insulin treatment		
Streptozotocin	Aorta	Peptide (RKRTLRRL)	Unchanged activity	88% Increased activity	3

Model	Tissue	Substrate	Cytosolic	Particulate	Ref
Streptozotocin	Small intestine	Histone	60% Decreased activity	10% Decreased activity	8
			Total activity restored by insulin therapy		
Streptozotocin	Glomeruli	Histone	Decreased activity	Increased activity	9
			Prevented by insulin treatment		
Streptozotocin	Prostate	Histone	Decreased activity	Increased activity	10
Elevated glucose	Vascular smooth muscle	Peptide (VRKRTLRRL)	Increased overall activity		11
Starvation-induced insulin resistance	Liver	Histone	200% Increased overall activity Increased expression		
Healthy / Type II NIDDM humans	Mononuclear cells	Peptide (RKRTLRRL)	Decreased overall activity		12
Insulin treatment	Adipocytes	n/a	Decreased expression	Increased expression	13
Insulin treatment	Adipocytes	Histone	Increased overall activity		14

Figure 4.1. Profile of protein kinase C activity in normal rat liver after partial purification on DE52 ion exchange chromatography

Protein kinase C activity was assayed (as described in Section 2.1.12) in each fraction collected after chromatography using the following three assay conditions: phosphatidylserine, diacylglycerol and Ca^{2+} (■); phosphatidylserine, diacylglycerol and EGTA (○); and EGTA only (◇). Total activity in each fraction is expressed in units of pmoles of phosphate transferred from the γ position of ATP onto the substrate histone per minute, and corrected per gram of liver (wet weight) used to allow comparison between animals.

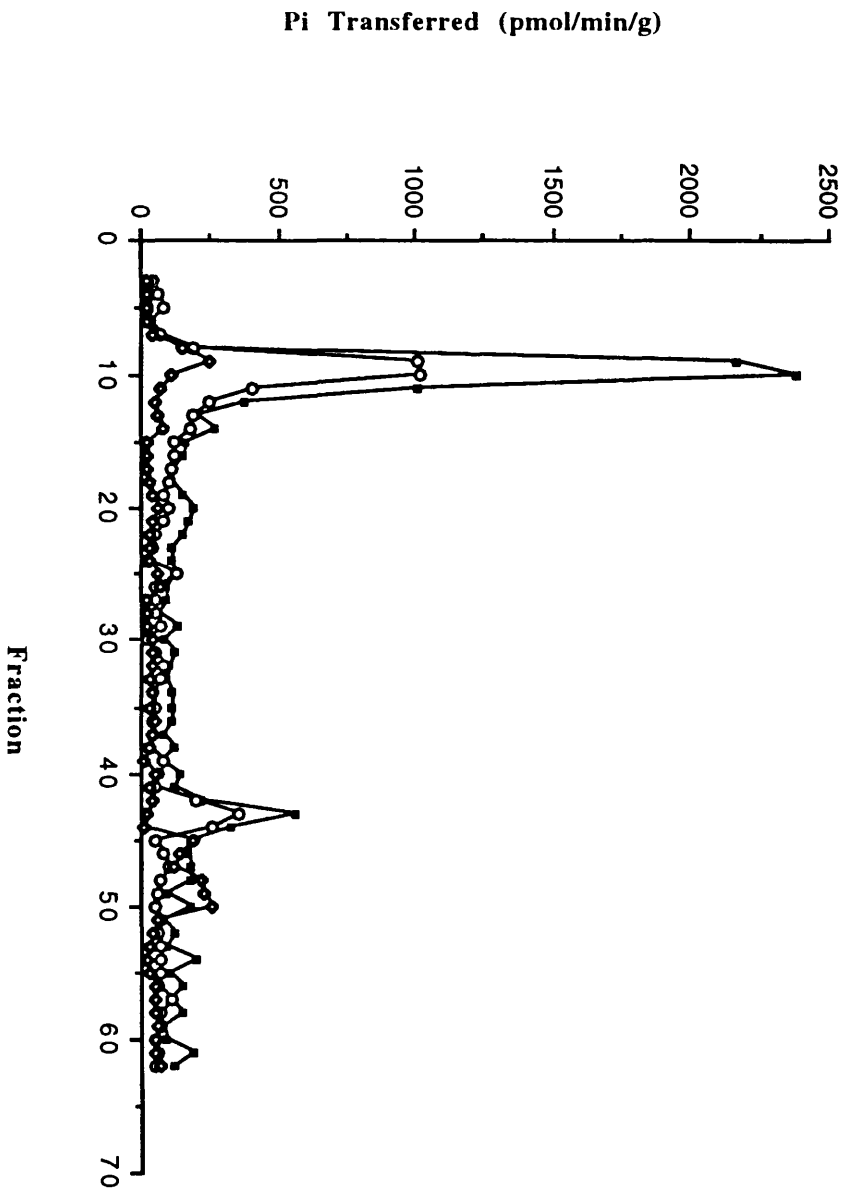

Pi Transferred (pmol/min/g)
0
500
1000
1500
2000
2500
0
10
20
30
40
50
60
70
Fraction

Figure 4.2. Profile of protein kinase C activity in diabetic rat liver after partial purification on DE52 ion exchange chromatography

Protein kinase C activity was assayed (as described in Section 2.1.12) in each fraction collected after chromatography using the following three assay conditions: phosphatidylserine, diacylglycerol and Ca^{2+} (■); phosphatidylserine, diacylglycerol and EGTA (○); and EGTA only (◇). Total activity in each fraction is expressed in units of pmoles of phosphate transferred from the γ position of ATP onto the substrate histone per minute, and corrected per gram of liver (wet weight) used to allow comparison between animals.

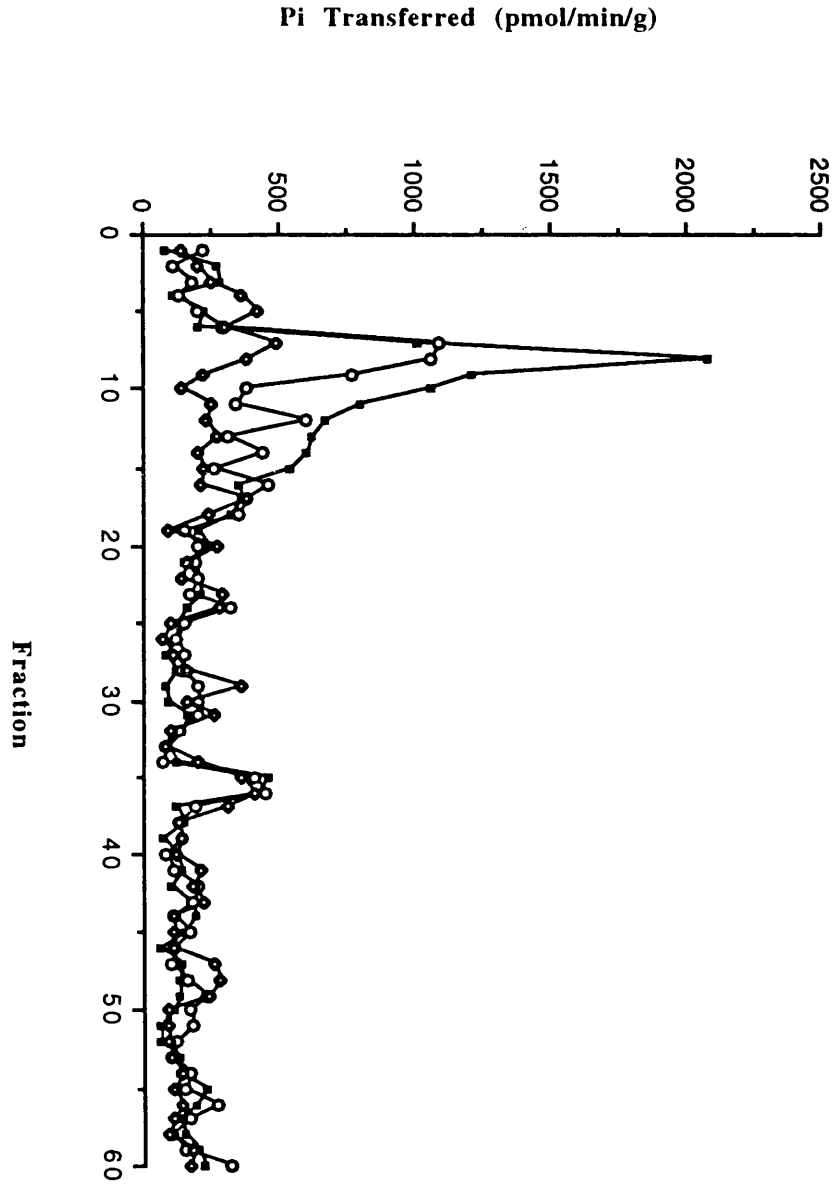

Pi Transferred (pmol/min/g)
0
500
1000
1500
2000
2500
Fraction
0
10
20
30
40
50
60

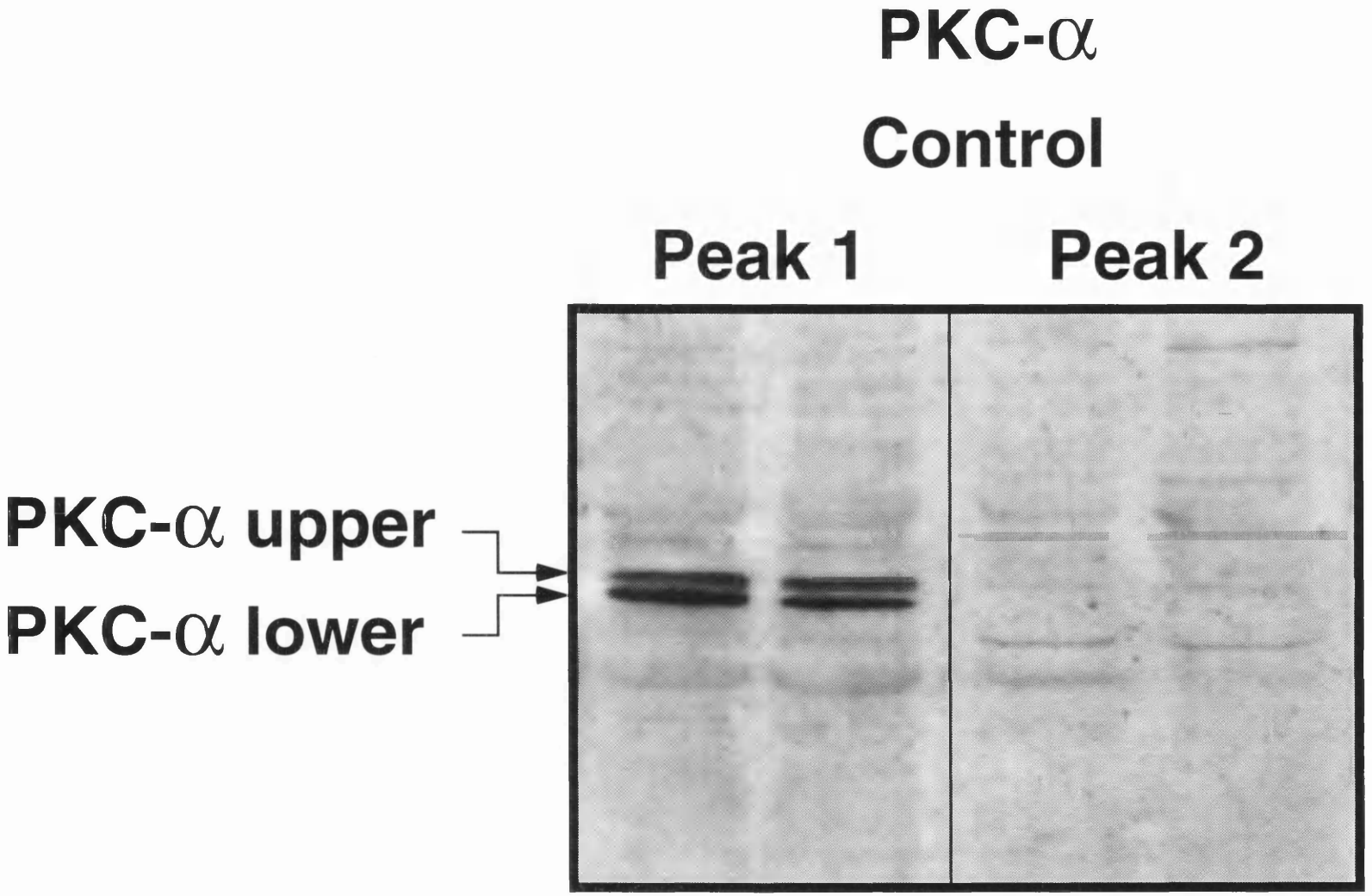
Table 4.2. Analysis of partially purified protein kinase C activity in normal and streptozotocin induced diabetic rat liver

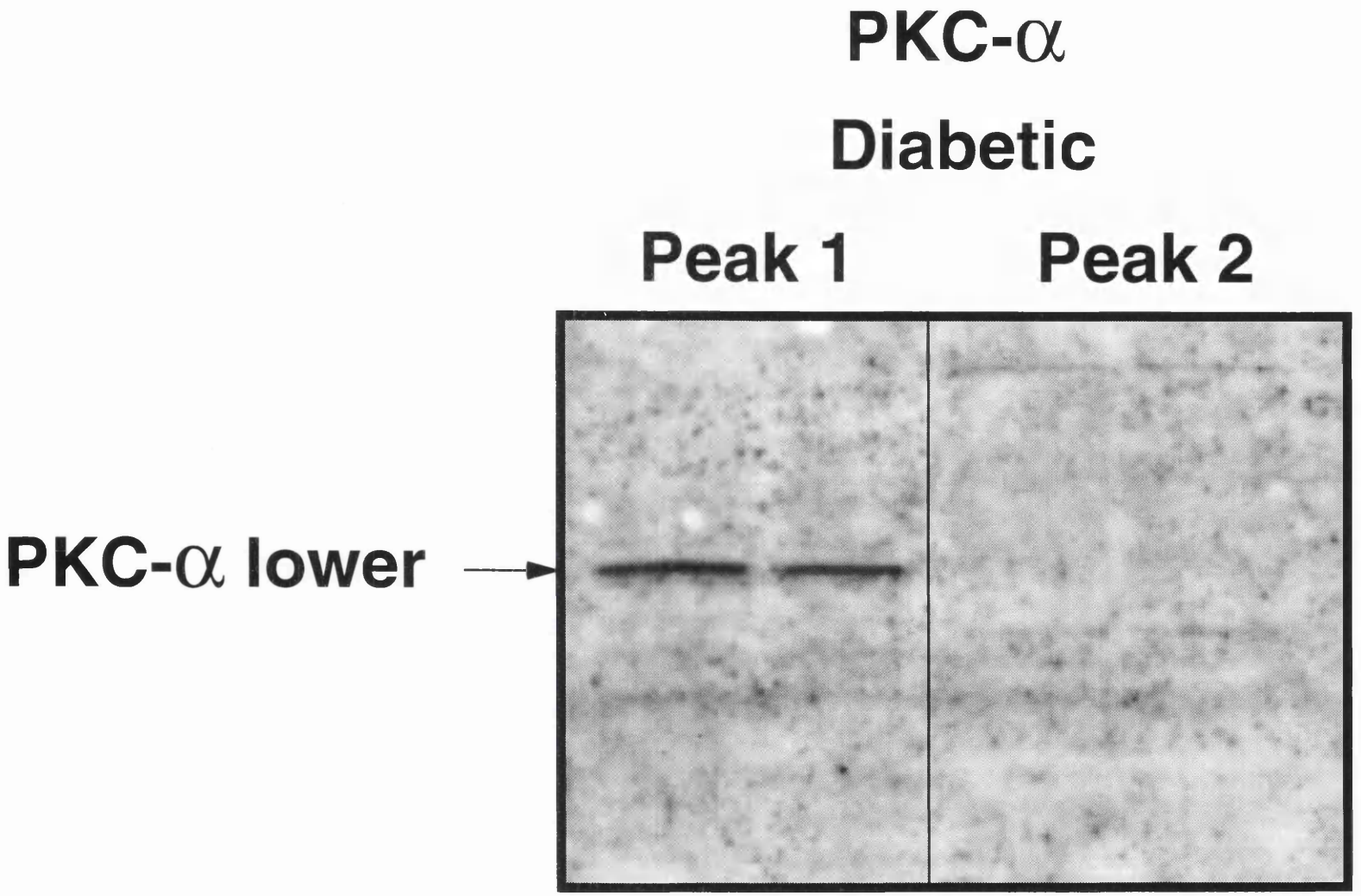
Protein kinase C activity was assayed as described in Section 2.1.12 and total activity in each peak calculated as the area under the peak of activity observed in the presence of PS/DAG and Ca^{2+} (see Figures 4.1 and 4.2). Ca^{2+}-dependent activity was measured as total activity minus the area under the peak of activity observed in the presence of PS/DAG and EGTA. PS/DAG-dependent activity was calculated as the difference in the area under the peaks observed in the presence of PS/DAG and EGTA and in the presence of EGTA alone. Results are expressed in arbitrary units (±S.D) and are representative of three normal and three diabetic subjects. Results were statistically assessed using a student's t-test and relevant p values shown in the table (nsd = no significant difference).

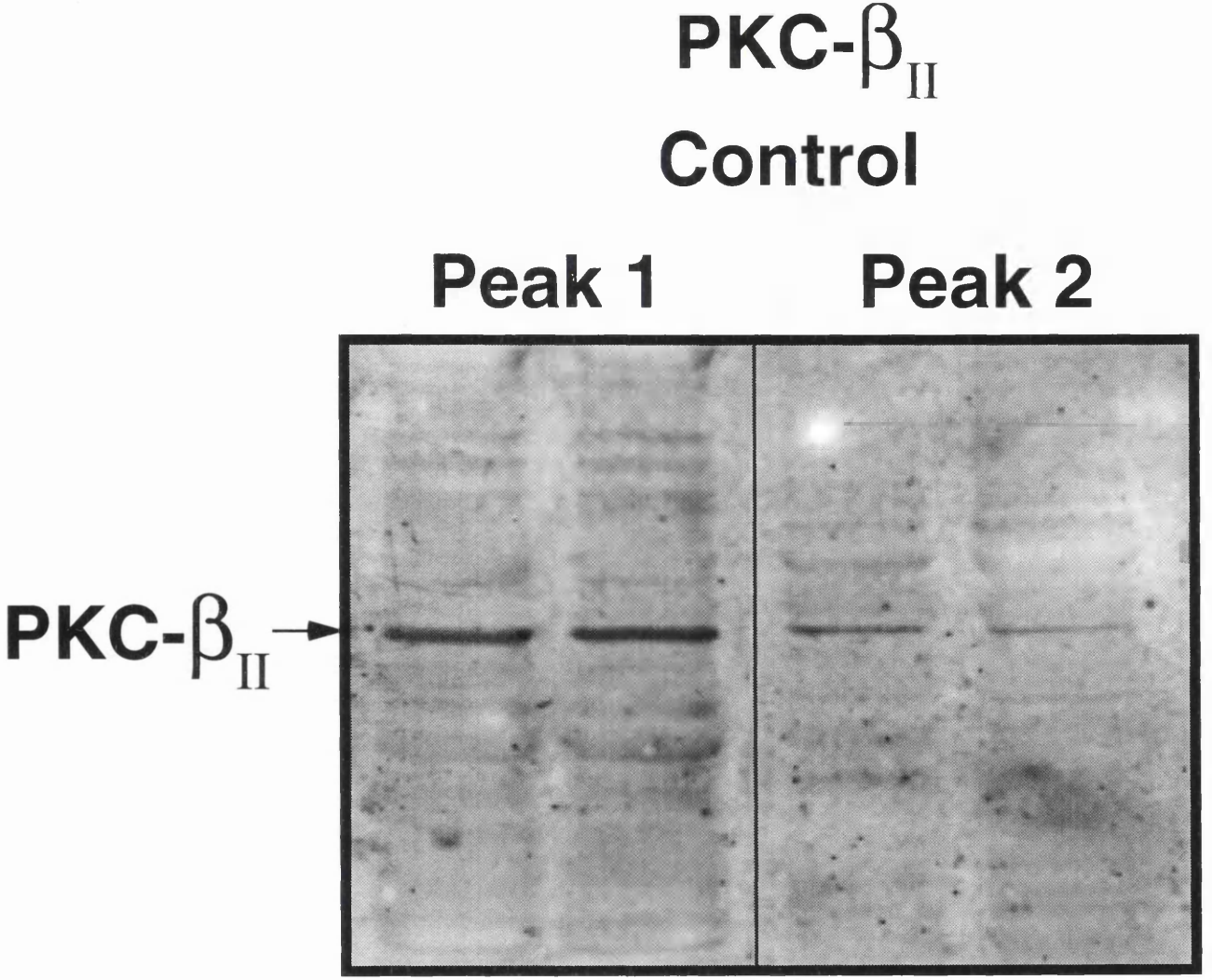
	Normal (n=3)	Diabetic (n=3)	
Total Activity (peak 1)	679 ± 44	504 ± 50	p=0.01
Total Activity (peak 2)	46 ± 23	59 ± 10	nsd
Ca^{2+}-dependent activity (peak 1)	360 ± 54	258 ± 25	p=0.03
Ca^{2+}-dependent activity (peak 2)	9 ± 8	0 ±11	nsd
PS/DAG-dependent activity (peak 1)	332 ± 20	183 ± 7	p=0.001
PS/DAG-dependent activity (peak 2)	-35 ± 40	-7 ± 7	nsd

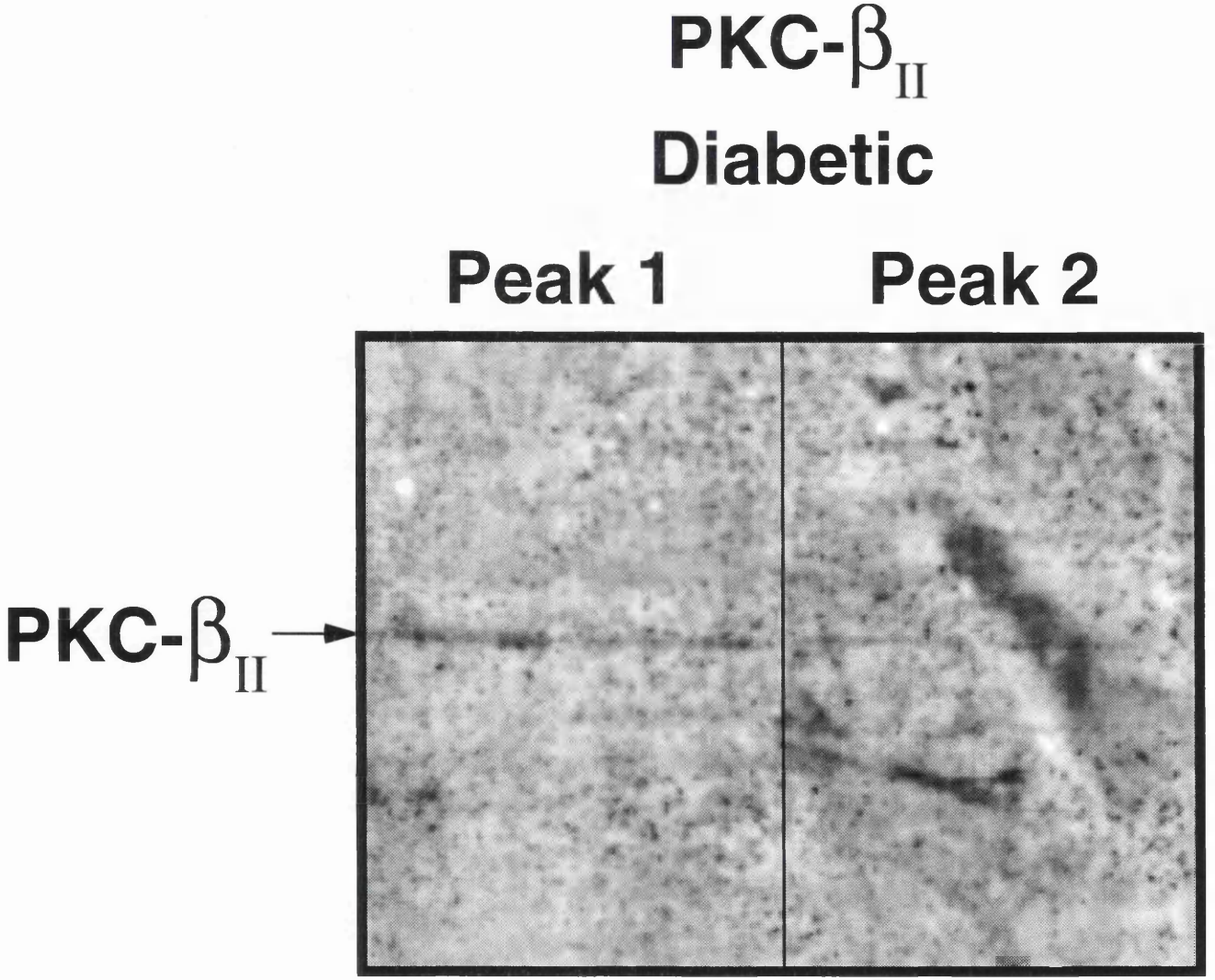
Figures 4.3. - 4.10. Expression of protein kinase C isoforms corresponding to observed peaks of activity after partial purification of the enzyme from normal and streptozotocin induced diabetic rat liver

Antiserum specifically recognizing the α, β_{II}, ε and ζ isoforms of protein kinase C were employed in studying expression of these isoforms in fractions exhibiting protein kinase C activity. Samples were taken from individual fractions and volumes corresponding to 100μg total protein content were mixed with an equivalent volume of x2 sample buffer (Section 2.1.8.1) and boiled before being analysed by SDS-PAGE in each study. Results representing isoform expression in peak 1 and peak 2 are presented here as Figure 4.3 (PKC-α normal), Figure 4.4 (PKC-α diabetic), Figure 4.5 (PKC-β_{II} normal), Figure 4.6 (PKC-β_{II} diabetic), Figure 4.7 (PKC-ε normal), Figure 4.8 (PKC-ε diabetic), Figure 4.9 (PKC-ζ normal) and Figure 4.10 (PKC-ζ diabetic).


PKC-α
Control
Peak 1
Peak 2
PKC-α upper
PKC-α lower


PKC-α
Diabetic
Peak 1
Peak 2
PKC-α lower

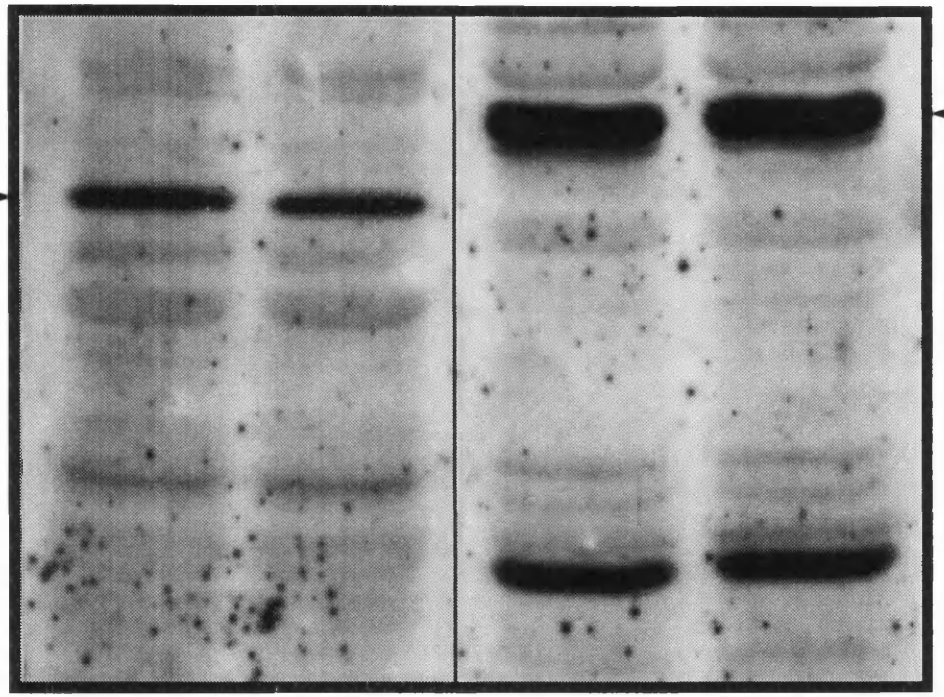

PKC-βII
Control
Peak 1
Peak 2
PKC-βII


PKC-βII
Diabetic
Peak 1
Peak 2
PKC-βII

PKC-Σ

Control

Peak 1 Peak 2

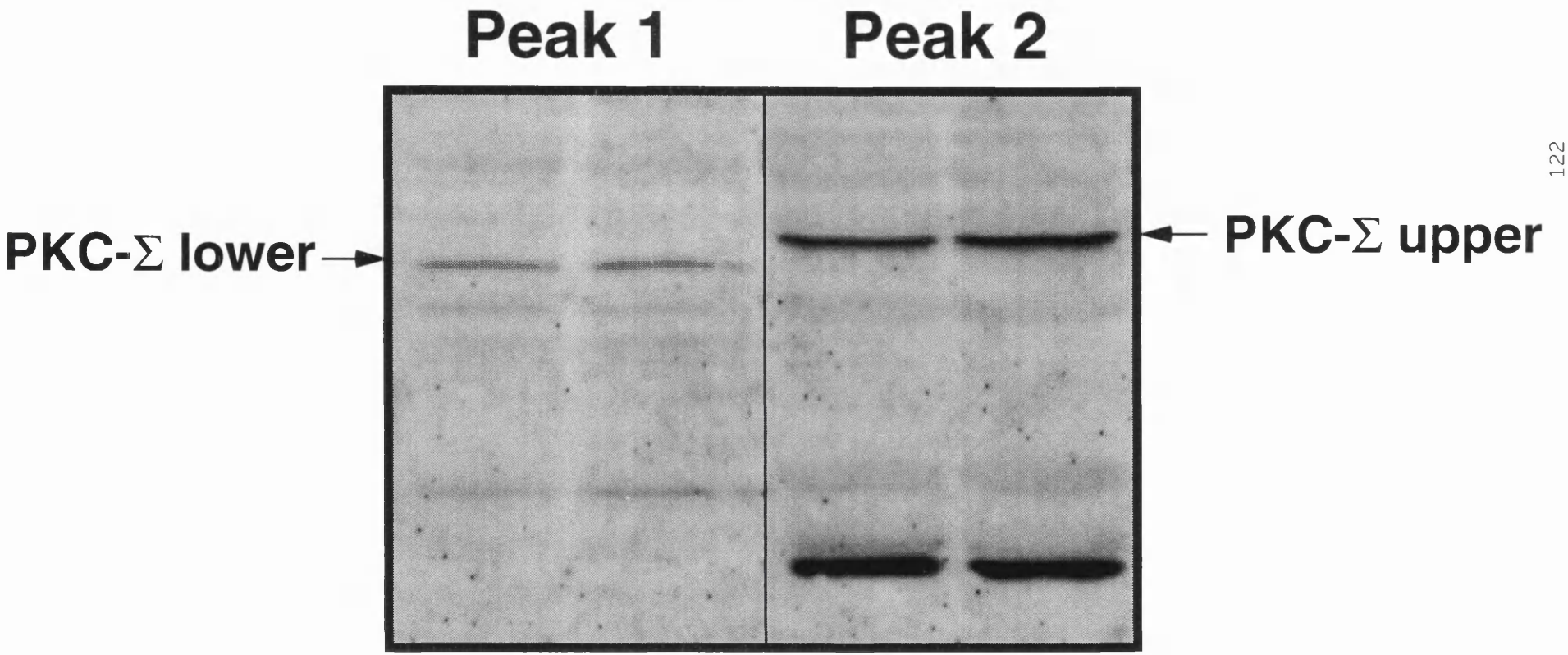


PKC-Σ lower

PKC-Σ upper

PKC-Σ

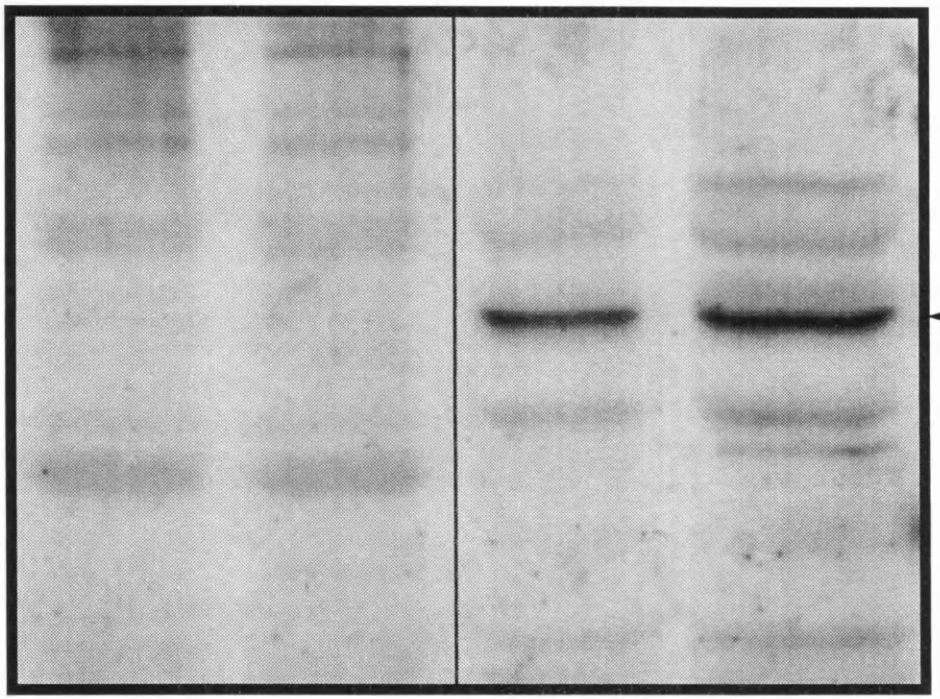
Diabetic



PKC-ζ

Control

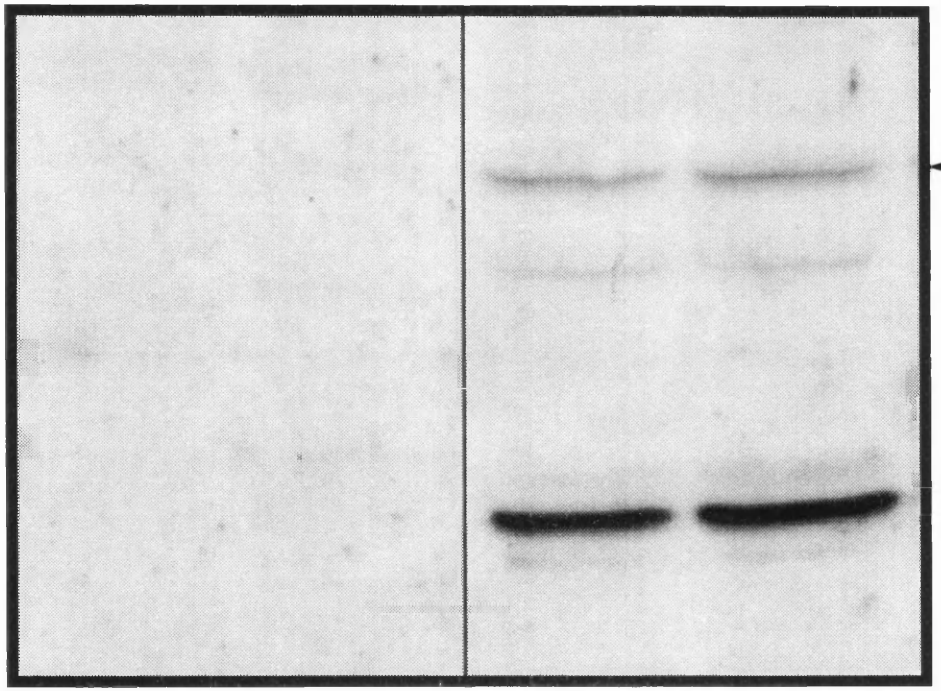
Peak 1 Peak 2



PKC-ζ

Diabetic

Peak 1 Peak 2



Chapter 5

Control of intracellular cyclic AMP metabolism in CHO cells

5.1. Introduction

The role of cyclic AMP as a second messenger was first revealed by the work of Sutherland and colleagues several decades ago (Sutherland, *et al.*, 1962). This nucleotide, synthesized in cells from ATP by the membrane bound enzyme adenylate cyclase, is constantly turning over in cells, its production being counteracted by hydrolysis to 5'-AMP through the action of phosphodiesterases. Both cyclic AMP and adenylate cyclase exist in every tissue of higher organisms.

Many hormones bind to specific receptors on the cell surface to elicit changes in intracellular cyclic AMP concentrations. These changes can be brought about by changes in either the rate of synthesis or the rate of degradation of cyclic AMP. Examples of ligands which increase intracellular cyclic AMP concentrations include β-adrenoceptor agonists, histamine (via H2 receptors), prostaglandins, vasopressin and glucagon (via GR2 receptors). Ligands such as muscarinic receptor agonists, α_2-adrenoceptor agonists, thromboxanes and opioids act to decrease intracelluar cyclic AMP concentrations. Both the number of ligands impinging upon this system and their physiological role again stress the fundamental importantance of cyclic AMP in the cell.

Binding of the agonist isoprenaline to the β-adrenergic receptor results in the activation of adenylate cyclase with consequent intracellular formation of cyclic AMP. Transduction of this signal involves sequential interaction of the β-adrenergic receptor and the stimulatory guanine nucleotide-binding protein G_s with consequent activation of the enzyme adenylate cyclase. As mentioned previously, other ligands acting via their own specific receptors can also stimulate adenylate cyclase. Two such agonists also used in these studies are PGE_1 and glucagon. As discussed in Section 1.10, insulin is known to influence cyclic AMP metabolism in

various systems. Results presented in this chapter assessed this activity in CHO cells and particularly the part played by specific protein kinase C isoforms.

In the work presented in this Chapter and in Chapter 6, several Chinese hamster ovary-derived cell clones were used. These were as follows;

CHO-K (wild type cells),

CHO-T (CHO-K cells transfected such that they stably overexpressed the human insulin receptor),

CHO-α (overexpressed human insulin receptor and PKC-α),

CHO-β (overexpressed human insulin receptor and PKC-β),

CHO-γ (overexpressed human insulin receptor and PKC-γ),

CHO-ε (overexpressed human insulin receptor and PKC-ε).

5.2. Results

The first set of experiments set out to examine cyclic AMP levels under various conditions in the six CHO cell-clones available. Intracellular cyclic AMP levels in cells under normal growth conditions are shown in Figure 5.1. Only CHO-γ cells and CHO-T cells showed significantly lower values than those of wild type control cells. None of the other cell types showed any significant difference in their basal cyclic AMP levels as compared to CHO-K cells.

Isoprenaline stimulated increases (or in CHO-γ cells decrease) in intracellular cyclic AMP levels were then measured for all cell clones. Results are expressed in Table 5.1 as a percentage of the basal intracellular cyclic AMP concentration in that cell type, with basal values being designated as 100%. In all cell clones, with the exception of CHO-γ cells, isoprenaline elicited a less than two fold increase in intracellular cyclic AMP levels (Table 5.1). CHO-γ cells were different in that

isoprenaline did not cause any intracellular cyclic AMP accumulation, and in fact a large reduction was observed (Table 5.1).

Also displayed in Table 5.1 are results of studies examining the effect of insulin upon the ability of isoprenaline to elicit changes in intracellular cyclic AMP concentration. It was observed that only in those cells transfected with PKC-ε, in addition to the human insulin receptor, did insulin exert an effect, namely to attenuate isoprenaline stimulated increases in intracellular cyclic AMP concentration.

This result made analysis of the influence of IBMX, a phosphodiesterase inhibitor, worthy of evaluation. As can be seen from Table 5.1 IBMX increased basal intracellular cyclic AMP concentrations between two and three fold, as well as consistently increasing such levels when used in conjunction with isoprenaline and insulin. In experiments where CHO-ε cells were treated with IBMX, insulin could, to a certain extent, still act to reduce the increased intracellular cyclic AMP levels elicited by isoprenaline. Note, however, that treating cells with IBMX as well as isoprenaline and insulin increased intracellular cyclic AMP concentrations over that observed with either isoprenaline and insulin treatment. . In all other cell clones examined insulin, again, had no significant effect on intracellular cyclic AMP concentrations.

Figure 5.2 represents data obtained upon the stimulation of cyclic AMP accumulation in CHO-ε cells by using various agonists. Isoprenaline, acting via β-adrenergic receptors, PGE_1, acting via its own specific receptors, and glucagon, acting via GR2 receptors, all stimulated adenylate cyclase to increase intracellular cyclic AMP levels. Under normal conditions, insulin had a dramatic effect upon intracellular cyclic AMP accumulation in response to these agonists. In the case of all three agonists, insulin attenuated their ability to increase intracellular cyclic AMP, and in each case caused cyclic AMP concentrations to fall below

basal levels. This effect of insulin was lost, however, if cells were preincubated for 16 hours with 100nM TPA to down-regulate protein kinase C prior to agonist challenge.

5.3. Discussion

All six CHO cell-clones were characterised in terms of basal intracellular cyclic AMP concentration as well as isoprenaline stimulated responses. Similar basal intracellular cyclic AMP levels were detected in CHO-α, CHO-β, CHO-ε and CHO-K cells. With reference to results presented in Chapter 6, where phosphodiesterase activity was measured in cells under similar conditions and found to be elevated in CHO-ε cells, it is surprising that CHO-ε cells did not exhibit lower basal intracellular cyclic AMP concentrations than wild type control cells. One explanation for this may be that despite a clearly elevated phosphodiesterase activity in CHO-ε cells, there was a compensatory up-regulation of adenylate cyclase activity under basal conditions. Thus, they would be expected to maintain the basal intracellular cyclic AMP concentration indicated. Indeed, a basal cyclic AMP level of this magnitude may be essential for viability of this particular cell clone. CHO-γ cells, together with CHO-T cells, had a significantly lower intracellular cyclic AMP concentration compared with wild type cells. Therefore, the proteins overexpressed by these cells must somehow be affecting intracellular cyclic AMP metabolism, either by impairing adenylate cyclase activity or increasing phosphodiesterase activity. Overexpression of PKC-γ could conceivably, for example, alter the phosphorylation state of either a relevant G-protein (Katada, *et al.*, 1985), adenylate cyclase (Jacobowitz, *et al.*, 1993) or phosphodiesterase(s) (Irvine, *et al.*, 1986; Abou-Samra, *et al.*, 1987) such that their activity is altered. Why transfection of insulin receptor alone should bring about these changes is more difficult to explain. It may be that since growth

factors present in serum in cell growth medium will include insulin, which acts via these receptors, some exaggerated input upon cyclic AMP metabolism from the overexpressed insulin receptor might explain these changes. If this is the case, it must be assumed that transfection of PKC-α, -β and -ε in the other cell types somehow alleviated this problem.

With the exception of CHO-γ cells, all other cell lines proved to be sensitive to isoprenaline stimulation by exhibiting an increased intracellular cyclic AMP concentration. Surprisingly, not only did isoprenaline fail to elicit increased intracellular cyclic AMP levels in CHO-γ cells, intracellular cyclic AMP was consistently found to be reduced from basal levels. An explanation for why PKC-γ transfection should cause such an unlikely effect is difficult to rationalise. This could be hypothesized to be due to elevated phosphodiesterase activity, but other experiments (Table 6.1) suggest that alterations in phosphodiesterase activity do not account for this observation. Therefore, a defect in cyclic AMP generation, ie. at some stage between the receptor and catalytic unit of adenylate cyclase, seems more likely as PKC-γ could potentially phosphorylate and attenuate the function of one of the components involved in cyclic AMP synthesis. Nevertheless, it is possible that using isoprenaline to stimulate adenylate cyclase in this cell type provides a system where the effects of various other ligands or inhibitors can be usefully investigated. This allows examination of, for example, whether observed phenomenon are common to all, or only receptor specific, adenylate cyclase mediated responses.

Having established the response to isoprenaline in each of the cell types, the effect of insulin upon this effect was then examined. Upon doing so, one exciting observation stood out. Only in CHO-ε cells did insulin dramatically attenuate the ability of isoprenaline to elevate intracellular cyclic AMP, such that its concentration remained around

basal levels after isoprenaline treatment. This dramatic effect represents one of the few clear demonstrations known where insulin directly influences intracellular cyclic AMP metabolism. The mechanisms underlying this effect may involve stimulation of a phosphodiesterase or inhibition of cyclic AMP synthesis, and these will be discussed further as results both in this and the following chapter contribute towards their understanding.

As expected, if cells were pretreated with IBMX, intracellular cyclic AMP concentrations were correspondingly increased two to three fold in all cases, a typical response in many cell types, albeit a small one. A slightly greater increase was observed in CHO-ε cells. This suggests a higher basal phosphodiesterase activity in this cell type, as inhibition of this activity would be expected to result in a more pronounced increase in intracellular cyclic AMP concentration within these cells. Considering the difference between isoprenaline and combined isoprenaline/IBMX treatment of cells again shows that IBMX apparently causes a more significant increase in intracellular cyclic AMP in CHO-ε, and to a slightly lesser extent in CHO-α, cells when compared with wild type cells. This, again, is in agreement with data presented in Table 6.1 showing an increased basal phosphodiesterase activity does indeed exist in CHO-ε cells.

Looking specifically at the effect of IBMX on insulin's ability to alter intracellular cyclic AMP levels in CHO-ε cells, it would be expected that if insulin was acting by stimulating phosphodiesterase activity then IBMX would prevent insulins action. Infact, IBMX appeared only to partly block the ability of insulin to attenuate isoprenaline stimulated increases in intracellular cyclic AMP concentration. This speaks in favour of an additional action of insulin at the level of cyclic AMP generation. Again results presented in Table 6.1 suggest that insulin stimulates

phosphodiesterase activity in these cells. Therefore, only a partial block of insulin action here by IBMX means either that insulin may have a dual action both upon adenylate cyclase and cyclic AMP phosphodiesterase or that the type of phosphodiesterase(s) stimulated by insulin are somewhat resistant to IBMX inhibition, at least under these experimental conditions. Other possibilities include an effect at the level of the stimulatory ligand receptor or G_s, where in both cases insulin induced alteration in, for example, their phosphorylation state may cause inhibition. Similarly, some modification at the level of G_i could be influential. Indeed, insulin has been noted to reduce basal G_i-2 phosphorylation in rat hepatocytes (N. Morris, personal communication), an effect which would increase the proposed 'tonic' inhibition exerted by this G-protein on adenylate cyclase, and hence ultimately result in decreased intracellular cyclic AMP levels.

Finally, a separate set of experiments was conducted with the ultimate aim of further clarifying the molecular basis of the observed effect of insulin on agonist stimulated intracellular cyclic AMP accumulation in CHO-ε cells. Three agonists known to stimulate adenylate cyclase and therefore to increase intracellular cyclic AMP concentrations were used to challenge CHO-ε cells and the responses characterised. As can be seen from the results shown in Figure 5.2.Y, each of the three agonists induced an approximately similar degree of elevation of intracellular cyclic AMP concentration, with PGE_1 being slightly the more potent of the three agonists.

Also shown in Figure 5.2.Y is the effect observed upon pretreating the cells with insulin and using the same agonists to repeat the above stimulations. After insulin treatment, these agonists were no longer capable of eliciting an overall increase in intracellular cyclic AMP concentration, with levels now falling below basal values, as was observed in previous experiments using isoprenaline. In identifying the molecular

basis of this effect, the use of a cell-line such as CHO-ε allows the opportunity to investigate the role played not just by protein kinase C in this effect, but by a specific isoform of the enzyme.

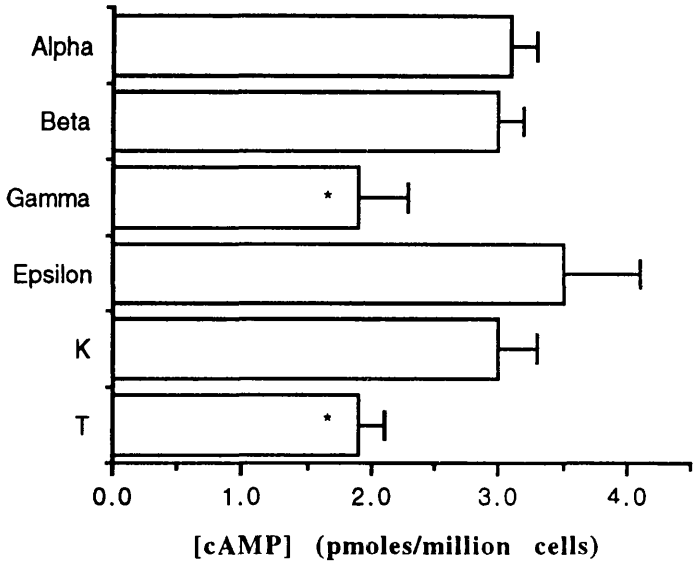
With this in mind, the experiments presented in Figure 5.2.Y were repeated in cells which had been treated with the phorbol ester TPA to down-regulate protein kinase C. The main observation was that after treating cells with TPA for 16 hours, insulin was no longer able to cause the dramatic reduction in intracellular cyclic AMP concentration seen previously (Figure 5.2.Z). This suggests that PKC-ε is involved in insulin's action on agonist stimulated increases in intracellular cyclic AMP in this cell type. Whether this is via causing increased phosphodiesterase activity or by inhibiting synthesis will be further addressed in Chapter 6. It should be noted at this stage, however, that this loss of insulin action is less striking in the case of PGE_1 stimulated increases. This may be explained by hypothesizing that insulin reduces intracellular cyclic AMP concentration in these cells by two mechanisms, stimulation of a cyclic AMP phosphodiesterase and inhibition of a component involved in synthesis. If we assume that only phosphodiesterase stimulation is PKC-ε dependent then phorbol ester treatment and PKC-ε down-regulation will abolish this effect no matter what agonist is used. The other portion of insulin's effect may be PKC-ε independent, hence a slight reduction in intracellular cyclic AMP is still elicited after phorbol ester treatment in all cases. This only appears to be significant in the case of PGE_1 induced increases. This suggests that the location of this portion of the insulin effect could be at the level of the receptor, since it appears to be fairly specific for one agonist compared to the others used. Nevertheless, a subtle modification of G_s that influences receptor interaction, with the PGE_1 receptor binding site being most greatly affected by this, is also possible.

In summarizing these results it must be concluded that PKC-ε plays a crucial role at some stage in the pathway whereby insulin attenuates agonist stimulated increases in intracellular cyclic AMP concentration. Exactly how insulin's input upon control of cyclic AMP metabolism is achieved may be more complex than envisaged, such that it allows for fine tuning in antagonism to specific ligands. It is suggested that in CHO-ε cells, under the experimental conditions imposed here, insulin acts both by causing phosphorylation, and stimulation, of a cyclic AMP phosphodiesterase and by inducing ligand receptor phosphorylation, with attenuation of activity. PKC-ε appears to play a crucial role in the former explanation, the latter effect perhaps being achieved via a protein kinase C-independent pathway.

Finally, one other potential reason for the apparently agonist specific effects of PKC down-regulation observed could simply be due to the times that agonists were allowed to act after insulin pretreatment. For example, loss of insulin action upon PKC down-regulation is less marked in the case of PGE_1 stimulated increases in intracellular cyclic AMP, and PGE_1 is added only for 2 minutes as opposed to 3 minutes for glucagon and 5 minutes for isoprenaline.

Figure 5.1. Analysis of intracellular cyclic AMP levels in CHO cells

Results to determine basal intracellular cyclic AMP levels for each of six cell clones (designated CHO-α, $-\beta$, $-\gamma$, $-\epsilon$, -K and -T) are summarized ($n \geq 3$) in both graphic and tabular form. Values represent pmoles of cyclic AMP per million cells. Statistically significant results are indicated as $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***).



	CHO-α	CHO-β	CHO-γ	CHO-ε	CHO-K	CHO-T
[cAMP]	3.1±0.2	3.0±0.2	1.9±0.4	3.5±0.6	3.0±0.3	1.9±0.2
(pmoles/million cells)			*			*

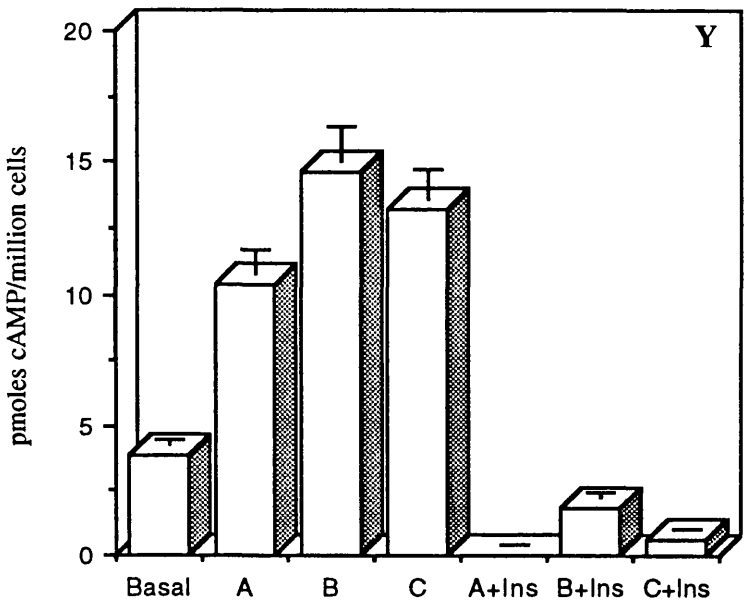
Table 5.1. Control of intracellular cyclic AMP levels in CHO cells

Results for each of six cell types (designated CHO-α, -β, -γ, -ε, -K and -T) are summarized for the conditions listed ($n \geq 3$). All values are expressed as a percentage of the basal value (designated 100%) for each individual cell clone, unless otherwise stated. Basal specific activities in each clone was as follows; 3.1±0.2 (CHO-α), 3.0±0.2 (CHO-β), 1.9±0.4 (CHO-γ), 3.5±0.6 (CHO-ε), 3.0±0.3 (CHO-K) and 1.9±0.2 (CHO-T). Isoprenaline stimulation in each case was over 5 minutes at 10^{-4}M, while insulin pretreatment was for 5min at 10^{-8}M and IBMX pretreatment for 15min at 10^{-3}M. Statistically significant results are indicated as $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***).

Basal	3.1±0.2	3.0±0.2	1.9±0.4	3.5±0.6	3.0±0.3	1.9±0.2
	CHO-α	CHO-β	CHO-γ	CHO-ε	CHO-K	CHO-T
Isoprenaline	197±5	148±22	60±8	198±12	190±21	168±16
Isoprenaline +Insulin	192±5	136±3	64±1	107±4	167±9	163±9
% Change due to insulin	-5%	-12%	+4%	-91% ***	-23%	-5%
IBMX	247±5	242±8	227±15	275±18	233±23	220±20
Ratio of change due to IBMX *cf.* CHO-K cells	1.1	1.1	1.0	1.3	(1)	0.9
Isoprenaline +IBMX	329±35	259±11	182±10	365±33	294±22	282±25
Ratio of change due to IBMX *cf.* CHO-K cells	1.3	1.1	1.2	1.6 *	(1)	1.1
Isoprenaline +IBMX +Ins	286±20	270±6	199±26	240±3	249±12	234±13
Ratio of change due to IBMX *cf.* CHO-K cells	1.1	1.6 *	1.6	1.6 *	(1)	0.9

Figure 5.2. Agonist induced increases in intracellular cyclic AMP levels in CHO-ε cells and the effects of insulin and phorbol ester

Results in this figure display intracellular cyclic AMP concentrations (expressed as pmoles cAMP per 10^6 cells) in CHO-ε cells under basal conditions and after treatment with various ligands (n=1). Three agonists were used to stimulate cyclic AMP formation; (A) isoprenaline (5min, 10^{-4}M), (B) PGE_1 (2min, 10^{-5}M) and (C) glucagon (3min, 10^{-6}M). Treatment of cells with each of these three agonists was also carried out after insulin pre-treatment (A, B or C +ins), (5min, 10^{-8}M). These results are expressed in Figure 5.1.Y. Finally, all of these conditions were repeated on cells which had been treated with the phorbol ester TPA (100nM) for 16 hours to down-regulate protein kinase C (see Figure 6.2), results of which are shown in Figure 5.1.Z.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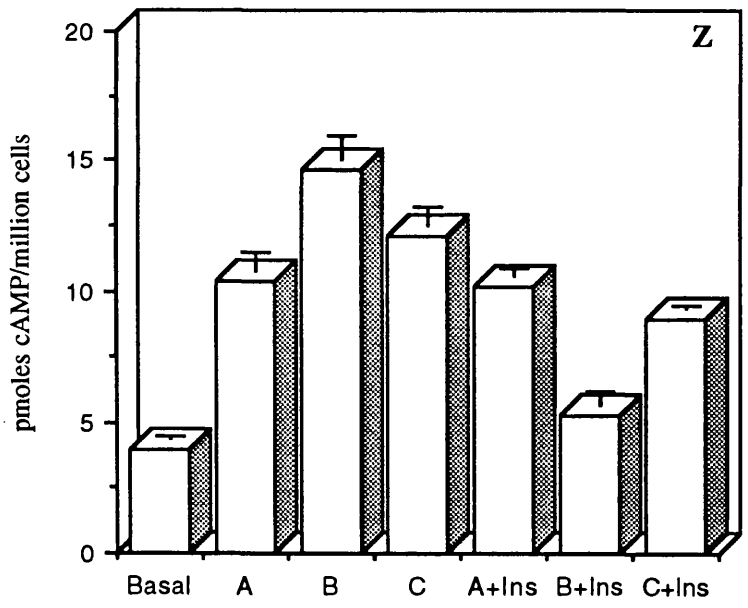
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Chapter 6

Phosphodiesterase activity in CHO cells

6.1. Introduction.

The only known enzymatic reaction for the degradation of cyclic nucleotides is their conversion to the corresponding 5'-nucleotide via hydrolysis of the 3', 5'-phosphodiester bond. This is catalyzed by cyclic nucleotide phosphodiesterases, as discussed in Section 1.9.

Results presented in this chapter were obtained using the phosphodiesterase assay described in detail in Section 2.1.2. Chinese hamster ovary cell clones transfected with insulin receptor alone or combined with a specific protein kinase C isoform (see Section 5.1), were used in all experiments and compared to wild type, non-transfected CHO cells as an appropriate control. This allowed analysis of the role of individual protein kinase C isoforms in regulating cyclic AMP phosphodiesterases, under both basal and insulin stimulated conditions. This was determined in order to help clarify the mechanism by which insulin attenuated agonist induced increases in intracellular cyclic AMP concentrations in CHO-ε cells (Chapter 5).

Given the significant importance of cyclic nucleotide second messengers, hormonal control of phosphodiesterase activity is of great importance within cells. Regulation of phosphodiesterase activity by insulin was established in 1985 (Beebe, *et al.*, 1985) and has been reinforced by many subsequent studies (Lonroth and Smith, 1986; Kather and Scheureer, 1987). More specifically these studies suggest that, in liver, insulin specifically regulates activity of individual phosphodiesterase isoforms. Activation of both a type III ('dense vesicle') and a type IV ('peripheral plasma membrane') phosphodiesterase have been observed (Heyworth, *et al.*, 1983; Marchmont and Houslay, 1980).

Evidence that phosphodiesterase activity can be controlled by protein kinase C has now been suggested by several observations. For example, protein kinase C phosphorylates the rod photoreceptor

phosphodiesterase *in vitro* such that the inactive form is stabilized (Udovichenko, *et al.*, 1994). Protein kinase C mediated inhibition of phosphodiesterase activity has also been shown in rat hepatocytes (Irvine, *et al.*, 1986) and anterior pituitary cells (Abou-Samra, *et al.*, 1987). Altered expression of phosphodiesterases is another way in which their overall activity may be regulated. There is evidence, for example in L6 cells, to show that insulin can increase protein expression, an effect which it mediates via protein kinase C activation (Thompson, *et al.*, 1993). Indeed, differential control of transcription factors by individual protein kinase C isoforms has been established, at least *in vitro* (Goode, *et al.*, 1993). Hence, the possibility exists that insulin and protein kinase C might combine to regulate phosphodiesterase expression.

6.2. Results

Basal phosphodiesterase activity in each cell clone was first assessed and results presented in Table 6.1. Values are expressed both as specific activity (pmol/min/mg protein) and relative to basal activity in wild type cells, which were assigned an arbitrary value of 1. Significantly, CHO-ε cells exhibited a higher basal phosphodiesterase activity than control cells. Activity in all other cell clones was not significantly different from that of control cells.

Phosphodiesterase activity was next assessed after insulin treatment of cells. These results are shown in Table 6.1. Insulin treatment had no effect on phosphodiesterase activity in CHO-γ, CHO-K and CHO-T cells. It did, however, stimulate an increased phosphodiesterase activity in CHO-ε, CHO-α and CHO-β cells, the effect in CHO-ε cells being much greater than that observed in the others.

The effect of acute phorbol ester treatment of cells on phosphodiesterase activity was then investigated. Preliminary results are presented in Table 6.1. It does appear, however, that such treatment may stimulate phosphodiesterase activity with the most significant effects appearing to be in CHO-α, CHO-β and CHO-γ cells.

Phosphodiesterase activity was then assessed after long-term phorbol ester treatment of cells to down-regulate protein kinase C. Results are presented in Table 6.1. The most striking consequence of such treatment was that it caused a marked decrease in phosphodiesterase activity in CHO-ε cells. This effect was no longer evident when insulin treatment was combined with TPA induced down-regulation. In all other cells, long-term phorbol ester treatment resulted in an increased phosphodiesterase activity. When combining insulin treatment with TPA induced down-regulation the increase in phosphodiesterase activity was still observed in CHO-α, CHO-β, CHO-γ and CHO-K cells.

Figure 6.1 shows the inhibition of phosphodiesterase activity by the non-specific phosphodiesterase inhibitor IBMX in all cells. Under the assay conditions used, approximately 90% of phosphodiesterase activity was inhibited by IBMX in all cells with the exception of CHO-γ cells, which exhibited an inhibition of ~80%.

The effects of various activators and inhibitors which exhibit selectivity for particular isoforms were then assessed. It was noted (Table 6.2) that Ca^{2+}/calmodulin acted to stimulate phosphodiesterase activity in all cell clones, and rolipram, cilostamide and cyclic GMP inhibited activity in all cases. Analysing the results in more detail highlighted the fact that Ca^{2+}/calmodulin had a greatly enhanced stimulatory effect on phosphodiesterase activity in CHO-α cells. In addition, cilostamide and cyclic GMP mediated inhibition of phosphodiesterase activity was

significantly enhanced only in CHO-ε cells. Cyclic GMP mediated inhibition was also enhanced in CHO-β cells.

The same parameters of stimulation/inhibition were then investigated in cells which had been pretreated with either insulin, or TPA (16hours) or TPA (16hours) plus insulin. These results are displayed in Tables 6.3-6.6.

Firstly, insulin pretreatment alone had no effect on Ca^{2+}/calmodulin stimulated phosphodiesterase activity in any of the cell clones (Table 6.3). Long-term phorbol ester treatment had a significant inhibitory effect upon Ca^{2+}/calmodulin stimulated phosphodiesterase activity, such that stimulation was almost totally abolished. This effect was still apparent when insulin treatment was combined with long-term phorbol ester treatment.

Rolipram mediated inhibition was found to be enhanced by long-term phorbol ester treatment in CHO-α, CHO-β and CHO-γ cells (Table 6.4). This observation was still apparent if insulin treatment was combined with long-term phorbol ester treatment. Insulin pretreatment alone had no effect on rolipram sensitive activity compared with that in untreated cells.

From results shown in Table 6.5, the only significant change in cilostamide sensitive phosphodiesterase activity occurred in CHO-β cells upon long-term TPA treatment, where an increased inhibitory effect of cilostamide was observed. Cyclic GMP sensitive phosphodiesterase activity was unaffected by insulin or long-term TPA treatment of cells whether alone or combined together (Table 6.6).

Antisera specific to the α, β, γ and ε isoforms of protein kinase C were used in immunoblotting studies to monitor down-regulation of protein kinase C in cells treated with TPA. Results are displayed in Figure 6.2. They show that TPA treatment for 16 hours was sufficient to cause down-regulation of protein kinase C.

6.3. Discussion

By calculating basal phosphodiesterase activity in each of the cell types, an assessment of the influence of overexpression of the human insulin receptor, with or without each of the protein kinase C isoforms, was possible. On doing so, overexpression of PKC-ε (CHO-ε cells) resulted in a 2.3 fold increase in basal phosphodiesterase activity. Overexpression of the human insulin receptor alone (CHO-T cells), however, did not alter basal phosphodiesterase activity. This suggests that the observed increase in CHO-ε cells arose solely as a consequence of PKC-ε overexpression, the endogenous concentration of PKC-ε in CHO-T cells not being sufficient to allow a functional effect (see Figure 3.4). A possible explanation for this may be that under normal cell growth conditions some activation of PKC-ε is apparent and that this allows phosphorylation and activation of a phosphodiesterase by PKC-ε. Indeed another kinase, or phosphatase, located downstream of PKC-ε, whose activity is regulated by PKC-ε , may be responsible for the ultimate effect on a phosphodiesterase. Another possible explanation for this increased activity in CHO-ε cells may be that PKC-ε phosphorylates and activates a transcription factor, such that increased phosphodiesterase expression results. Both of these theories assume that under normal growth conditions basal diacylglycerol levels in CHO-ε cells are sufficiently high enough to allow activation of PKC-ε.

The observation of increased phosphodiesterase activity in CHO-ε cells confirms the increased basal phosphodiesterase activity, suggested by the fact that IBMX treatment of cells, whether alone or combined with isoprenaline, caused a greater increase in intracellular cyclic AMP concentration in CHO-ε cells than in any of the other cell clones (Table 5.1). However, basal intracellular cyclic AMP concentration

was not lower in CHO-ε cells compared with control cells, as would be expected.

Upon insulin treatment of cells, some stimulation of phosphodiesterase activity was seen in CHO-α, CHO-β and CHO-ε cells. Interestingly, however, the most substantial increase occurred in PKC-ε cells. This would at least partly explain the observed ability of insulin to attenuate agonist stimulated increases in intracellular cyclic AMP concentrations in these cells (Chapter 5). A possible explanation of this may be that insulin interacts with its receptor to initiate a signalling cascade, in which PKC-ε is involved at some stage, the ultimate result of which is activation of an insulin-stimulated protein kinase. This might then phosphorylate a phosphodiesterase such that activation of the enzyme results, or phosphorylate a transcription factor such that increased expression of the enzyme results.

Short-term phorbol ester treatment of cells, and hence activation of protein kinase C, appeared in general to increase phosphodiesterase activity, especially in CHO-α, CHO-β, CHO-γ and CHO-ε cells. This would suggest that in cells transfected with these particular protein kinase C isoforms there is the capacity for protein kinase C stimulation to increase phosphodiesterase activity. If the increased activity seen in CHO-ε cells upon, for example, insulin treatment was due to direct phosphorylation and activation of the enzyme, then short-term phorbol ester treatment of cells would be expected to result in an increased activity. These results, therefore, confirm that direct phosphorylation of a phosphodiesterase enzyme appears to be at least partly responsible for the stimulatory effect of PKC-ε upon phosphodiesterase activity.

The contribution of PKC-ε in mediating phosphodiesterase activity within CHO-ε cells was further highlighted upon assessement of phosphodiesterase activity after long-term phorbol ester treatment of cells,

which results in the down-regulation of protein kinase C. Such treatment resulted in a significantly reduced phosphodiesterase activity compared with that in untreated cells. Combining insulin treatment with long-term phorbol ester treatment elevated phosphodiesterase activity to around basal levels in untreated cells. This small increase due to insulin, however, was only approximately one third of that which insulin elicited in untreated cells, confirming that a large part of the insulin effect is mediated via PKC-ε.

These results suggest that as well as being essential in regulating, for example, insulin induced stimulation of phosphodiesterease activity, PKC-ε plays a vital role in controlling endogenous phosphodiesterase activity within CHO-ε cells, as was suggested by the increased basal phosphodiesterase activity seen in this particular cell clone. This does not appear to be the case for any other protein kinase C isoforms studied here, as in each case they exhibited an increased phosphodiesterase activity upon down-regulation of protein kinase C. This suggests that there may be some degree of negative control over phosphodiesterase activity in these cells which is relieved upon protein kinase C down-regulation.

The availability of activators and inhibitors specific for individual phosphodiesterase species allowed the phosphodiesterase isoenzyme profile in each cell to be established. They also allowed an assessment of individual isoenzyme activities and their contribution to overall activity changes upon treatment of cells with insulin, long-term phorbol ester treatment, and a combination of these. They included: Ca^{2+}/calmodulin, which stimulates type I phosphodiesterase activity; rolipram, which inhibits type IV activity; cilostamide, which inhibits type III activity; and cyclic GMP, which stimulates type II and inhibits type III activity.

Using Ca^{2+}/calmodulin established the fact that all cell clones contain some class I phosphodiesterase activity since Ca^{2+}/calmodulin

increased activity in all cases. Ca^{2+}/calmodulin stimulated activity in untreated cells was greater in CHO-α cells than in any other clone used. This suggests that PKC-α might be involved in regulating class I phosphodiesterase activity. Confirming this role of PKC-α was the observation that phorbol ester induced down-regulation of protein kinase C blocked the stimulatory effect of Ca^{2+}/calmodulin in CHO-α (and CHO-β) cells.

All clones appear to contain a similar amount of type IV phosphodiesterase activity, rolipram causing a similar degree of inhibition of activity in each case. This was unaffected by treating cells with insulin. Rolipram mediated inhibition was enhanced in CHO-α, CHO-β and CHO-γ cells upon down-regulation of protein kinase C, suggesting that the degree of negative control exerted upon phosphodiesterase activity in these cells discussed earlier may have been due to some extent to a specific effect on class IV phosphodiesterases, (type III effect also evident in CHO-β cells - see below). This enhanced inhibition persisted after combining long term phorbol ester treatment with insulin treatment, which was in agreement with the fact that insulin alone had no effect on type IV activity in any of the cell clones.

All cell clones contained type III phosphodiesterase activity. The increased cilostamide mediated inhibition of activity seen in CHO-ε cells suggests a greater amount of type III activity in this cell clone. Increased total phosphodiesterase activity in CHO-ε cells was still observed after insulin treatment of cells, but not after phorbol ester induced protein kinase C down-regulation. The enhanced cilostamide mediated inhibition observed in CHO-β cells after long-term phorbol ester treatment suggests that an increased type III activity contributes to the overall increased phosphodiesterase activity observed in this cell clone after long-term phorbol ester treatment.

Since cyclic GMP inhibited phosphodiesterase activity in all cell clones under various conditions it appears that all cell clones contain more type III than type II phosphodiesterase activity. Ratios of the levels of these isoenzymes in all cells seem to be similar, with the possible exception of CHO-β cells where cyclic GMP caused an increased inhibition of activity. This suggests either an increased type III activity or an even lesser type II activity in these cells. No specific type II inhibitor was used to directly determine the contribution of this isoenzyme to total activity in each clone.

It seems that activity of individual classes of phosphodiesterase isoenzymes is regulated by intracellular factors, including protein kinase C, which can, in turn, be regulated by distinct signals impinging upon the cell. This provides another example of the fine tuning within cells, where the existence of a group of related isoenzymes allows for the influence of different extracellular ligands to be integrated at a similar intracellular regulatory system in a synergistic or antagonistic manner.

In conclusion, there is an elevated basal phosphodiesterase activity only in CHO-ε cells, and this appears to be at least partly due to increased type III activity. The fact that down-regulation of protein kinase C by phorbol ester eliminates this observation further suggests a critical role for PKC-ε in mediating this effect. Similarly, only in CHO-α cells is an elevated type I activity observed and this observation is also lost upon protein kinase C down-regulation, highlighting the role played by PKC-α.

Insulin was found to stimulate phosphodiesterase activity principally in CHO-ε, but also in CHO-α and CHO-β cells. However, insulin apparently had no significant effects on individual phosphodiesterase isoforms, hence a cumulative general stimulatory effect across phosphodiesterase isoforms seems a more likely explanation of insulin's effect than an effect targetted specifically at one individual class.

The observation that insulin no longer had the same magnitude of effect on phosphodiesterase activity in CHO-ε cells after protein kinase C down-regulation again demonstrates the importance of this protein kinase C isoform in controlling phosphodiesterase activity.

Phorbol ester induced down-regulation of protein kinase C, however, resulted in an increased phosphodiesterase activity in all cells other than CHO-ε. It is suggested that one explanation for this effect could be the relieving of an inhibitory constraint upon phosphodiesterase activity in these cells. More specifically, an elevated type IV activity, with some suggestion of an elevated type III activity in CHO-β cells, seems to be responsible.

Table 6.1. Phosphodiesterase activity in Chinese hamster ovary cell clones: the influence of insulin and phorbol ester on total activity

Phosphodiesterase activity was measured in six Chinese hamster ovary cell clones including control cells (CHO-K), cells overexpressing human insulin receptor (CHO-T) and cells overexpressing both the human insulin receptor and a specific protein kinase C isoform (CHO-α, -β, -γ or -ε). Activity was first assessed in untreated cells. The resulting specific activities (pmol/min/mg protein) are expressed here. These results are also expressed relative to basal activity in wild type cells (CHO-K), designated an arbitrary basal value of 1. Activities were then assessed in cells after insulin treatment (10nM, 5minutes), acute phorbol ester treatment (100nM, 10minutes), long-term phorbol ester treatment (100nM, 16hours) and a combination of long-term phorbol ester and insulin treatment. Specific activities are again presented in each case. All results represent means for the number of experiments indicated and include S.D. values. Statistically significant results are highlighted as $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***).

	CHO-α	CHO-β	CHO-γ	CHO-ε	CHO-K	CHO-T
Untreated cells (n≥2)	8.9 ±1.3	7.3 ±0.4	4.9 ±3.4	19.4 ±2.1 *	8.6 ±3.7	7.7 ±1.6
(Ratio relative to CHO-K) (CHO-K =1)	(1.0)	(0.9)	(0.6)	(2.3)	(1.0)	(0.9)
Insulin-treated (n≥2)	18.5 ±1.8 *	15.7 ±1.2 *	9.7 ±1.7	36.7 ±4.6 **	12.6 ±3.7	13.9 ±1.0
% Change from basal	(108)	(115)	(98)	(89)	(47)	(81)
TPA (10min) (n=1)	23.3	15.7	10.3	32.9	11.7	10.9
% Change from basal	(162)	(115)	(110)	(70)	(36)	(42)
TPA (16hrs) (n≥2)	31.2 ±6.5 *	28.9 ±0.5 ***	22.9 ±0.1 *	13.1 ±2.5 #	18.8 ±2.6 *	21.1 ±0.1 **
% Change from basal	(250)	(296)	(367)	(-32)	(119)	(174)
TPA (16hrs) + Insulin (n≥2)	36.4 ±2.7 **	29.3 ±3.6 *	18.5 ±1.3 *	20.4 ±2.7	21.2 ±4.6 *	15.6 ±3.5
% Change from basal	(309)	(300)	(278)	(5)	(147)	(103)

Figure 6.1. Inhibition of phosphodiesterase activity in CHO cells by IBMX

The inhibitory effect of IBMX on phosphodiesterase activity in CHO-α (A), CHO-β (B), CHO-γ (C), CHO-ε (D), CHO-K (E) and CHO-T (F) cells was investigated and results expressed as the percentage of total enzyme activity which was IBMX sensitive (▫) and IBMX insensitive (▪). IBMX was used in the assay at a final concentration of 1mM.

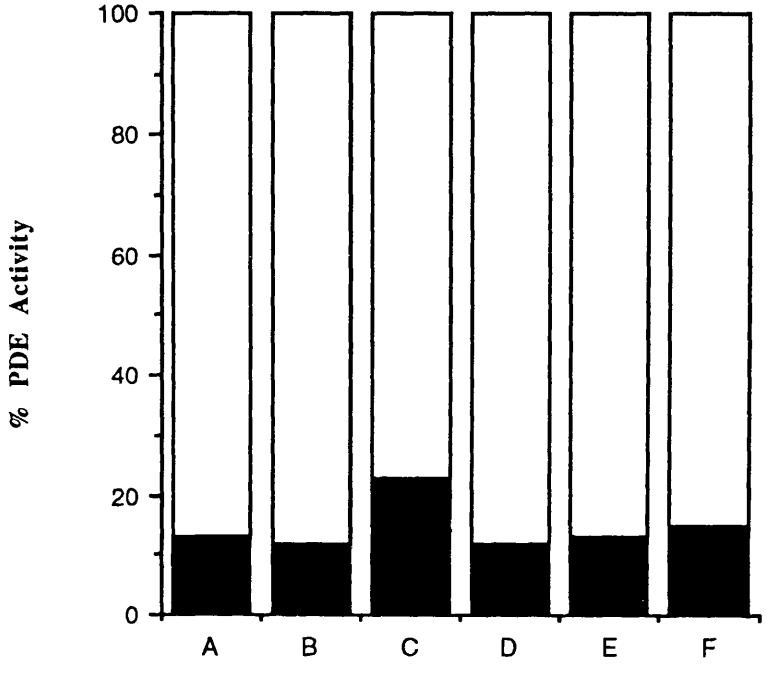

% PDE Activity
100
80
60
40
20
0
A
B
C
D
E
F

Figure 6.2. The effects of TPA on expression of protein kinase C isoforms in CHO cells

Antiserum specifically recognizing the α, β, γ and ε isoforms of protein kinase C were employed (final dilution 1:100) in studying expression of these isoforms in CHO-α (PKC-α), CHO-β (PKC-β), CHO-γ (PKC-γ) and CHO-ε (PKC-ε) cells under basal conditions (A), after TPA treatment (100nM) for 10 minutes (B) and after TPA treatment (100nM) for 16 hours (C). Samples were prepared from cells as described in Section 2.1.2.1. Volumes of cell extract corresponding to 100μg total protein content were mixed with an equivalent volume of x2 sample buffer (Section 2.1.8.1) and boiled for 5 minutes before being analysed by SDS-PAGE in each case.

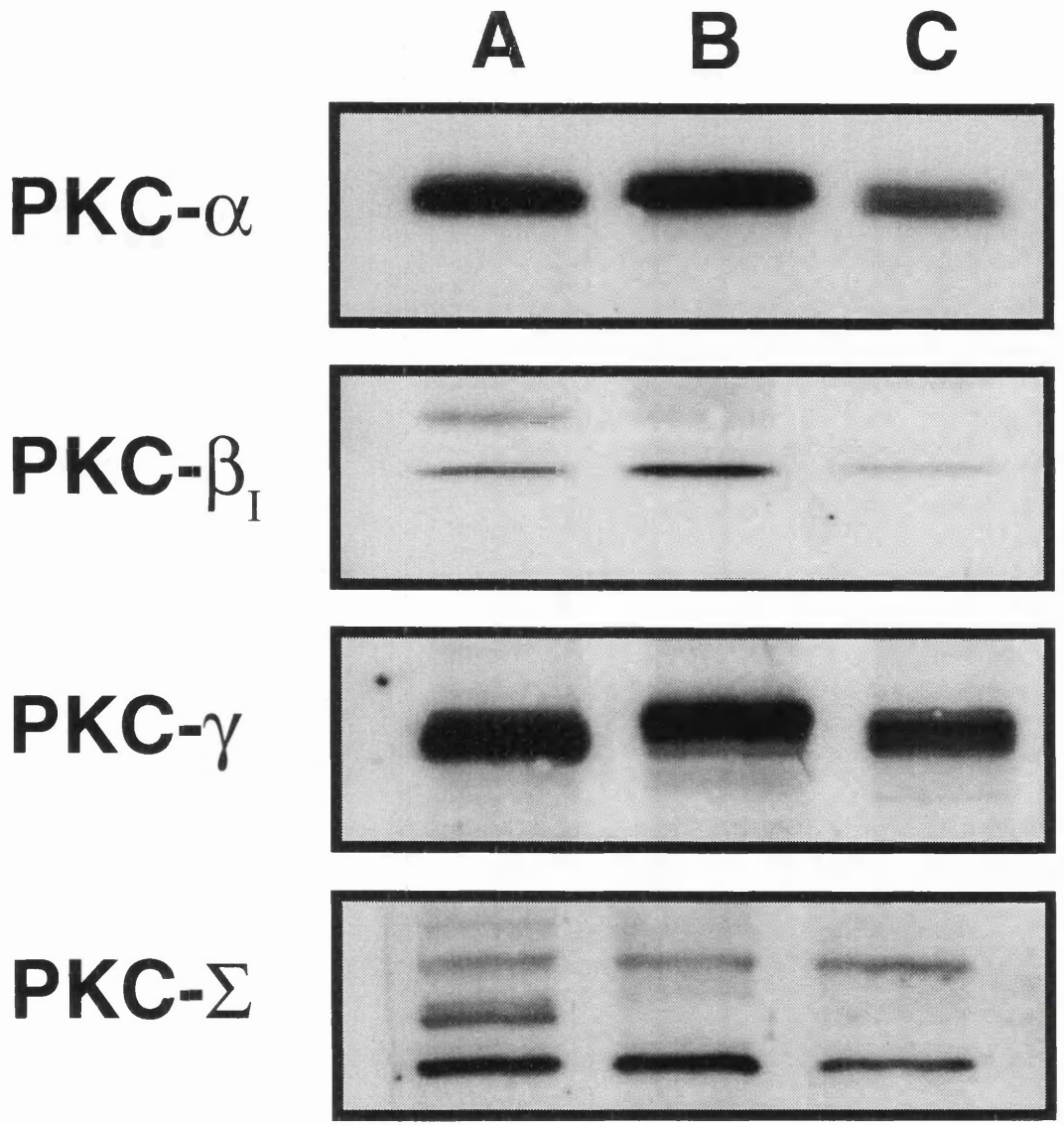

A
B
C
PKC-α
PKC-β$_I$
PKC-γ
PKC-Σ

Table 6.2. Phosphodiesterase activity in Chinese hamster ovary cell clones; characterization of the isoenzyme profile present in each

Phosphodiesterase activity was measured in six Chinese hamster ovary cell clones including control cells (CHO-K), cells overexpressing human insulin receptor (CHO-T) and cells overexpressing the human insulin receptor and a specific protein kinase C isoform (CHO-α, -β, -γ and -ε). All values in this table represent the amount (change in units of specific activity) of stimulation caused by Ca^{2+}/calmodulin (100μM final concentration and 200ng/assay, respectively) or the amount of inhibition caused by rolipram (10μM final concentration), cilostamide (10μM final concentration) or cyclic GMP (10μM final concentration). Basal specific activities for each cell clone were 8.9 ±1.3 pmol/min/mg protein (CHO-α), 7.3 ±0.4 (CHO-β), 4.9 ±3.4 (CHO-γ), 19.4 ±2.1 (CHO-ε), 8.6 ±3.7 (CHO-K) and 7.7 ±1.6 (CHO-T). All results represent means for the number of samples indicated and include S.D. values. Statistically significant results are highlighted as $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***).

	CHO-α	CHO-β	CHO-γ	CHO-ε	CHO-K	CHO-T
Untreated cells (n≥2)	8.9 ±1.3	7.3 ±0.4	4.9 ±3.4	19.4 ±2.1	8.6 ±3.7	7.7 ±1.6
Ca^{2+}/calmodulin (n≥2)	+14.6 ±4.5 * (+164%)	+5.9 ±1.2 (+80%)	+2.8 ±0.9 (+57%)	+8.5 ±6.0 (+44%)	+2.1 ±0.2 (+24%)	+2.0 ±0.8 (+26%)
Rolipram (n≥2)	-3.8 ±2.4 (-42%)	-5.1 ±2.1 (-69%)	-2.4 ±1.0 (-49%)	-9.0 ±5.5 (-46%)	-5.9 ±2.5 (-68%)	-4.4 ±1.1 (-57%)
Cilostamide (n≥2)	-5.0 ±2.1 (-56%)	-7.1 ±3.4 (-97%)	-1.5 ±0.9 (-30%)	-7.7 ±2.1 * (-39%)	-2.9 ±1.4 (-34%)	-5.9 ±2.4 (-77%)
Cyclic GMP (n≥2)	-4.3 ±1.3 (-48%)	-6.1 ±1.7 * (-83%)	-1.5 ±0.3 (-30%)	-7.3 ±1.7 * (-38%)	-2.9 ±0.5 (-34%)	-4.5 ±0.9 (-58%)

Table 6.3. Phosphodiesterase activity in Chinese hamster ovary cell clones: the influence of insulin and phorbol ester on calcium/calmodulin sensitive activity

Ca^{2+}/calmodulin stimulated phosphodiesterase activity was measured in six Chinese hamster ovary cell clones including control cells (CHO-K), cells overexpressing human insulin receptor (CHO-T) and cells overexpressing both the human insulin receptor and a specific protein kinase C isoform (CHO-α, -β, -γ or -ε). Activity was first assessed in untreated cells then in cells after insulin treatment (10nM, 5minutes), long-term phorbol ester treatment (100nM, 16hours) and a combination of long-term phorbol ester and insulin treatment. Results are expressed in the table as the degree of stimulation (change in units of specific activity) caused by Ca^{2+}/calmodulin (100μM final concentration and 200ng/assay, respectively) under normal conditions and after insulin or TPA treatment of cells. Basal specific activities for each cell clone were 8.9 ±1.3 pmol/min/mg protein (CHO-α), 7.3 ±0.4 (CHO-β), 4.9 ±3.4 (CHO-γ), 19.4 ±2.1 (CHO-ε), 8.6 ±3.7 (CHO-K) and 7.7 ±1.6 (CHO-T). All results represent means for the number of samples indicated and include S.D. values. Statistically significant results are highlighted as $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***).

Changes in phosphodiesterase activity listed for insulin, TPA or insulin and TPA treated cells refers to the appropriate controls as illustrated in Table 6.1.

	CHO-α	CHO-β	CHO-γ	CHO-ε	CHO-K	CHO-T
Calcium/calmodulin Untreated cells (n≥2)	+14.6 ±4.5 +164%	+5.9 ±1.2 +80%	+2.8 ±0.9 +57%	+8.5 ±6.0 +43%	+2.1 ±0.2 +24%	+2.0 ±0.8 +26%
Calcium/calmodulin Insulin-treated (n≥2)	+15.7 ±4.5 +85%	+5.4 ±1.2 +34%	+3.7 ±0.9 +38%	+8.9 ±6.0 +25%	+2.5 ±0.4 +20%	+1.9 ±0.2 +14%
Calcium/calmodulin TPA (16hrs) (n≥2)	+2.4 ±0.9 * +7%	+2.0 ±0.8 * +6%	+1.2 ±0.6 +5%	+2.0 ±0.4 +15%	+2.5 ±0.8 +13%	+1.2 ±0.7 +6%
Calcium/calmodulin TPA (16hrs) + Insulin (n≥2)	+4.7 ±1.3 +13%	+9.2 ±8.2 +31%	+1.2 ±0.7 +6%	+5.9 ±3.5 +29%	+2.5 ±1.2 +12%	+2.7 ±2.5 +17%

Table 6.4. Phosphodiesterase activity in Chinese hamster ovary cell clones: the influence of insulin and phorbol ester on rolipram sensitive activity

Rolipram inhibited phosphodiesterase activity was measured in six Chinese hamster ovary cell clones including control cells (CHO-K), cells overexpressing human insulin receptor (CHO-T) and cells overexpressing both the human insulin receptor and a specific protein kinase C isoform (CHO-α, -β, -γ or -ε). Activity was first assessed in untreated cells then in cells after insulin treatment (10nM, 5minutes), long-term phorbol ester treatment (100nM, 16hours) and a combination of long-term phorbol ester and insulin treatment. Results are expressed in the table as the degree of inhibition (change in units of specific activity) caused by rolipram (10μM final concentration) under normal conditions or after insulin and TPA treatment of cells. Basal specific activities for each cell clone were 8.9 ±1.3 pmol/min/mg protein (CHO-α), 7.3 ±0.4 (CHO-β), 4.9 ±3.4 (CHO-γ), 19.4 ±2.1 (CHO-ε), 8.6 ±3.7 (CHO-K) and 7.7 ±1.6 (CHO-T). All results represent means for the number of samples indicated and include S.D. values. Statistically significant results are highlighted as $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***).

Changes in phosphodiesterase activity listed for insulin, TPA or insulin and TPA treated cells refers to the appropriate controls as illustrated in Table 6.1.

	CHO-α	CHO-β	CHO-γ	CHO-ε	CHO-K	CHO-T
Rolipram Untreated cells (n≥2)	-3.8 ±2.4	-5.1 ±2.1	-2.4 ±1.0	-9.0 ±5.5	-5.9 ±2.5	-4.4 ±1.1
	-43%	-70%	-49%	-46%	-69%	-57%
Rolipram Insulin-treated (n≥2)	-7.0 ±4.7	-8.4 ±1.8	-4.3 ±2.3	-15.8 ±8.6	-6.6 ±5.0	-8.8 ±0.5
	-38%	-53%	-44%	-43%	-52%	-63%
Rolipram TPA (16hrs) (n≥2)	-11.1 ±3.6 *	-14.4 ±2.8 *	-17.5 ±3.3 *	-7.6 ±2.9	-11.7 ±3.0	-16.7 ±4.1
	-36%	-50%	-76%	-58%	-62%	-79%
Rolipram TPA (16hrs) + Insulin (n≥2)	-12.0 ±3.3 *	-17.2 ±3.1 *	-11.9 ±1.8 *	-10.1 ±4.5	-12.6 ±3.0	-8.4 ±4.0
	-33%	-59%	-64%	-49%	-59%	-54%

Table 6.5. Phosphodiesterase activity in Chinese hamster ovary cell clones: the influence of insulin and phorbol ester on cilostamide sensitive activity

Cilostamide inhibited phosphodiesterase activity was measured in six Chinese hamster ovary cell clones including control cells (CHO-K), cells overexpressing human insulin receptor (CHO-T) and cells overexpressing both the human insulin receptor and a specific protein kinase C isoform (CHO-α, -β, -γ or -ε). Activity was first assessed in untreated cells then in cells after insulin treatment (10nM, 5minutes), long-term phorbol ester treatment (100nM, 16hours) and a combination of long-term phorbol ester and insulin treatment. Results are expressed in the table as the degree of inhibition (change in units of specific activity) caused by cilostamide (10μM final concentration) under normal conditions and after insulin or TPA treatment of cells. Basal specific activities for each cell clone were 8.9 ±1.3 pmol/min/mg protein (CHO-α), 7.3 ±0.4 (CHO-β), 4.9 ±3.4 (CHO-γ), 19.4 ±2.1 (CHO-ε), 8.6 ±3.7 (CHO-K) and 7.7 ±1.6 (CHO-T). All results represent means for the number of samples indicated and include S.D. values. Statistically significant results are highlighted as $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***).

Changes in phosphodiesterase activity listed for insulin, TPA or insulin and TPA treated cells refers to the appropriate controls as illustrated in Table 6.1.

	CHO-α	CHO-β	CHO-γ	CHO-ε	CHO-K	CHO-T
Cilostamide Untreated cells (n≥2)	-5.0 ±2.1	-7.1 ±3.4	-3.1 ±0.9	-7.7 ±2.1	-2.9 ±1.4	-5.9 ±2.4
	-56%	-97%	-63%	-40%	-34%	-76%
Cilostamide Insulin-treated (n≥2)	-6.6 ±3.3	-11.1 ±0.2	-2.7 ±0.8	-10.5 ±3.8	-2.4 ±1.5	-4.3 ±0.8
	-36%	-71%	-28%	-29%	-19%	-31%
Cilostamide TPA (16hrs) (n≥2)	-9.3 ±2.4	-16.3 ±0.9 *	-11.8 ±5.8	-5.0 ±2.1	-6.2 ±4.1	-9.3 ±2.3
	-30%	-56%	-52%	-38%	-33%	-44%
Cilostamide TPA (16hrs) + Insulin (n≥2) ±5.9	-11.8 ±4.1	-8.0 ±2.6	-4.1 ±4.0	-5.6 ±3.7	-4.6 ±0.6	-4.3
	-32%	-27%	-22%	-27%	-22%	-27%

Table 6.6. Phosphodiesterase activity in Chinese hamster ovary cell clones: the influence of insulin and phorbol ester on cyclic GMP sensitive activity

Cyclic GMP inhibited phosphodiesterase activity was measured in six Chinese hamster ovary cell clones including control cells (CHO-K), cells overexpressing human insulin receptor (CHO-T) and cells overexpressing both the human insulin receptor and a specific protein kinase C isoform (CHO-α, -β, -γ or -ε). Activity was first assessed in untreated cells then in cells after insulin treatment (10nM, 5minutes), long-term phorbol ester treatment (100nM, 16hours) and a combination of long-term phorbol ester and insulin treatment. Results are expressed in the table as the degree of inhibition (change in units of specific activity) caused by cyclic GMP (10μM final concentration) under normal conditions and after insulin or TPA treatment of cells. Basal specific activities for each cell clone were 8.9 ±1.3 pmol/min/mg protein (CHO-α), 7.3 ±0.4 (CHO-β), 4.9 ±3.4 (CHO-γ), 19.4 ±2.1 (CHO-ε), 8.6 ±3.7 (CHO-K) and 7.7 ±1.6 (CHO-T). All results represent means for the number of samples indicated and include S.D. values. Statistically significant results are highlighted as $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***).

Changes in phosphodiesterase activity listed for insulin, TPA or insulin and TPA treated cells refers to the appropriate controls as illustrated in Table 6.1.

	CHO-α	CHO-β	CHO-γ	CHO-ε	CHO-K	CHO-T
Cyclic GMP						
Untreated cells	-4.3	-6.1	-1.5	-7.3	-2.9	-4.5
(n≥2)	±1.3	±1.7	±0.3	±1.7	±0.5	±0.9
	-48%	-83%	-31%	-38%	-34%	-58%
Cyclic GMP						
Insulin-treated	-7.9	-5.0	-1.9	-9.9	-1.9	-2.1
(n≥2)	±1.9	±2.6	±0.9	±3.0	±0.4	±0.3
	-43%	-32%	-20%	-27%	-15%	-15%
Cyclic GMP						
TPA (16hrs)	-9.5	-9.8	-8.8	-6.9	-3.5	-8.8
(n≥2)	±3.1	±2.9	±4.5	±1.2	±1.8	±4.1
	-30%	-34%	-38%	-52%	-19%	-42%
Cyclic GMP						
TPA (16hrs) + Insulin	-4.5	-8.4	-4.3	-5.5	-3.3	-2.3
(n≥2)	±0.9	±7.4	±0.7 *	±1.9	±2.1	±2.3
	-12%	-29%	-23%	-27%	-16%	-15%

Chapter 7

Kinase activity of partially purified insulin receptor

7.1. Introduction

The majority of proposed models for the transduction of the insulin signal across the membrane are based on the nature of the insulin receptor as a tyrosine kinase. According to these, insulin binds to the extracellular α-subunit of the receptor, activating the tyrosine kinase activity of the transmembrane β-subunit. Many effects of insulin ultimately involve altered phosphorylation of target proteins, usually on serine residues, including, for example, phosphorylation of acetylCoA carboxylase and dephosphorylation of glycogen synthase. This model has been supported by studies with mutant receptors lacking kinase activity (Fujita-Yamaguchi, *et al.*, 1983) and by antibodies that inhibit receptor tyrosine kinase activity (Morgan and Roth, 1987), both of which lead to loss of insulin action.

In intact cells the insulin receptor is phosphorylated on serine residues in response to insulin (Stadtmauer and Rosen, 1986a) although most partially purified preparations of insulin receptor lack serine kinase activity. When amino acids are analyzed by two-dimensional tryptic phosphopeptide mapping using intact cells, this results in the appearance of two phosphopeptides not evident using soluble receptor preparations (Smith, *et al.*, 1988). However a purification protocol has been devised which allows receptor purification with associated serine kinase activity (Smith, *et al.*, 1988).

Other studies have suggested that autophosphorylation is not a necessary feature of insulin signalling. For example, antibodies to the insulin receptor can stimulate its kinase activity towards exogenous substrates without inducing receptor autophosphorylation (Tavare, *et al.*, 1988). It may be, however, that in such cases antibodies induce a conformational change akin to that resulting from autophosphorylation, hence allowing signal transduction.

The range of substrates phosphorylated *in vivo* upon interaction of insulin with its receptor are becoming more clearly established (Kasuga, *et al.*, 1990). Several of these substrates have proved ideal for *in vitro* work. In addition, a synthetic peptide repesenting part of the receptor β-subunit sequence which contains all three major insulin receptor autophosphorylation sites has been specifically designed for such *in vitro* assays.

The influence of insulin on G-proteins is one theory to explain certain actions of this hormone (Houslay, 1990). For example, studies using the streptozotocin-induced diabetic rat show that, in liver tissue, there is a decreased inhibitory constraint upon adenylate cyclase (Gawler, *et al.*, 1987). This loss of activity is associated with a reduction in the amount of the α-subunit of G_i in liver membranes measured by immunochemical techniques, an effect partially reversed by insulin therapy.

Work presented in this chapter will investigate various aspects of insulin receptor function using a partially purified preparation of the receptor.

7.2. Results and discussion

7.2.1. Studies on insulin binding to its receptor

The binding of ^{125}I-insulin to the insulin receptor was assessed as detailed in Section 2.1.4. In order to find the linear range of insulin binding to its receptor, samples of crude placental extract were used to construct a 2-fold dilution series. For such a dilution series the total insulin binding activity was calculated by determining the sample fold dilution required to bind 5% of the total added counts then multiplying the reciprocal of this dilution by the total volume. From Figure 7.1, which

depicts insulin binding at various dilutions of crude placental extract, the number of units of insulin binding activity in this preparation was calculated to be 8064 (192x42).

Fold purification can be assessed by examining the percentage of total insulin binding activity of crude placental extract exhibited by the preparation after incubation with IMMAD. Results presented in Table 7.1 show that a yield of 49% and a fold purification of 113 were achieved.

Table 7.1. Partial purification of human placental insulin receptor

	Protein Concn mg	% Total Insulin Binding Activity	per mg protein
Crude placental extract	155	100	0.645
Pooled discarded supernatant fractions	-----	53	-----
IMMAD after incubation with CPE	0.67	49	73.1

In terms of the yield obtained, this purification procedure compared favourably with other methods designed for the same purpose (Fujita-Yamaguchi, *et al.*, 1983). When attempting to elute the receptor from the IMMAD, using a low pH sodium acetate buffer (pH 5.0), a loss of receptor activity was found, hence in subsequent experiments an immobilised receptor preparation was used.

7.2.2. Characterization of the parameters of insulin receptor autophosphorylation

To characterize the partially purified insulin receptor preparation, autophosphorylation was first examined. Phosphorylation assays were conducted as described in Section 2.1.5. Firstly, autophosphorylation over a one hour time period in the presence and absence of insulin was examined (Figure 7.2), and secondly an insulin dose response curve of receptor autophosphorylation was carried out (Figure 7.3). These show a clear insulin and time dependency for autophosphorylation of the receptor.

7.2.3. Substrate phosphorylation using the partially purified insulin receptor

7.2.3.1. Studies using the peptide IRβ

The peptide IRβ corresponds to residues 1142-1153 of the insulin receptor β-subunit. Its amino acid composition is as follows:

COOH NH_2

Thr-Arg-Asp-Ile-Tyr-Glu-Thr-Asp-Tyr-Tyr-Arg-Lys

As it contains all three major autophosphorylation sites of the insulin receptor β-subunit it is the ideal artificial substrate for the insulin receptor kinase, as shown in Figure 7.4. The insulin dependent nature of this phosphorylation is confirmed in Figure 7.5. Work by Dickens *et al.* (Dickens, *et al.*, 1991) has shown that under appropriate conditions all three tyrosine residues in this peptide are phosphorylated. Using a series of peptide analogues in which each tyrosine was substituted by phenylalanine they predicted the order of tyrosine phosphorylation to be tyrosine residue 9, followed by tyrosine 10, then finally tyrosine 5 (Levine, *et al.*, 1991). This confirmed earlier work suggesting that tyrosine 1152 (residue 9) was preferentailly phosphorylated (Stadtmauer and Rosen, 1986b). Note, however, that bis- and tris-phosphopeptides do not

bind to phosphocellulose paper. This order of phosphorylation contrasts with that deduced (residues 1148 (5), 1152 (9) then 1153 (10)) in another study (Flores-Riveros, *et al.*, 1989) where digests of insulin receptor were analysed. Results from the latter study also suggested that the transition from the doubly to the triply phosphorylated forms was primarily responsible for the activation of IRS-1 phosphorylation. This result was contradicted by other work which suggests that phosphorylation of residues 1152 and 1153 was the only requirement to induce full kinase activity of the receptor (Zhang, *et al.*, 1991).

7.2.3.2. Studies using the purified inhibitory G-protein Gi

It has been suggested that insulin can interact with the guanine nucleotide regulatory protein system based upon its ability to inhibit adenylate cyclase (Heyworth and Houslay, 1983) and activate specific species of cyclic AMP phosphodiesterase (Heyworth, *et al.*, 1983a).

Phosphorylation of the inhibitory guanine nucleotide binding protein G_i by insulin receptors purified from human placental membranes has previously been shown, in an analagous way to ADP-ribosylation, to be dependent on the protein being in its GDP bound holomeric form (O'Brien, *et al.*, 1987a). Thus, its activation by the non-hydrolyzable analogue GTPγS was able to attenuate phosphorylation. High concentrations of Mg^{2+}, a physiological regulator of G-protein function, were also noted to abolish this phosphorylation. A conformational change induced in the G-protein by this divalent ion was proposed as a logical explanation (Houslay, 1990). In addition, this phosphorylation of G_i was found to occur exclusively on tyrosine residues.

Some studies suggest that insulin can influence G-protein function by a noncovalent interaction (Luttrell, *et al.*, 1990). These authors suggest a direct interaction, with the tyrosine kinase activity of the receptor not

playing a significant role. A functional interaction between insulin and G_i-proteins has also been shown in the plasma membrane of pancreatic acinar cells (Profock, *et al.*, 1991), a tissue where insulin potentiates cholecystokinin stimulated amylase release. These experiments, which examined the effect of insulin on GTPγS binding (increased) and pertussis toxin induced ADP ribosylation (decreased) again suggested a direct association of receptor and G-protein as opposed to a phosphorylation mediated control.

Figure 7.6 clearly illustrates the relationship between insulin receptor autophosphorylation and phosphorylation of G_i purified from bovine brain. In this case the insulin receptor was not pre-activated with 'cold' ATP prior to addition of substrate, partly explaining the lag seen between the rates of receptor autophosphorylation and G_i phosphorylation. Another reason for this lag might, understandably, be due to the receptor kinase having a much lesser affinity for G_i than it does for the insulin receptor β-subunit. Determination of the molecular weight associated with the bands detected by autoradiography showed values of 91 (insulin receptor β-subunit) and 39.5 (G-protein α-subunit). These results clearly demonstrated that under appropriate conditions the purified insulin receptor could cause the phosphorylation of the α-subunit of G_i.

7.2.4. Effect of the tyrosine kinase inhibitor ST271 on substrate phosphorylation by the insulin receptor kinase

ST271 was first identified as an inhibitor of the epidermal growth factor receptor kinase (Shiraishi, *et al.*, 1989). These studies showed ST271 to be a relatively specific inhibitor of tyrosine kinase, as opposed to serine/threonine kinases.

Figure 7.7 shows the inhibitory effect of ST271 on substrate phosphorylation, and highlights the differences observed when the insulin

receptor was not previously activated by incubation with 'cold' ATP. Using pre-activated receptor the effect of ST271 was apparent after several minutes, with a minimal increase in peptide phosphorylation over a ten minute period in the presence of the inhibitor. Without previously activating the insulin receptor preparation there was a lag of some 10 minutes before there was a discernable difference in peptide phosphorylation in the presence and absence of ST271. After this time, however, a dramatic inhibitory effect was seen as before. In this case an initial increase in peptide phosphorylation was observed over the first two minutes before a plateau phase was reached.

7.3. Conclusions

A valuable tool in examining insulin receptor phosphorylation using purified receptor has been synthetic peptides representing distinct domains of the receptor β-subunit. The peptide used in the work presented here was an ideal substrate for the insulin receptor kinase, showing definite insulin- and time-dependency for phosphorylation. Unlike other studies, the stoichiometry of phosphorylation was not assessed here, although it was assumed that the assay used in this work measured the level of monophosphorylated peptide since bis- and tris-phosphopeptides have been reported not to bind to phosphocellulose paper. From analysis of the literature it is apparent that similar purification protocols can yield receptor preparations that either possess or lack an associated serine kinase activity. However, since phosphoaminoacid analysis was not conducted in this particular study, it is unclear if G_i phosphorylation was due to phosphate incorporation into tyrosine or serine, although previous work suggested phosphorylation exclusively on tyrosine residues (O'Brien, *et al.*, 1987a).

Using the tyrosine kinase inhibitor ST271 produced a significant reduction in insulin receptor mediated phosphorylation of the peptide IRβ. Interestingly, however, without pre-activation of the insulin receptor before assaying phosphorylation there was a period of approximately 10 minutes required before the inhibitory effect of ST271 was apparent. In addition, over the first two minutes a small but definite initial increase in peptide phosphorylation was observed. In explaining these observations, we must concede from previous observations that a delay of several minutes would be expected to occur before peptide phosphorylation was apparent if the receptor was not pre-activated. Even when the receptor became activated it is apparent from the data presented here that several minutes more elapsed before normal and ST271 inhibited phosphorylation patterns were significantly different. This reasoning accounts for the plateau phase seen in Figure 7.7(B). The initial slight increase in phosphorylation, however, was unexpected. The explanation may be that, for this purified receptor preparation, challenge by insulin immediately elevated the receptor to a state of partial kinase activity, with full kinase activity ensuing after approximately 2 minutes exposure to insulin as expected. Finally, this being the case why should phosphorylation of IRβ plateau and not continue to increase slowly unless the initial burst of kinase activity exhibited by the receptor was transient?

Previous work in this laboratory established that insulin inhibits adenylate cyclase activity in rat hepatocytes, an effect lost in cells taken from streptozotocin diabetic animals. Although evidence has been somewhat contradictory, it seems that G_i is not involved in transducing this insulin mediated inhibition. However the GTP-dependent nature of this effect does suggest that the action of insulin is mediated at some point by a guanine nucleotide binding protein, perhaps p21ras or a novel G-protein (G_{ins}). Results presented here confirm the findings of other groups

which suggested that, under appropriate conditions, the purified insulin receptor can infact phosphorylate a preparation of purified G_i. The functional significance of this G_i phosphorylation by the insulin receptor kinase however is still debatable and it will be interesting to establish whether such a phosphorylation event occurs *in situ* in the intact cell. In conclusion, it would seem that it is possible to phosphorylate G_i by the insulin receptor using purified preparations of both of these proteins, although it is doubtful if this is of major physiological relevance.

Figure 7.1. Measurement of total insulin binding activity in crude placental extract.

Samples of Triton X-100 solubilized human placental membranes were serially diluted two fold and ^{125}I-insulin binding activity assayed as described in Section 2.1.4. The dilution factor at which 5% of added counts were bound was used to calculate the total insulin binding activity.

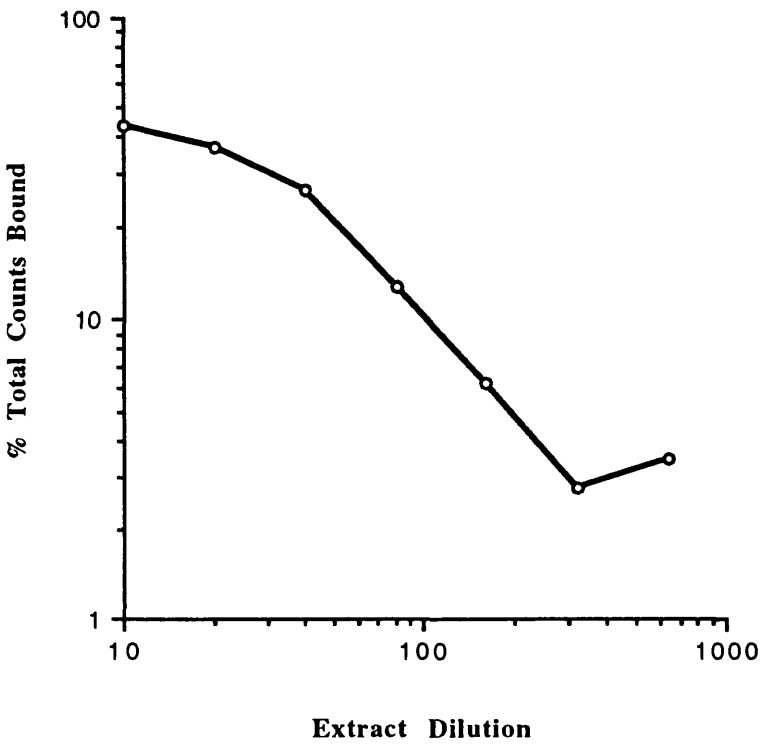

100
10
1
% Total Counts Bound
10
100
1000
Extract Dilution

Figure 7.2. Time-course of immobilised insulin receptor autophosphorylation

This graph shows the level of insulin receptor autophosphorylation in the presence (—o—) and absence (—•—) of insulin. Phosphorylation assays were performed as described in Section 2.1.5. Values represent means and errors are expressed as ± S.D. in a typical experiment. Incorporation of phosphate into insulin receptor was quantified by excision of the appropriate SDS-PAGE gel chip and Cerenkov counting.

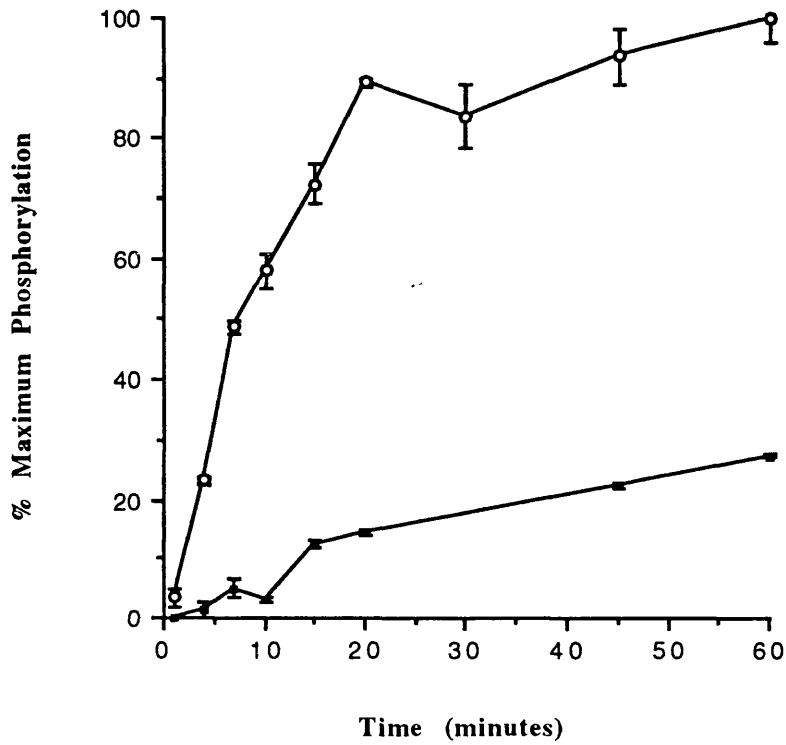

% Maximum Phosphorylation
100
80
60
40
20
0
0
10
20
30
40
50
60
Time (minutes)

Figure 7.3. Insulin dose response curve of immobilised insulin receptor autophosphorylation

Phosphorylation assays were performed as described in Section 2.1.5. In this case, samples were incubated at 23°C for 1 hour. Values represent means and errors are expressed as ± S.D. in a typical experiment. Incorporation of phosphate into insulin receptor was quantified by excision of the appropriate SDS-PAGE gel chip and Cerenkov counting.

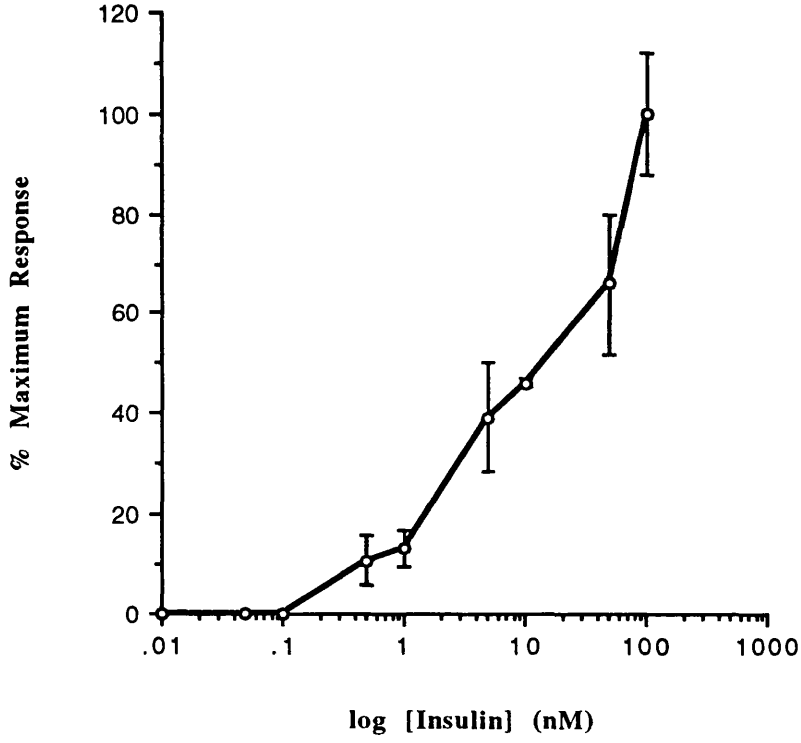

120
100
80
60
40
20
0
% Maximum Response
.01
.1
1
10
100
1000
log [Insulin] (nM)

Figure 7.4. Time course of phosphorylation of the peptide IRβ by the partially purified insulin receptor kinase

Phosphorylation assays were performed as described in Section 2.1.7. Values represent means and errors are expressed as ± S.D. in a typical experiment. Incorporation of phosphate into the peptide IRβ (final concentration 0.33mM) was measured by absorbing the peptide onto P81 paper and Cerenkov counting.

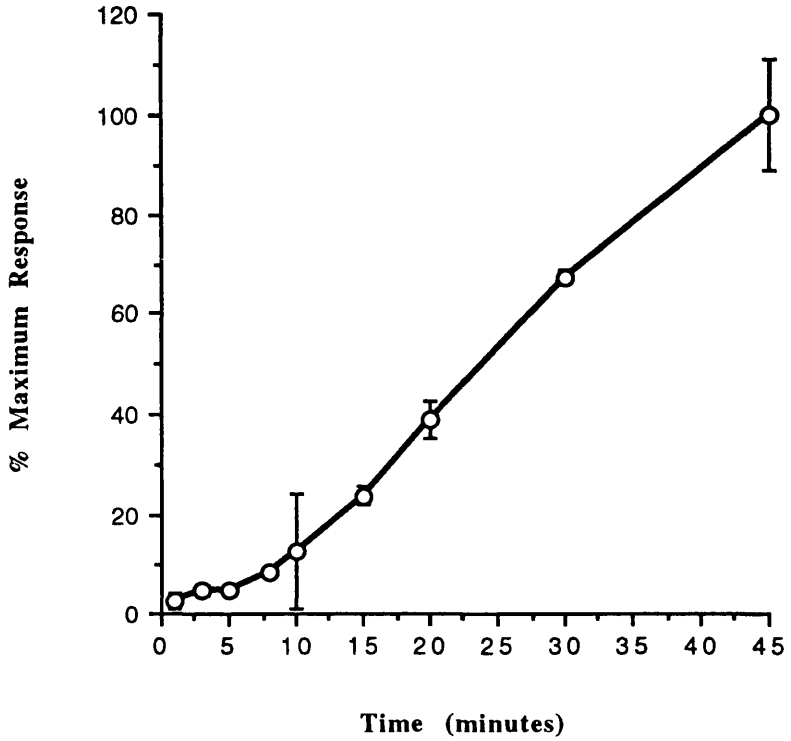

120
100
80
60
40
20
0
% Maximum Response
0
5
10
15
20
25
30
35
40
45
Time (minutes)

Figure 7.5. Insulin dependence of phosphorylation of the peptide IRβ by the immobilised insulin receptor kinase

Insulin dependency of IRβ phosphorylation by the insulin receptor is shown by phosphate incorporation in the presence of 100nM insulin (—o—) and in the presence of 0.1nM insulin (—•—). Phosphorylation assays were performed as described in Section 2.1.7. Values represent means and errors are expressed as ± S.D. in a typical experiment. Incorporation of phosphate into the peptide IRβ (final concentration 0.33mM) was measured by absorbing the peptide onto P81 paper and Cerenkov counting.

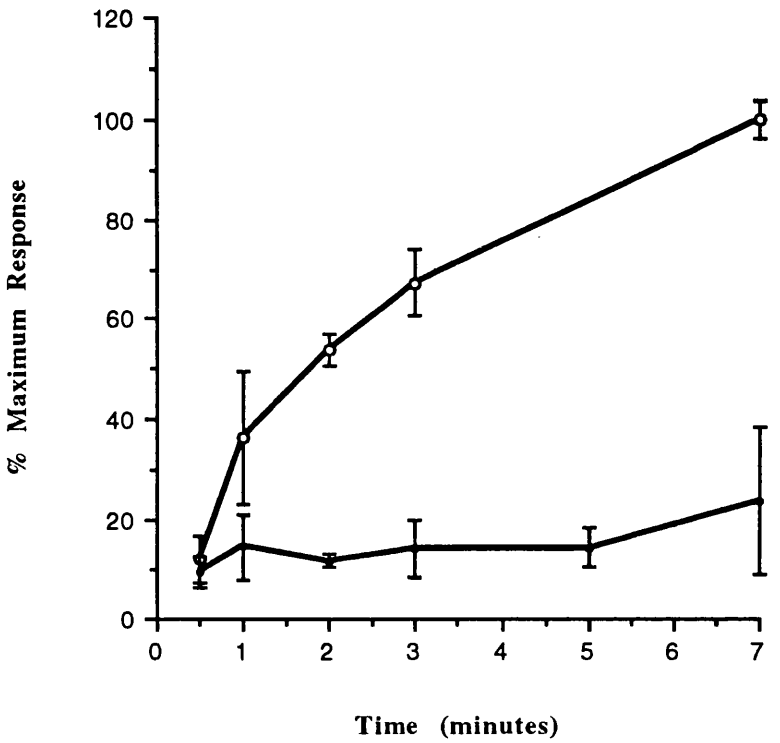

120
100
80
60
40
20
0
% Maximum Response
0
1
2
3
4
5
6
7
Time (minutes)

Figure 7.6. Phosphorylation of the purified G-protein G_i by partially purified insulin receptor

Purified G_i was examined as a substrate for the partially purified insulin receptor as described in Section 2.1.6. The graph presented shows simultaneous analysis of insulin receptor autophosphorylation (—o—) and G_i phosphorylation (—■—) in a typical experiment. Incorporation of phosphate into insulin receptor was quantified by scanning densitometry of the resulting autoradiograph. Values represent data from a typical experiment.

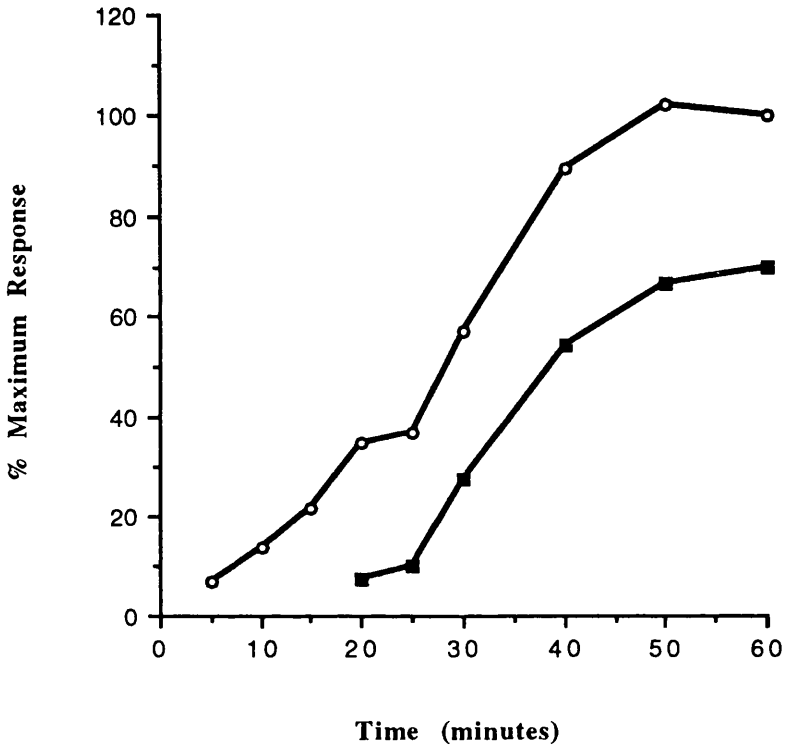
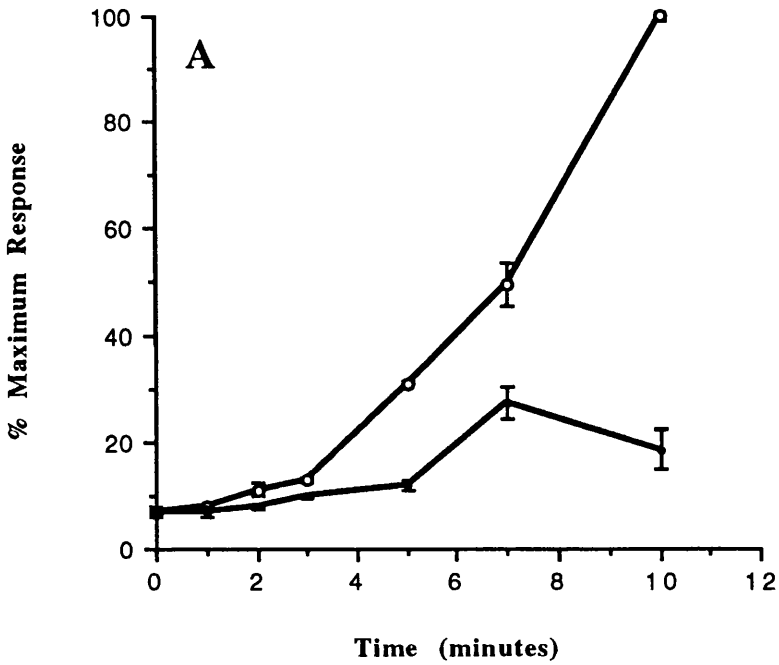
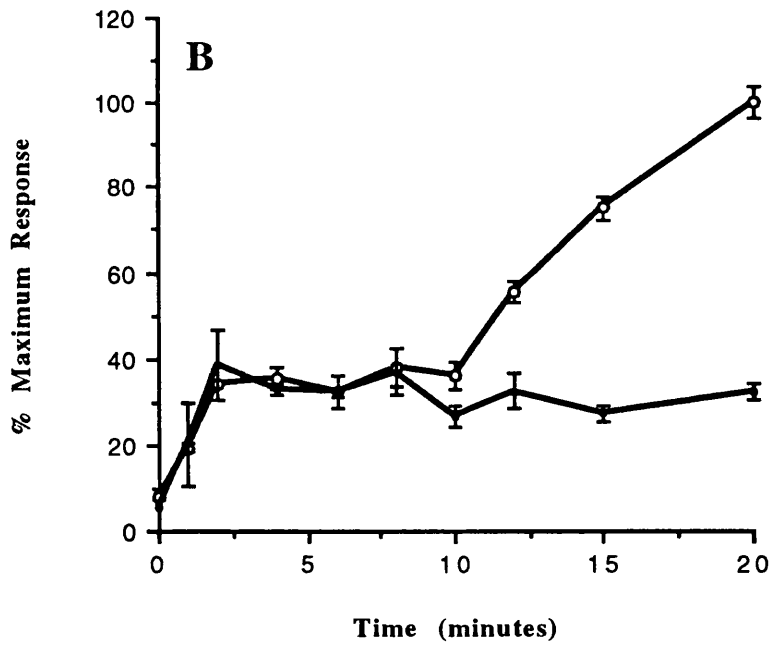

120
100
80
60
40
20
0
0
10
20
30
40
50
60
% Maximum Response
Time (minutes)

Figure 7.7. Effect of the tyrosine kinase inhibitor ST271 upon phosphorylation of the peptide IRβ by the partially purified insulin receptor

Phosphorylation assays were performed as described in Section 2.1.7, except that in (A) partially purified insulin receptor was activated by pre-incubation with 10nM insulin, whereas in (B) no such pre-incubation was carried out. The graphs show phosphate incorporation into the peptide under normal assay conditions (—o—) and in the presence of the tyrosine kinase inhibitor ST271 (—•—). Values represent means and errors are expressed as ± S.D. in a typical experiment. Incorporation of phosphate into the peptide IRβ (final concentration 0.33mM) was measured by absorbing the peptide onto P81 paper and Cerenkov counting.


A
% Maximum Response
100
80
60
40
20
0
0
2
4
6
8
10
12
Time (minutes)


B
% Maximum Response
120
100
80
60
40
20
0
0
5
10
15
20
Time (minutes)

Chapter 8

Discussion

The protein kinase C family is a widespread group of kinases responsible for many diverse and critical cellular functions. It is now appreciated, however, that individual protein kinase C isoforms might have quite separate and well defined actions. Thus, with the advent of activators and inhibitors for specific protein kinase C isoforms, the understanding of the complexity of protein kinase C function is becoming better understood. As yet, however, there is no conclusive evidence for a specific protein kinase C isoform being activated in a distinct cellular compartment leading to phosphorylation of one defined physiologically relevant substrate. There have been indications that certain substrates, for example the EGF receptor, can be phosphorylated to varying degrees by individual protein kinase C isoforms (Ido, *et al.*, 1987). More importantly with reference to work presented in this thesis, this was also recently shown to apply to the insulin receptor where PKC-α, -β_I and -γ but not PKC-ε could attenuate insulin receptor function via causing its phosphorylation (Chin, *et al.*, 1993). In the case of PKC-α, phosphorylation of the insulin receptor inhibited insulin-stimulated tyrosine phosphorylation of IRS-1 and PI 3-kinase (Chin, *et al.*, 1994).

The phenomenon of protein kinase C regulating insulin receptor function by altering its phosphorylation state has been extensively studied, as detailed in earlier text, and is now widely accepted. The suggestion that protein kinase C was part of the insulin signalling pathway has been more controversial. I feel, however, that enough evidence now exists to suggest that this is definitely the case, at least for certain insulin mediated responses. For example, treatment of adipocytes with insulin elicited translocation of protein kinase C to the membrane, indicative of activation (Farese, *et al.*, 1992b). More recent evidence has also shown that insulin stimulation of the Na^+-K^+ pump in skeletal muscle occurred via activation of protein kinase C (Sampson, *et al.*, 1994). In addition, my own work has

shown that protein kinase C, and more specifically PKC-ε, appears to play a central role in insulin's ability to regulate intracellular cyclic AMP metabolism in CHO cells.

In some of the work presented in this thesis prolonged treatment with phorbol esters was used to down-regulate protein kinase C. This is a common procedure which invariably has been shown to decrease expression of protein kinase C and result in decreased functional responsiveness of cells to subsequent stimulation with the relevant ligand. However, some doubt has been cast upon the reliability of this criteria in investigating the role of protein kinase C (Wilkinson and Hallam, 1994). Reservations principally concern the fact that more recently identified isoforms, such as PKC-ζ do not down-regulate in response to TPA. The isoforms studied in my work, however, do respond to phorbol ester by down-regulating and so their function can usefully be examined in this way.

Care must also be taken in using phorbol ester stimulation as a yardstick of protein kinase C function as other proteins are now known to be capable of binding phorbol ester. Neuronal chimaerin, for example, can bind phorbol esters, allowing this protein to regulate p21rac-GTPase activity (Ahmed, *et al.*, 1993). Hence, activation of the ras cascade leading to activation of MAP kinase might mediate the effects of phorbol esters in some systems.

The development of phorbol ester-derived isoenzyme specific agents will facilitate future investigation of the role of individual protein kinase C isoforms in cell signalling pathways. For example, the TPA analogue sapintoxin has been developed which will activate PKC-α, -β_I, -γ and -ε to the exclusion of other isoforms (Ryves, *et al.*, 1991). The use of specific protein kinase C activators and inhibitors would further studies presented in this thesis, and thus represents a potential future development

in this research. This would, for example, circumvent the doubts cast upon the reliability of phorbol esters, as discussed above.

On a broader perspective, protein kinase C inhibitors might prove to be of substantial clinical importance. Their possible therapeutic uses include the treatment of chronic inflammatory or auto-immune diseases, such as rheumatoid arthritis, as well as for the prevention of graft rejection and in treatment of cancers. Due to the fairly ubiquitous distribution and fundamental importance of protein kinase C to many physiological processes, systemic effects of such agents are likely to inhibit their therapeutic prospects. Progress has been made in designing inhibitors selective for protein kinase C over a broad range of serine/threonine and tyrosine specific protein kinases, one of the best being Ro318425 (Bradshaw, *et al.*, 1993). Furthermore, it is hoped that agents selective for the individual protein kinase C isoform of interest in specific cases might circumvent this problem to an extent which will allow their therapeutic exploitation and, indeed, initial results are surprisingly encouraging (Birchall, 1994).

Results presented here indicate that reduced protein kinase C activity occurs upon streptozotocin-induced diabetes. In these studies, no assessment of the corresponding activity of the insulin receptor was made. One recent study has suggested, however, that under hyperglycaemic conditions, as would be expected in hypoinsulinaemic streptozotocin-diabetic rats, a reduced insulin receptor tyrosine kinase activity is observed (Berti, *et al.*, 1994). This was found to be mediated by protein kinase C activation and serine phosphorylation of the insulin receptor (Berti, *et al.*, 1994). However, my work showed that in the streptozotocin-diabetic rat model a reduced protein kinase C activity occurred. This contradiction in results seems typical of the literature published in this area of research. Why this should be quite so marked is surprising, although since most

studies have identified changes in total protein kinase C activity, rather than in individual isoforms, it is likely that the varying complement of protein kinase C isoforms present in different tissues, and their differential regulation in diabetic states, underlies the disparity of published results.

With the elucidation of more precise details of various intracellular signalling pathways, the extent of cross-talk between different signalling systems in cells is becoming appreciated. For example, protein kinase C and cyclic AMP-dependent kinase can act antagonistically: they are known to phosphorylate the glycine receptor *in vivo* with opposite functional consequences (Vaello, *et al.*, 1994). This means that in controlling intracellular cyclic AMP levels, protein kinase C might be envisaged as being able to switch off or reduce cyclic AMP-dependent kinase signalling and enhance its own effect. Conversely, the potential for a negative feedback control of protein kinase C activated responses exists.

An important point at which the convergence of cyclic AMP and protein kinase C signal transduction occurs is at the level of adenylate cyclase. The influence of protein kinase C on adenylate cyclase has been investigated in many instances. For example, phorbol esters were recently shown to synergistically increase cyclic AMP production when adrenaline simultaneously activated receptors via an effect on adenylate cyclase (Morimoto and Koshland, 1994). Another study suggests that protein kinase C is directly involved in mediating bradykinin-dependent activation of adenylate cyclase (Stevens, *et al.*, 1994). Control of adenylate cyclase by protein kinase C has been examined further by Jacobowitz and co-workers who provided evidence that protein kinase C could selectively stimulate specific types of adenylate cyclase (Jacobowitz, *et al.*, 1993).

Individual signalling pathways may also act synergistically within cells. Indeed, it appears that hormonal signal transduction systems often have a specialized kinase for signalling at high concentrations of a ligand

and another second messenger regulated kinase to mediate signalling at low concentrations of ligand. With reference to insulin action, for example, it appears that, in controlling Na^+-H^+ antiport function in hepatocytes, insulin acts at high concentrations via protein kinase C and at low concentrations via a tyrosine kinase pathway (Incerpi, *et al.*, 1994).

Stable overexpression of protein kinase C isoforms has recently become used to examine the roles of particular isoforms. Overexpression provides a useful indication of which isoforms are involved in individual receptor mediated processes, yet signalling via non-physiological pathways may arise due to unrepresentatively exaggerated levels of these proteins. The availability to us of the CHO cell line overexpressing specific protein kinase C isoforms together with the human insulin receptor allowed investigation of responses to insulin and parallel analysis of the contribution of protein kinase C isoforms to insulin's action.

Using this system, I have shown that PKC-ε plays a central role in regulating both basal and insulin stimulated phosphodiesterase activity within CHO-ε cells and in allowing insulin to attenuate agonist induced increases in intracellular cyclic AMP concentrations in these cells. These results suggest that expression of PKC-ε in individual tissues is of importance in mediating at least part of insulin's ability to control intracellular cyclic AMP levels. The influence of protein kinase C on cyclic AMP metabolism was investigated in another recent study in PC18 cells (Yingling, *et al.*, 1994). It was also concluded from these studies that protein kinase C controlled cyclic AMP metabolism at both the level of synthesis and degradation. On a broader perspective, the concept of such specific intracellular regulatory mechanisms means that certain effects of ligands can be confined to particular tissues where the appropriate signalling molecules are expressed, despite the ligand interacting with receptors in other tissues.

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