Studies on the Regulation of Inositol Lipid-Specific Phospholipase C

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ABSTRACT

Several inositol-lipid-specific phospholipase C (PtdIns-PLC) isozymes have been cloned and divided into 3 classes, β, γ and δ, on the basis of structural homology. The γ isozymes are activated through stimulation of tyrosine kinase receptors, however the β and δ isozymes do not appear to be activated in this way. Stimulation of 7-transmembrane receptors also activates phosphatidylinositol 4, 5-bisphosphate (PtdIns(4,5)P2) hydrolysis and this receptor-coupled PtdIns-PLC activation is sensitive to GTP, implying that G-proteins are involved in receptor mediated PtdIns-PLC activation. This G-protein coupled activation of PtdIns-PLC is both sensitive and insensitive to Pertussis toxin (PTX), implying the existence of more than one G-protein-coupled PtdIns-PLC activation pathway. A family of PTX insensitive G-proteins, Gq, has been cloned, and purified α subunits from this family activate PtdIns-PLCβ1 in vitro.

To investigate the mechanisms involved in the regulation of the β and δ isozymes, both full length proteins and domains of the β isozymes have been expressed as fusion proteins in E.coli and purified. Using these proteins, the role of phosphorylation in the regulation of the different isozymes has been assessed and it is demonstrated that these isozymes are not targets of Protein kinase C (PKC) phosphorylation. PtdIns-PLCβ1 activation by Gqα subunits has been reconstituted in vitro and the ability of the different PtdIns-PLCβ domains to disrupt this activation has been assessed. Thus the carboxy terminus has been identified as the Gqα subunit-binding region of PtdIns-PLCβ1. This result is confirmed using an in vivo COS-1 cell expression system in which the binding of G-protein βγ subunits to the aminotermminus of PtdIns-PLCβ2 is indirectly demonstrated. These findings are discussed in the context of results from other laboratories.
ACKNOWLEDGEMENTS

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ABBREVIATIONS

Ach  Acetyl choline
ADP  Adenosine diphosphate
ARF  ADP-ribosylation factor
ATP  Adenosine triphosphate
βARK β-adrenergic receptor kinase
BSA  Bovine serum albumen
cAMP Cyclic adenosine monophosphate
cGMP Cyclic guanosine monophosphate
CTX  Cholera toxin
DAG sn-1,2 diacylglycerol
DTT  Dithiothreitol
dNTP Deoxynucleoside phosphate
DSP Dithiobis(succinimidyl propionate)
DSS Disuccinimidyl suberate
EGF  Epidermal growth Factor
EGFR EGF receptor
EGS Ethylene glycolbis(succinimidylsuccinate)
EDTA Ethylenediaminetetraacetic acid
EGTA Ethylene glycolbis(b-aminoethyl ether) N, N, N', N' Tetra-acetic acid
GAP GTP-ase activatory protein
GST  Glutathione-S-transferase
GPRK G-protein-coupled receptor kinase
G-protein Guanyl-nucleotide-binding protein
GTP  Guanosine triphosphate

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<td>IPTG</td>
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</tr>
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<td>LPA</td>
<td>Lysophosphatidic acid</td>
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<tr>
<td>M</td>
<td>Muscarinic</td>
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<tr>
<td>PA</td>
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<td>PBS</td>
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<td>Phosphodiesterase</td>
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<td>Platelet derived growth factor</td>
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<td>PDGF receptor</td>
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<td>Phenylmethylsulfonyl fluoride</td>
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<tr>
<td>PP</td>
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<tr>
<td>PSS</td>
<td>Pseudosubstrate short</td>
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CHAPTER 1 - INTRODUCTION

Introduction

When cells communicate with one another, in some cases over large distances, the signals that are transmitted have to cross the plasma membrane barrier of the receiving cell. The mechanisms by which this is achieved range from simple diffusion of lipid soluble molecules such as the steroid hormones, to direct contact between the communicating cells through gap junctions [Bennett et al., 1985], allowing the passage of inorganic ions and small water-soluble molecules from the cytoplasm of one cell to the cytoplasm of the other. Both of these mechanisms are limiting, the first restricting the molecular design of the signal to lipid soluble molecules, the second excluding long-distance signalling. A third mechanism which overcomes both of these problems, employs cell surface receptors to relay the signals across the otherwise impenetrable plasma membrane.

The mechanisms by which cell surface receptors transfer signals inside the cell from the exterior can be divided into 3 categories. One mechanism involves the receptor being directly linked to ion channels in the cell membrane, which respond to the signal received with conformational changes, thus regulating the passage of specific ions into and out of the cell. Such receptors belong to a class of homologous proteins with multiple α helices which pass through the plasma membrane to form the channels. This mechanism is mainly used in the central nervous system in response to neurotransmitters such as acetylcholine (Ach), which binds to the nicotinic class (N) of Ach receptors [Guy and Hucho, 1987] during the propagation of action potentials from one nerve cell to the next.

The other direct mechanism of signal relay across the plasma membrane is that involving receptors with intrinsic catalytic activity, such as the receptor tyrosine kinases. When this class of receptors is activated, by the binding of agonists such as Epidermal Growth Factor (EGF), the receptor protein itself acts directly upon a signalling enzyme such as PtdIns-PLCy, in this case catalysing the phosphorylation of the substrate enzyme on selected tyrosine residues. These receptors are generally composed of a single membrane spanning region, with a large cytoplasmic carboxyterminal tail containing the
regions required for substrate interactions and catalytic activity. A more indirect mechanism of receptor mediated signalling involves another class of proteins, the heterotrimeric G-proteins, which associate with 7-transmembrane receptors. When agonist binds to these receptors, the membrane bound G-proteins are activated so that they can interact with and regulate membrane bound enzymes such as adenylate cyclase, or ion channels such as those coupled to the muscarinic (M) Ach receptors.

In this chapter, mechanisms of receptor mediated signal transduction involving the receptor tyrosine kinases and G-protein coupled receptors will be discussed as an introduction to the regulation of the PtdIns-PLC enzymes.

1.1 The elucidation of a model signalling pathway

The pathway through which the hormones adrenaline and noradrenaline regulate glycogen metabolism in cells was the first receptor mediated signalling pathway involving heterotrimeric G-protein coupling to be elucidated. The general mechanisms involved in this pathway have been studied in great depth and similar processes have been observed since in many other pathways, including those involving the PtdIns-PLC isozymes.

Biochemical studies on the breakdown of glycogen revealed that the production of glucose-1-phosphate catalysed by phosphorylase, was the rate-limiting step in the production of glucose, prior to glycolysis. Whilst studying the mechanisms that regulated this enzyme, it was discovered that treatment of fresh liver slices with adrenaline caused a measurable increase in the levels of active phosphorylase, accompanied by a rapid incorporation of radiolabelled phosphate into the liver phosphorylase [Sutherland et al., 1955]. Having previously demonstrated that treatment of purified phosphate-radiolabelled phosphorylase with a purified 'phosphorylase inactivating enzyme' caused release of the labelled phosphate, they concluded that the observed enzymatic release of phosphate from purified liver phosphorylase was related to a mechanism of activation and inactivation of the phosphorylase. By testing different fractions of liver homogenates in a broken cell system, they demonstrated that the response to hormones could be separated into two phases [Rall et al., 1956]. In the first phase, hormone stimulation led to the formation of an
active factor in the particulate fraction of the cells. This was followed in the second phase by the stimulation of liver phosphorylase in the supernatant fractions, by the heat stable factor. This factor was subsequently identified as cAMP and later found to be produced during stimulation of the enzyme adenylate cyclase by adrenaline [Davoren and Sutherland, 1963]. A further link in the pathway was made when a protein kinase which catalysed the cAMP dependent phosphorylation of phosphorylase kinase [Walsh et al., 1968] was purified. This cAMP dependent kinase, now known as protein kinase A (PKA), was shown to catalyse the phosphorylation and activation of the activator of liver phosphorylase, phosphorylase kinase. This pathway is shown in Fig 1.1.

It became clear that the membrane-bound enzyme, adenylate cyclase was part of a multimeric enzyme complex and it was demonstrated that five different hormones could stimulate a single adenylate cyclase, through separate and independent binding sites or receptors [Rodbell et al., 1970]. The mechanism by which hormone-directed signals were relayed across the plasma membrane was divided into 3 processes - discrimination, transduction and amplification. Initial models suggested that all 3 functions were performed by different subunits of the same protein, however the separate identities of receptor and effector (in this case adenylate cyclase) were confirmed in cell fusion studies performed by Orly and Schramm [1976]. They successfully transferred a functional receptor from a cell containing defective adenylate cyclase, to a cell with a defective receptor, restoring the hormone stimulated adenylate cyclase activation in that cell. The β-adrenergic receptor was fully purified by Shorr et al., [1981] and many other receptors that couple to adenylate cyclase have since been identified.

The component responsible for 'transduction' was identified as a GTP dependent protein after Rodbell et al., [1971] reported that the binding of glucagon to liver membranes was regulated by GTP. Other laboratories later demonstrated similar results with other hormones and cell types, [Krishna et al., 1972; Glossmann et al., 1974; Berrie et al., 1979], indicating that this GTP regulated mechanism was generally applicable to many signalling pathways.
FIGURE 1.1 The Control of Glycogen Metabolism by Adrenaline.

The pathway described in 1.1 is shown. An asterisk indicates that the enzyme is in its activated state. The de-phosphorylation reactions which are paired with the phosphorylations are not shown (excepting the de-phosphorylation of glycogen synthase, which is activated by PKA).
Rodbell demonstrated that the GTP analogue GMP-P(NH)P could cause slow stimulation of adenylate cyclase, which was vastly increased in the presence of hormone [Londos et al., 1974] and it became obvious that it was the hormone which regulated the GTP effect on adenylate cyclase and not the reverse.

The site of GTP action was identified as a GTPase by Cassel and Selinger [1976], who measured the hormonal stimulation of GTP hydrolysis and demonstrated that the previously observed activation of adenylate cyclase upon treatment with cholera toxin was accompanied by inhibition of the hormone stimulated GTP hydrolysis. Having also demonstrated that the binding of agonist to the receptor caused GDP release, they proposed that a GTPase regulatory cycle controlled the activation of adenylate cyclase. Pfeuffer et al., [1975] demonstrated that a 40-kDa protein distinct from the receptor or adenylate cyclase was involved in this GTP cycle. The removal of this protein resulted in a system which was no longer stimulated by GMP-P(NH)P or fluoride ions [Ross et al 1978]. Another report revealed that a detergent extract from normal cell membranes could be used to restore GMP-P(NH)P stimulatable adenylate cyclase activity in cyc- cells which were defective in the stimulation of adenylate cyclase and thought to lack the enzyme. Thus with the isolation of the GTP binding protein (G-protein), later denoted Gs, because it stimulated adenylate cyclase, (distinguishing it from the adenylate cyclase inhibitory G-protein Gi), a pathway from a cell surface signal to an intracellular metabolic response was completely delineated.

It was later possible to reconstitute a hormone-sensitive adenylate cyclase activity in phospholipid vesicles containing the 3 purified components - the β-adrenergic receptor (discriminator), the G-protein Gs (transducer) and the adenylate cyclase (amplifier) [Pederson and Ross, 1982]. The success of this reconstitution confirmed that these three proteins were sufficient to achieve a primary pathway for hormonal stimulation of cAMP synthesis [May et al., 1985]. The components of this pathway have since been studied in great detail, giving much insight into the workings of other similar G-protein coupled pathways and the fundamental elements of the pathway are described in the following paragraphs.
1.1.1 The 7-transmembrane receptors

Purification of the β-adrenergic receptor was followed by the cloning and sequencing of the gene [Dixon et al., 1986 and Frielle et al., 1987] encoding this 64-kDa integral membrane protein. Analysis of the protein sequence [Yarden et al., 1986] using hydrophobicity plots predicted that the protein contained 7 potential transmembrane helices. This type of structure had previously been identified in rhodopsin [Hargrave et al., 1983] which had been modelled on the structure of bacteriorhodopsin from *Halobacter halobium*, obtained from electron diffraction data and sequence analysis [Unwin and Henderson, 1975; Engelman et al., 1980]. With the elucidation of many more protein sequences, this structure has now been predicted in more than 74 different receptor proteins [Probst et al., 1992]. Many of these are known to be G-protein coupled, although a few, including the original bacteriorhodopsin are not.

A schematic representation of a 7-transmembrane receptor is shown below:

![Schematic representation of a 7-transmembrane receptor](image)

The transmembrane regions are numbered from the aminoterminus to the carboxyterminus.

These proteins comprise an extracellular aminoterminus of variable length, 7 α-helical domains separated by 3 extracellular and 3 cytoplasmic loops, terminated by a long cytoplasmic carboxy-terminus. They are single polypeptide chains, ranging in size from the 324 amino acids of the rat *mas* oncogene to the 744 amino acids of the thyroid-stimulating hormone receptor. Many techniques including photoaffinity labelling, fluorescence emission
spectroscopy, deletion mutagenesis, site-directed mutagenesis, synthetic peptide competition and production of chimeras have enabled identification of regions and individual residues responsible for the different functions of the receptor proteins [reviewed by Probst et al., 1992].

Proteolytic cleavage studies have confirmed the existence of seven regions of hydrophobic residues in these proteins [Dohlman et al., 1988]. The least conserved part of the protein sequence is the extracellular aminoterminus, however almost all G-protein coupled receptors contain sequences for N-terminal-glycosylation [Kornfeld and Kornfeld, 1985]. In the β2-adrenergic receptor this modification is thought to be required for efficient expression of the receptor protein [Dixon et al., 1987]. Alignment of the α-helices, such that hydrophilic residues face inwards and hydrophobic residues face outwards, is thought to allow formation of a polar ligand binding site in the centre of the structure. The third transmembrane helix of the α and β-adrenergic and M1 receptors contains a conserved acidic residue, aspartate, which has been found to be critical for agonist binding to the receptor. It has been proposed that this residue interacts with agonists such as adrenaline and Ach through the positively charged amine head groups of these cationic amines. The involvement of the extracellular aminoterminus varies in the different receptors and in the β-adrenergic receptor is not required for ligand binding. Deletion of the aminoterminal domain in other receptors caused ligand binding to occur at lower affinity, suggesting the existence of low and high affinity agonist binding sites on the receptor [Ji and Ji, 1991].

All of the cytoplasmic regions, in particular the third cytoplasmic loop and the carboxyterminal tail, have been implicated in G-protein interaction. Mutation of the aspartate in a highly conserved DRY sequence in the second cytoplasmic loop of the β-adrenergic receptor had no effect upon agonist binding, but reduced or completely abolished G-protein coupling [Dixon et al., 1988]. Detailed deletion studies of the third cytoplasmic loop of the β-adrenergic receptor identified specific regions which were necessary for propagation of the signal. Deletion of 7 residues in the same region of the α1-adrenergic receptor caused a large reduction in hormone stimulated PtdIns-PLC activity.
Studies of the cytoplasmic carboxyterminal tail have revealed potential sites of modification by palmitoylation and phosphorylation. Deletion of this region from the β2-adrenergic receptors reduces their ability to activate G-proteins, leading [O'Dowd et al., 1989] to suggest that palmitoylation of this region would permit membrane anchorage and formation of a fourth cytoplasmic loop, which could also bind to and activate G-proteins together with the other cytoplasmic loops. The phosphorylation of these receptors has been identified as part of the de-sensitisation mechanism (see 1.1.6).

1.1.2 The Heterotrimeric GTP binding proteins (G-proteins).

The heterotrimeric G-proteins are composed of 3 subunits - α, β and γ which associate with the non-ligand-bound receptor as a complex. The sizes of the α subunits range from 39 to 50-kDa, the β subunits from 35 to 36-kDa and the smaller γ subunits between 6 and 11-kDa. Multiple forms of each subunit were identified and classified [Simon et al., 1991], although the numbers of subunits (16 α, 4 β and 5 γ) have been increased substantially since that review. It is not known how many different combinations of these subunits play physiological roles in vivo. The G-protein Gs, originally identified as the 'transducer' between the β-adrenergic receptor and adenylate cyclase stimulation, appears to be a mixture of splice variants, when purified.

Since the discovery of the GTP dependent activation of adenylate cyclase through Gs, many other pathways have been identified in which these heterotrimeric G-proteins are involved. The different G-proteins can be sub-divided on the basis of their sensitivity or insensitivity to modification by pertussis toxin (PTX) or cholera toxin (CT). Both toxins, acting through different mechanisms and targeting different substrates, modify certain G-proteins by ADP ribosylation. In the case of PTX, the modification occurs at a cysteine residue, 4 residues from the carboxyterminus of the α subunit. The ADP ribosylation blocks the activation of the G-protein by agonist-bound receptor, preventing the release of the potentially active α-GTP and βγ complexes. The action of CT is more complex, requiring the presence of a small G-protein from the Ras superfamily, ADP-ribosylation factor (ARF). CT modifies an arginine residue in the α subunit, which disrupts the GTPase
activity, leading to constitutive activation of the G-protein and its effector. These toxins have been useful in matching particular G-proteins to specific signalling pathways and can indicate where more than one pathway is signalling through a single enzyme. This appears to be the case in PtdIns-PLC activation, which can be sensitive or insensitive to PTX treatment (see 1.3.6).

The table below shows the currently known G-protein families:

<table>
<thead>
<tr>
<th>G-protein Family</th>
<th>Modified by Toxin ?</th>
<th>Known Effectors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gs</td>
<td>Cholera Toxin</td>
<td>Adenylate cyclase activation</td>
</tr>
<tr>
<td>Gi (including Go and Gt)</td>
<td>Pertussis Toxin</td>
<td>Adenylate cyclase inhibition</td>
</tr>
<tr>
<td>Gq</td>
<td>No modification</td>
<td>Ion Channel Modulation</td>
</tr>
<tr>
<td>No modification</td>
<td>cGMP PDE activation</td>
<td></td>
</tr>
<tr>
<td>G12</td>
<td>No Modification</td>
<td>PtdIns-PLC activation</td>
</tr>
<tr>
<td>Unknown</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The G-proteins families have been defined on the basis sequence similarity between the α subunits [Simon et al., 1991]. There are several members in each family, the largest being Gi, which includes the Go and Gt sub-groups as well as at least 3 αi subunits. The multiple members of each family demonstrate different patterns of expression, enabling the G-protein coupled receptors to signal a great diversity of responses. The effectors of the newly discovered G12 family members have yet to be defined, although they may play a role in Drosophila embryonic development [Parks and Weichaus, 1991]. The 5 members of the Gq family (αq, α11, α14, α15, α16) are discussed in more detail in chapter 9.
The full mechanism by which the heterotrimeric G-proteins transduce signals is still emerging, however, it is based upon the simple cycle shown below.

\[
\begin{align*}
\text{Pi} & \quad \text{GDP-} \alpha \beta \gamma \\
\text{Hydrolysis} & \quad \text{GTP-} \alpha + \beta\gamma \\
\text{H}_2\text{O} & \quad \text{Exchange} \\
\text{GTP} & \quad \text{GDP} \\
\downarrow & \\
\text{SIGNAL}
\end{align*}
\]

Before the receptor is activated by agonist binding, GDP is strongly bound to the G-protein \( \alpha \) subunit. In this state the \( \alpha \) subunit has a high affinity for the \( \beta\gamma \) subunits, which are attached to the membrane through the isoprenylated (geranylgeranyl) residues of the \( \gamma \) subunit [Simonds et al., 1991]. When bound to GDP the \( \alpha \) subunit is inactive with respect to its effector enzyme. Upon agonist binding, the G-protein associates more strongly with the receptor [Limbird et al., 1980] which causes the dissociation of GDP in exchange for GTP on the G-protein \( \alpha \) subunit [Brandt and Ross, 1986]. With GTP bound, the \( \alpha \) subunit no longer has a strong affinity for the \( \beta\gamma \) subunits or the hormone-receptor complex and is able to dissociate from them. Whilst GTP is bound, the \( \alpha \) subunit is able to interact with and modulate its effector. The intrinsic GTPase activity of the \( \alpha \) subunit is low and therefore, the subunit retains its activity for several seconds, however, once the GTP has been hydrolysed to GDP, the \( \alpha \) subunit becomes inactive and again binds to the \( \beta\gamma \) subunits, to complete the cycle. The interaction between the activated G-protein \( \alpha \) subunit and the effector adenylate cyclase is known to be direct, indeed the enzyme can be purified to homogeneity as a complex with GP-P(NH)P bound Gs [Pfeuffer et al., 1985].

The mechanism by which \( \alpha \)s activates its effector, adenylate cyclase is not fully understood, although deletion and site-directed mutagenesis have indicated that the carboxyterminal region of the G-protein subunit is involved in this process [Weiss et al., 1988]. Ridelot and Bourne [1992] used a technique known as scanning mutagenesis, in
conjunction with the production of chimeric α<sub>i</sub>-α<sub>s</sub> proteins, to identify the residues of α<sub>s</sub> that determine the specificity of its interaction with adenylate cyclase. They established that a region of 122 amino acids in the carboxyterminus of α<sub>s</sub> was sufficient to mediate cAMP synthesis in the chimeras. By modelling the 3-D structure of α<sub>s</sub> on the crystal structure obtained for p21<sup>ras</sup> [Milburn et al., 1990], they predicted that 3 of the effector-activating regions identified would be located on the same side of the protein, facing the membrane. Similar studies on the G-protein α subunit of transducin (Gα<sub>t</sub>) have also uncovered regions necessary and sufficient for effector activation within the carboxyterminal region of the protein [Rarick et al., 1992].

The above mechanism assumes that the GTP bound α subunit is solely responsible for transmitting the signal onto the effector. Until quite recently it was generally held that the βγ subunits performed a more passive role, facilitating the interaction of the α subunit with the receptor [Kanaho et al., 1984], anchoring the G-protein into the membrane [Sternweis, 1986], stabilising the GDP bound α subunit complex [Iyengar and Birnbaumer, 1982] and enabling the continual cycling of G-protein α subunits. However, several lines of evidence have suggested that the βγ subunits may play a more active role in signal transduction.

The mechanism by which the G-protein Gi causes inhibition of adenylate cyclase has been hotly disputed [Gilman, 1987]. One suggested mechanism is that the α<sub>i</sub> subunit directly interacts with and inhibits adenylate cyclase. Reconstitution of this G-protein subunit in S49 cell membranes revealed only a modest inhibitory effect of Gisα-GTP on the adenylate cyclase. However, a recent report that transfection of cells with constitutively active mutant forms of Gisα caused the inhibition of cAMP accumulation [Wong et al., 1992], confirmed that α<sub>i</sub> can transduce the inhibitory signal to adenylate cyclase directly. In contrast, many observations have been made of the strong inhibitory effect of βγ subunits on adenylate cyclase. However, this effect is dependent on the presence of Gs [Smigel, 1986], leading to suggestions that the βγ subunits do not exert the inhibition by direct interaction with the effector. As Gi is more abundant than Gs in many tissues, including brain, a mechanism was proposed whereby the βγ subunits, released upon the activation of
Gi, acted as a reservoir to 'lock up' the α subunits of Gs. The excess βγ subunits could then accelerate the GTPase-driven deactivation of the αs subunit to its GDP-bound state, thus inhibiting adenylate cyclase activation. There is evidence to suggest that the 'reservoir' model cannot explain all of the adenylate cyclase inhibitory effects of Gi. Katada et al., [1984] showed that adenylate cyclase could be inhibited in response to hormonal stimulation in cyc— cells, which are known to lack Gs. This result was explained on the basis of the small inhibitory effect of αi already mentioned.

Aside from the mechanism of action of Gi, another example of direct signalling through βγ subunits is the propagation of the mating pheromone signal in *S.cerevisiae*. In this lower eukaryote, the G-protein α subunit (GPA1) does not appear to directly transduce the signal from the mating factor receptors STE2 and STE3, as elimination of it leads to constitutive activation of the response pathway [Kurjan and Dietzel 1988]. However null mutants of the β and γ homologous proteins (STE4 and STE18 respectively) are non responsive to mating pheromone [Nakayama et al., 1988]. This suggests that the α subunit serves as a negative regulator of the pathway, by binding to the βγ subunits which propagate the signal. Receptor activation leads to GDP-GTP exchange and dissociation of the regulatory α subunit, allowing the released βγ subunits to activate their target effector. Other pathways in which βγ subunits appear to directly activate the effector include the activation of the cardiac K+ channel regulated by the muscarinic Ach receptor [Codina et al., 1987], the activation of phospholipase A2 in response to light activation of rod outer segments through transducin [Jelsema and Axelrod., 1987a] and the activation of several different adenylate cyclases [Tang and Gilman., 1991].

The general mechanisms by which heterotrimeric G-proteins transduce signals from the receptor to the effector enable amplification of the initial signal into an effective response whilst at the same time permitting the generation of diverse responses to a wide variety of agonists. Amplification of the signal can occur at two points in the G-protein coupling mechanism; it has been observed that one molecule of hormone-activated receptor can interact with as many as 10 molecules of G-protein, providing an initial point of signal amplification in the pathway: a second signal amplification is achieved at the point of
adenylate cyclase activation, as the hydrolysis of GTP bound to Gs is slow (seconds) in comparison with the turnover rate for adenylate cyclase (1000/min), therefore many cAMP molecules can be made during the life-time of one Gs-GTP complex. Response diversity can be introduced at the level of the agonist, with several receptors, such as the adrenergic receptor family, binding the same agonist. At the level of the receptor, there exists the possibility for one receptor type to interact with several G-protein types [Ewald et al., 1989 and Cotecchia et al., 1990]. The G-proteins themselves can potentially be constructed from many different combinations of subunits, giving them different specificities for receptors and effectors, although evidently some combinations are favoured [Pronin et al., 1992].

The sharing of a basic mechanism of signal transduction amongst many pathways also provides opportunities for cross-talk between pathways and therefore subunit specificity is an important factor in regulating the interactions between different pathways. The dissociation of these heterotrimeric proteins into two potential signal bearing-complexes (βγ and GTP-α) provides a further opportunity to regulate two signalling pathways through one receptor. These properties of the heterotrimeric G-protein coupled receptor pathways make them an ideal system for signal transduction, hence their wide use in many different eukaryote cells.

1.1.2.1 The small GTP-binding proteins

Another family of G-proteins has also been identified in eukaryotic cells, which unlike the heterotrimeric G-proteins discussed above, are composed of single polypeptides of approximately 21-kDa. Like the heterotrimeric G-proteins, the small GTP-binding proteins are able to bind guanine nucleotides and possess an intrinsic GTPase activity. This enables them to cycle between an active GTP bound state and an inactive GDP bound state, in the same manner as the α subunits of the heterotrimeric G-proteins. Although they do not complex with βγ subunits, they are regulated by proteins which accelerate the exchange of guanine nucleotides (exchange factors) and the hydrolysis of GTP (GTPase activators or GAPs). They contain a carboxyterminal site for isoprenylation, enabling them to associate with the plasma membrane in the same manner as the heterotrimeric γ subunits. They can
be further divided into four sub-families, Ras, Rho, Rab and Arf, based upon sequence similarity and function [Hall, 1993], with members of the Rab and Arf families mainly involved in membrane trafficking [Balch, 1990] and the Rho family involved in the regulation of actin reorganisation in response to extracellular factors [Ridley and Hall, 1992]. Ras has been shown to be required for many intracellular responses to agonist stimulation of cell surface receptors, although only a few direct effectors of Ras activation have so far been identified, including adenylate cyclase (see 1.1.3) in S. cerevisiae [Toda et al., 1985], the mammalian Raf kinase [Vojtek et al., 1993] and phosphatidylinositol-3-OH kinase [Rodriguez-Viciania et al., 1994; Kodaki et al., 1994]. Recent work expressing mutant forms of Ras has suggested that it may in part function by shuttling proteins, such as the Raf kinase, to the cell membrane [Leevers et al., 1994], performing a similar role to that played by the heterotrimeric G-protein βγ subunits in βARK activation (see 1.1.6). The extensive literature pertaining to the small G-proteins has recently been reviewed [Boguski and McCormick, 1993] and will not be discussed further in this thesis.

1.1.3 Adenylate cyclases

As is typical for many of the enzymes involved in signal transduction, the effector enzyme of Gs, adenylate cyclase, is in fact a large family of enzymes. The mammalian proteins are membrane associated, which is important for close proximity to the activatory G-proteins and receptors. The most common structure for the enzymes from higher eukaryotes comprises a short cytoplasmic aminoterminus, followed by two hydrophobic regions, thought to contain six trans-membrane helices, interdigitated by two cytoplasmic domains. Initial estimates of the size of these proteins, based on hydrophobic properties, suggested molecular weights in the region of 200-250-kDa. However, more accurate measurements have shown them to be expressed as 120-kDa proteins, in brain. This discrepancy can be explained by the presence of associated proteins, in complexes. Indeed, the activatory α subunit of Gs remains bound to adenylate cyclase, when preparations are performed on GP-P(NH)P treated cells. Levitski proposed that in vivo, the enzyme is permanently complexed with its G-protein [Arad et al., 1984], although this seems unlikely
as cells contain an excess of Gs over adenylate cyclase. If each activated G-protein were only permitted to interact with one adenylate cyclase molecule, such permanent complexes would restrict the amplification of the signal at this point in the pathway

The family of adenylate cyclases, like other effector enzymes, have a variable abundance and distribution [Bakalyar and Reed, 1990], suggesting that selective expression and localisation is another mechanism by which the specificity of responses can be achieved. All of the enzymes so far cloned can be activated by Gs and some can be activated by calmodulin, again providing scope for cross-talk between different signalling pathways. The recently demonstrated activation of adenylate cyclase by βγ subunits was also found to be type-specific, as only types II and IV are responsive to this form of activation [Tang and Gilman., 1991]. Interestingly, the concentration of βγ subunits required to activate these subtypes is much greater than that of Gsα, implying that Gs βγ subunits would not be responsible for this activation in vivo. The more abundant Gi or Go proteins are likely candidates for βγ subunit donors, although Gi has already been implicated in the inhibition of adenylate cyclase. There is an apparent contradiction in the ability of Giβγ subunits to stimulate and inhibit adenylate cyclase activation [Federman et al., 1992], which can be explained by different regulation of the various adenylate cyclase isoforms. Thus it seems that some adenylate cyclase isoforms can be activated by βγ subunits, some can be inhibited by αi and some are not directly regulated by either, but when activated by Gs are subject to an indirect inhibition by Giβγ subunits.

1.1.4 cAMP - an ideal signalling intermediate

The heat stable activator, cAMP, is used as an intermediate in many different signalling pathways and in a variety of organisms from lower eukaryotes such as S.cerevisiae and D.discoideum through higher eukaryotes such as D.melanogaster and in mammalian cells. It is ideally suited to the role of a second messenger, as it is very stable in the absence of a specific phosphodiesterase and it is not directly involved in the main metabolic pathways of the cell. Thus the cytoplasmic concentration of cAMP can be dramatically altered for the purpose of signalling, without affecting the rates of important
metabolic processes such as oxidative respiration. As the hydrolysis of cAMP by its phosphodiesterase is highly thermodynamically favourable, cAMP can be rapidly degraded allowing the concentration of this second messenger to be reduced to basal levels once the signal has been propagated, ensuring that the pathway is highly responsive to hormonal stimuli. Likewise the ability of adenylate cyclase to synthesise cAMP from a widely available source (ATP), rapidly, means that upon hormonal stimulation, levels of the intermediate can change manyfold in seconds. This provides the rapid response required, for example after adrenaline stimulation during the physiological 'fight or flight' mechanism. The role of cAMP in signalling pathways is that of an allosteric activator and in that respect it is comparable with another second messenger, Ca^{2+}, which combines with calmodulin to form a complex which regulates the activation of the calmodulin-dependent kinases.

1.1.5 cAMP-dependent protein kinase

The activation of protein kinases is common to many eukaryotic signalling pathways, as a vast array of proteins are regulated by phosphorylation. The cAMP-dependent protein kinase (PKA) catalyses the transfer of the γ-phosphate group from ATP to specific serine or threonine residues on selected protein substrates, in response to increased cAMP levels. In skeletal muscle, stimulation, through the β-adrenergic pathway, leads to the phosphorylation of both glycogen synthase and phosphorylase kinase by PKA. This dual regulation of more than one enzyme enables amplification of the original signal, as one signal results in the stimulation of glycogen breakdown and the inhibition of glycogen synthesis, doubling the effect of the signal on glucose metabolism.

The protein kinases can be divided into two large groups, the tyrosine kinases, which phosphorylate their substrates on tyrosine residues (see 1.3.5) and the serine-threonine kinases, which phosphorylate substrates on either of these two residues. An additional small group of "dual-specificity kinases", such as MAPkinase, have recently been identified [Lindberg et al., 1992]. These serine-threonine kinases are also able to phosphorylate tyrosine residues. Much work has been done to understand what makes
particular residues substrates for these kinases. Apparently, the suitability of a target residue is partly designated by residues in the immediate vicinity of that site. The sequence preferences of the different kinases (consensus sequences) have been determined using artificial peptides and by examining and comparing the sequences surrounding known in vivo phosphorylation sites [Kennelly and Krebs, 1991]. The identification of these sites in a protein does not guarantee that it is a target for the particular kinase, as secondary and tertiary structures are also important. The consensus sequence for PKA, which describes 95% of the sequences of known PKA substrates is R-R/K-X-S/T, where X can be any residue. Tests on peptide substrates indicate that an R in the second position is ideal, but such a sequence is only found in 33% of the in vivo sites examined. Clearly basic residues, in particular arginines are very important in the consensus sequence which also appears to be the case for other serine-threonine kinases.

PKA is a complex formed from two identical regulatory subunits and two identical catalytic subunits. Allosteric activation is achieved when two molecules of cAMP bind to each regulatory subunit, causing the dissociation of the two activated catalytic subunits, which are then able to act independently. The regulation of PKA through the association of inhibitory subunits with catalytic subunits is partially mirrored in other serine-threonine kinases, such as PKC (see 1.2.). Although PKC is composed of a single peptide, it contains a regulatory pseudosubstrate domain which inhibits the action of the catalytic domain, until the protein is activated.

For a signalling pathway to function efficiently, the constituent enzymes must be rapidly inactivated once the signal has ceased. Therefore, enzymes that have been phosphorylated must be dephosphorylated or degraded. To avoid the necessity for degradation and enable rapid responses, most phosphorylation reactions are paired with an antagonistic dephosphorylation in a type of 'substrate cycle'. Dephosphorylation is achieved by protein phosphatases [Ballou and Fischer, 1986] and in the adenylate cyclase pathway, several such phosphatases reverse the effects of phosphorylation by PKA. One of these phosphatases (PP1), like PKA, is regulated by cAMP. In the absence of cAMP the active phosphatase dephosphorylates phosphorylase kinase and its substrate glycogen.
phosphorylase, as well as reversing the PKA phosphorylation of glycogen synthase. The activity of the phosphatase itself is regulated by an inhibitor, which can be phosphorylated by PKA. This ensures that the hormone stimulation of PKA activity has a rapid effect on glycogen breakdown, by inactivating the inhibitory circuit as well as activating the phosphorylase kinase directly. Thus the propagation of the signal works through the activation of a kinase, coupled with the deactivation of the phosphatase. There is also evidence of signal propagation through the activation of phosphatases, for example in response to the hormone insulin, which causes the uptake of glucose and stimulation of glycogen production [Foulkes et al., 1982]

1.1.6 Control of the pathway

The phosphorylation of proteins by PKA, leading to the activation of enzymes such as phosphorylase kinase, is vital for propagation of the signal through the β-adrenergic pathway. The ability of kinases like PKA to act on many different targets is not only vital for amplification of the original signal, but enables them to participate in feedback control mechanisms and cross-talk between different pathways (see 1.3.7). One major point of control in the adenylate cyclase pathway is the coupling between receptor and effector through the G-protein Gs. As mentioned in 1.1.2 the inhibitory pathway mediated through Gi is thought to work in part by 'hijacking' this process.

One control mechanism common to all receptor mediated pathways is desensitisation, leading to the rapid reduction of effector stimulation, even in the presence of a stimulus. Long-term exposure to stimulus may lead to a slower but less easily reversed control process - down-regulation. In down-regulation, receptor numbers are reduced in concert with reduced effector stimulation, either by degradation or internalisation. Therefore the effects of such regulation can only be reversed by the synthesis of new receptor molecules or recycling of the internalised receptors to the cell surface. Desensitisation is often regulated by phosphorylation [Benovic et al., 1988] and in the adenylate cyclase pathway this is partly achieved by the second messenger kinases, PKA and PKC [Hausdorff et al., 1990]. Thus desensitisation through phosphorylation of the
receptor and G-proteins by these kinases constitutes a classic negative feed-back loop through PKA and a cross-linking to other pathways through PKC.

Desensitisation in the adenylate cyclase pathway is also known to be mediated through another family of serine-threonine kinases, the G-protein coupled receptor kinases (GPRKs) [Inglese et al., 1993]. One member of this family, β-adrenergic receptor kinase (βARK) is dependent upon an interaction with G-protein βγ subunits for its activation [Kameyama et al., 1993]. It has been shown that this interaction with βγ subunits enables the kinase to translocate to the plasma membrane [Pitcher et al., 1992], where it phosphorylates the β-adrenergic receptor [Benovic et al., 1986a], causing its inactivation. The ability of βγ subunits to stimulate βARK translocation to the membrane appears to be a result of the isoprenylation of the heterotrimeric G-protein γ subunit (see 1.1.2), which serves to anchor the βγ subunits in the membrane. The related GPRK, rhodopsin kinase (RK) does not require βγ subunits to phosphorylate activated rhodopsin. Instead, RK possesses a C-terminal CAAX motif which becomes farnesylated, enabling the protein to be targeted to the membrane upon stimulation by light [Inglese et al., 1992]. However, targeting to the membrane is not sufficient for light-stimulated activation of RK, as a mutant geranylgeranylated kinase, although targeted to the membrane, did not phosphorylate rhodopsin. βARK proteins which were modified by either isoprenyl group were however translocated to the membrane and activated in the absence of βγ subunits, but not in a light-stimulable fashion. Thus a similar mechanism is employed by two kinases which are regulated by quite different systems. The specificity in the RK response is achieved by the requirement for farnesylation, which enables translocation to the membrane and an unknown additional activation mechanism. βARK activation is simply controlled by the specificity of the βγ subunits interactions, enabling its translocation to the membrane, without the requirement for any additional activation mechanism. βARK has also been shown to phosphorylate other receptors in the same manner [Benovic et al., 1986b; Benovic et al., 1987; Kwatra et al., 1989], implying that this mechanism of desensitisation is widely used in many pathways.
Other potential regulators of this G-protein coupled pathway include β-arrestin, which can only bind to the receptor after it has been phosphorylated by βARK or a related kinase, [Lohse et al., 1992], and phosducin, a 33-kDa protein with GTPase inhibitory activity, which can be phosphorylated and hence inactivated by PKA. Clearly there are many complex regulatory networks controlling the G-protein coupled pathways in cells, which when dissected seem to be composed of distinct but related mechanisms.

1.2 Protein Kinase C

Like PKA, PKC has emerged as an enzyme of central importance to many signalling pathways. As well as being a modulator in the recently dissected Raf-kinase to MAPKinase pathway [Blumer et al., 1994] and of the responses to signals generated by PtdCh hydrolysis, PKC has also been shown to play an important part in the mediation and regulation of responses to agonist stimulated PtdIns hydrolysis by PLC isozymes. However few direct in vivo substrates of this kinase have been identified, its role in pathways having been determined indirectly through the use of PKC inhibitors and the phorbol ester activators. As the PKC family has been extensively reviewed [Stabel and Parker, 1991; Azzi et al., 1992; Hug and Sarre, T.F., 1993] only selected aspects of its structure and function will be discussed here.

To date, 11 different PKC isozymes have been identified, on the basis of sequence homologies. They have been divided into 3 groups on the basis of structual criteria and their modes of activation [Nishizuka, 1992]. The classical or cPKC isozymes, consisting of α, β and γ, require both the signalling intermediate DAG (or phorbol esters) and Ca²⁺ for their activation. The novel (nPKC) isozymes include δ, ε, η, θ and μ and can be activated by DAG or phorbol esters in the absence of Ca²⁺. The isozymes for which no mode of activation has yet been identified are classified as atypical (aPKC) and comprise ζ, λ and τ. One possible activator is PtdIns(3,4,5)P3 which was shown to have an effect on PKC ζ activation [Nakanishi et al., 1993]. This lipid is one of the products of the enzyme PtdIns 3-kinase, which is known to be activated in response to receptor tyrosine kinase agonists such as PDGF [Valius and Kazlauskas 1993]. Evidently the variety of PKC
isozymes, their different expression, substrate specificity and activation mechanisms enable this enzyme to perform an important role in many different signalling pathways within the cell.

The structure of all PKC isozymes consists of an amino-terminal regulatory region and a carboxy-terminal catalytic region. The cPKC sequence was originally divided into 4 conserved regions (C1-C4), alternately interlinked by 4 variable regions (V1-V4) (Fig 1.2.). No function of the kinases has been attributed to the variable regions, except that they could provide for flexibility within the molecule, which may be particularly important in the V3 region between the regulatory and catalytic halves of the protein as it is predicted to act like a hinge. Conserved domains C3 and C4 form the catalytic site in the kinase and have homology to the catalytic regions of other kinases. Restricted proteolytic cleavage of PKC by trypsin, causing the removal of the regulatory domain from the catalytic carboxy-terminus, has been shown to generate a kinase that is active in the absence of activators. This technique has been used to test the effect of PKC phosphorylation on potential substrates in vitro.
FIGURE 1.2 The PKC Families.

A representation of the domain structure of each PKC family is shown, indicating the conserved (C) domains and variable (V) domains. The function of each of the domains is outlined in the text. The Cysteine-rich repeats of C1 are present in the other isozyme family members, as is the ATP binding site of C3 and the kinase domain C4.
Very little data is available on the substrate specificity of the PKC isozymes, as there are few known physiological substrates for this kinase. However, using synthetic peptides, the α and β isozymes demonstrated very similar patterns of recognition, yielding a consensus XRXXSX, whereas PKC γ was shown to have a requirement for both amino and carboxyterminal basic residues [Marais et al., 1990], with a preference for arginine over lysine as the basic residues. In confirmation of this, a peptide based upon the sequence of the pseudosubstrate site with the sequence :- XRXXSXRX is a very good substrate for PKC phosphorylation.

The C2 region is only found in the cPKC enzymes and is thought to be responsible for the Ca^{2+} dependence of lipid interactions and catalytic activity observed for this class. A similar sequence has also been found in rasGAP, PtdIns-PLC γ1 and cPLA₂, which also shows Ca^{2+} dependent activation [Baker 1989; Clark et al., 1991]. The C1 region, found in all of the PKC isozymes to a greater or lesser degree, contains an 'extended' zinc-finger-like sequence may play a role in binding lipid and phorbol ester activators. The mechanism through which this occurs is not known, but the role of such a sequence is of great interest as similar regions have been identified in Raf kinase and DAG kinase [Rapp et al., 1988; Schaap et al., 1990]. Another region which has been identified in the amino-termini of all the PKC isozymes is the pseudosubstrate site, which contains a PKC phosphorylation site consensus sequence, with the serine substituted by a non-phosphorylatable alanine. It has been shown that this region holds the kinase in an inactive conformation, by binding to the catalytic domain in place of substrate, without being phosphorylated [Stabel and Parker, 1991]. Activation of PKC γ was shown to involve a conformational change, demonstrated by the changing pattern of available epitopes, using a panel of monoclonal antibodies [Cazaubon et al., 1990]. Much work has been done on establishing the role of this region as a regulator of co-factor dependence [reviewed in Dekker and Parker, 1994].

As well as being regulated by allosteric effectors, PKC activation is regulated by phosphorylation. It appears that PKC α is produced as an unphosphorylated inactive kinase of 76-kDa and that its activation requires at least two phosphorylation steps [Cazaubon and Parker, 1993], which generate an active 80-kDa protein. The first step,
performed by an as yet unidentified kinase, involves the phosphorylation of threonine 497 in the catalytic domain of PKC [Cazaubon et al., 1994]. Once this priming event has occurred, the PKC is then able to autophosphorylate, leading to the observed mobility shift of the kinase. When a mutation was introduced into the primary phosphorylation site, replacing threonine 495 with a negatively charged glutamic acid residue, the requirement for the priming phosphorylation was lost. When the mutant was expressed in *E.coli* and purified, it was catalytically active *in vitro*, in comparison with the bacterially expressed wild-type PKCa, which, unlike the same protein expressed in COS-1 cells, was unphosphorylated and catalytically inactive.

PKC has been shown to be involved in the mediation of many different responses and doubtless the variety of isozymes and their activation mechanisms contributes greatly to the diverse functions of this enzyme. Specificity of response is probably partly achieved through the selective expression and localisation of the different isozymes. The varying requirements of each group of isozymes for co-factors and effectors, enables the kinases to distinguish between different activation pathways. The activator, DAG, can be released through activation of a number of signalling pathways. When generated through PtdIns-PLC catalysed PtdIns(4,5)P2 hydrolysis, DAG release is accompanied by IP3 production which leads to the release of Ca^{2+} from intracellular stores (see 1.3.3). Therefore the cPKC isozymes will be activated. Alternatively, if the source of DAG is PtdCh hydrolysis by PLD or PtdCh-PLC, only the nPKC isozymes will be activated. If PtdIns(3,4,5)P3 is an activator of the aPKC isozymes, these will be activated in response to PI-3kinase activation, for example through the PDGF receptor (see 1.3.5). The requirement for a priming phosphorylation to allow activation of PKC provides another means by which PKC activity could be localised or even compartmentalised, simply through the selective expression and localisation of the 'priming kinase'.

1.3 Lipids as Intracellular messengers

DAG and PtdIns(3,4,5)P3 are thought to perform important roles in the selective activation of PKC isozymes and are part of large group of second messengers derived from phospholipids. The role of second messengers in signal transduction demands that the concentrations of such molecules can be rapidly changed inside the cell. It is therefore vital for their success as signal transducers that a source of these molecules is readily available. The second messengers cAMP and cGMP are synthesised from ATP and GTP, produced during the citric acid cycle. Ca^{2+} is readily available as it is taken up by the cell and stored in organelles such as the endoplasmic reticulum and calciosomes. The largest group of second messengers is derived from the abundant source of lipids in the plasma membrane. Aside from their role as a hydrophobic barrier between the cell cytoplasm and the exterior, the lipids of the eukaryotic cell membrane perform many important roles in signalling. Cholesterol and its derivatives are used in the production of steroid hormones and the glycolipids are also believed to be involved in signalling. Finally, the phospholipids form the basis for the production of many second messengers.

1.3.1 The Phospholipids

The phospholipids comprise the phosphoglycerides, which are formed from a glycerol backbone bonded to two fatty acid chains and a phosphorylated alcohol, and sphingomyelin, a lipid with a sphingosine backbone linked to a fatty acid chain (see Fig 1.2). The phosphoglycerides can be synthesised from a common intermediate, phosphatidic acid (PA), which is generated from glycerol 3-phosphate via lysophosphatidic acid (LPA). The PA is then converted to the phosphoglycerides through the synthesis of CDP-diacylglycerol, from which diacylglycerol phosphate is transferred to molecules such as inositol, to generate PtdIns. In mammals, PtdCh is synthesised via another pathway from choline obtained in the diet, and PtdSer is mostly produced from PtdCh and serine by transphosphatidylation [Nishijima et al., 1986].
1.3.2 The Phospholipases.

The production of signalling molecules from the phospholipids is achieved through the activation of several phospholipases, including phospholipase A\(_2\) (PLA\(_2\)), phospholipase C (PLC) and phospholipase D (PLD) and sphingomyelinase (SPM). The PLC enzymes can be further categorised, according to their substrate specificity, into those which hydrolyse PtdCh and PtdEth [Kiss and Anderson, 1989] and those which hydrolyse PtdIns and its derivatives. Each phospholipase class catalyses the hydrolysis of a specific bond, whatever the substrate phospholipid (see below).

![Diagram of phospholipid structure](image)

The generalised structure of a phosphoglyceride is shown above, with R representing the variable moieties. In phosphatidyl choline, R3 represents choline and R1 and R2 the fatty acids. The arrows indicate the specific bond preferentially hydrolysed by each of the phospholipases.

The roles and action of the SPM enzymes responsible for the conversion of sphingomyelin to ceramide and choline or their phosphates is only just emerging and will not be discussed further in this thesis. PtdCh is the most abundant of the phospholipids in mammalian cell membranes, accounting for up to 50% of the total cellular phospholipid content, and its hydrolysis by the phospholipases leads to the formation of several
molecules which have been shown to function as signalling intermediates. When PtdCh is hydrolysed by PLC in response to agonist, the products are choline phosphate and $s_n$-1,2 diacylglycerol (DAG) [Exton, 1990], whereas when PtdCh is hydrolysed by PLD, the resulting products are choline and PA. It has been suggested that PA may mediate certain signals and when added to fibroblasts causes rapid DNA synthesis and cell proliferation [Moolenaar et al., 1986; Yu et al., 1988]. When added to cells exogenously it causes $Ca^{2+}$ influx across the plasma membrane [Putney et al., 1980] and a role in secretory mechanisms has also been proposed [Holmsen et al., 1984]. In addition, it can be converted into DAG by a phosphohydrolase, making it difficult to distinguish the actions of PtdChPLC and PLD in a pathway simply by analysing the products of hydrolysis. However, PLD activation can be distinguished from PLC activation by assaying for transphosphatidylation, which is catalysed by PLD. Under aqueous conditions the hydrolysis of PtdCh leads to PA production through the transfer of the phosphatidyl group to a water molecule, however in the presence of an alcohol such as ethanol, the transphosphatidylation produces a detectable and distinct product, Ptdethanol. It has been suggested that this reaction is exploited \textit{in vivo}, to transfer the phosphatidyl moiety to selected proteins, providing a means of anchoring otherwise cytosolic molecules in the membrane [Billah and Anthes, 1990]

The production of $s_n$-1,2 diacylglycerol also occurs when PtdIns-PLC hydrolyses PtdIns and its derivatives (see 1.3) and therefore forms an important link between several signal transduction pathways. DAG is different from other second messengers, such as cAMP and $Ca^{2+}$, as, being a lipid it has to remain in the membrane and thus its diffusion is limited. It has been shown to activate several enzymes, including the serine-threonine kinase, PKC (see 1.2) [Nishizuka 1992], which can also be activated by the more stable phorbol esters.

When cultured fibroblasts were stimulated with $\alpha$-thrombin, DAG concentrations increased biphasically with time [Wright et al., 1988]. The initial rapid production of DAG, which peaked 15s after stimulation, was coincident with the production of inositol phosphates, whereas the second phase of DAG production peaked at 5 minutes and was
sustained for at least 1 hour, but was not accompanied by inositol phosphate release. Analysis of the molecular species of diacylglycerides in stimulated and unstimulated cultures led to the conclusion that the first phase of DAG production was achieved through the hydrolysis of PtdIns and the second, more sustained phase was generated by the hydrolysis of PtdCh [Pessin et al., 1989]. Many mitogens and growth factors have since been demonstrated to elicit a similar bi-phasic response [Leach et al., 1991] and it has been shown that the sustained activation of PKC, which follows from the second phase, is required for longterm responses such as the differentiation of HL-60 cells into macrophages [Aihara et al., 1991]. Careful analysis of the products of PtdCh hydrolysis in response to bombesin in Swiss 3T3 cells revealed that PLD was primarily responsible for the sustained production of DAG, as choline but not choline phosphate release was detected [Cook and Wakelam, 1989].

Neither PtdChPLC nor PLD have been fully purified and the genes encoding these proteins have not yet been isolated, therefore little is known about their activation and mechanisms of action. However, it has been suggested that tyrosine phosphorylation may be required for activation of PtdChPLC [Choudhury et al., 1991]. Other experiments in intact cell systems have demonstrated that phorbol esters can stimulate PtdCh breakdown, in some cases synergistically with Ca^{2+} ionophore [Quian and Drewes, 1990], implying a role for PKC in PLD activation. Reports that GTP analogues can cause the release of PA and stimulate transphosphatidylation, indicated that a G-protein might also be involved in PLD activation. This suggestion has recently been confirmed by the discovery that in HL60 cells PLD can be activated by the small GTP binding protein ARF [Brown et al., 1993; Bowman et al., 1993; Cockcroft et al., 1994].

PLA_2 appears to be activated in response to the same agonists that elicit inositol phospholipid hydrolysis and is particularly sensitive to Ca^{2+} concentrations. It is able to hydrolyse several of the phosphoglycerides, including PtdIns and PtdCh, and has been shown to cause the release of lysophospholipids and cis unsaturated fatty acids such as arachidonic acid (AA). LysoPtdCh is toxic to cells, causing membrane lysis and is therefore usually immediately converted to PtdCh or metabolised further, although it has
been shown to mediate several biological actions including chemotaxis [Hofman et al., 1982]. Arachidonic acid is an intermediate in the synthesis of eicosinoids, which are involved in inflammatory responses, but may also play a role as second messengers [Khan et al., 1992]. Both products of PLA2 directed hydrolysis may play a role in the potentiation of PKC activation by DAG [Chen and Murakami, 1992]. An intracellular cPLA2 has been cloned and characterised [Clark et al., 1990] and has been shown to contain a sequence homologous to the PKC C2 domain (see 1.2.3), GAP and PtdIns-PLC, which has been implicated in Ca^{2+}-dependent translocation of these proteins to the cell membrane [Clark et al., 1991]. The cDNA sequence of PLA2 contains several potential PKC phosphorylation consensus sequences and the protein is modified by serine phosphorylation in agonist treated cells. This phosphorylation, together with activation of the enzyme can be blocked by staurosporine, an inhibitor of PKC [Lin et al., 1992]. This evidence suggests that PKC may be involved in PLA2 activation, although PKC alone is not apparently sufficient for the activation. Evidence also exists for G-protein mediated activation of PLA2 from cell permeabilisation experiments in which GTPyS was able to potentiate AA production. Addition of neomycin inhibited inositol phosphate production, without altering AA production, indicating that in this case, the activation of PLA2 through a G-protein was independent of PtdInsPLC activation. A role for G-proteins in the activation of PLA2 was also suggested from work on the light stimulated activation of phosphodiesterase (PDE), through transducin. The stimulation of rhodopsin in rod outer segments (ROS) has been shown not only to cause the stimulation of cGMP-PDE, but also the stimulation of PLA2 activity through the ROS G-protein transducin [Jalsema 1987 b]. This G-protein dependent activation was subsequently attributed to the βγ subunits of transducin, and could be blocked by simultaneous addition of αt [Jelsema and Axelrod, 1987 a].
1.3.3 Inositol lipid hydrolysis

The phosphoinositides, containing a myo-inositol headgroup in place of the choline moiety of PtdCh, can be phosphorylated at multiple sites, potentially creating many different but related molecules. The predominant forms are phosphatidylinositol (PtdIns), phosphatidylinositol 4-monophosphate (PtdIns4P) and phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P$_2$), making up approximately 5% of the total membrane phospholipids. In the plasma membrane these phospholipids and their derivatives can be interconverted by a collection of kinases and phosphatases, therefore the metabolism of each phosphoinositide can influence that of the others. Fig 1.3 shows some of the pathways involving the breakdown and interconversion of these lipids. A role in signalling has been identified for some of these molecules, the others are apparently transitorily required for the recycling and generation of the phosphoinositides.

The observation that PtdIns metabolism could be stimulated in pancreatic tissue by the addition of Ach was the first demonstration of agonist stimulated PtdIns hydrolysis [Hokin and Hokin, 1953]. Similar observations were subsequently made in many other tissues, suggesting that the stimulation of inositide metabolism might occur through a receptor [Durell et al., 1969] and that it was involved in the mediation of signals from agonists which raised the concentration of intracellular calcium [Michell, 1975]. The identity of the second messenger responsible for mediation of the Ca$^{2+}$ rise was not uncovered until much later. PtdIns turnover had been observed in all tissues which demonstrated calcium fluxes in response to agonist stimulation [Michell and Kirk, 1981] and in vasopressin stimulated hepatocytes it had been observed that PtdIns(4,5)P$_2$ turnover preceded PtdIns turnover, in a calcium independent manner [Michell et al., 1981].
FIGURE 1.3 Inositol lipid metabolism.

A Selection of the pathways involved in inositol lipid metabolism are shown. These reactions are catalysed by many different enzymes, some of which are regulated through agonist sensitive mechanisms. The other pathways serve to regenerate these signalling molecules. The reactions catalysed by PtdIns-3 kinase, for example converting PtdIns to PtdIns(3)P, are not shown.
It was therefore proposed that inositol 1,4,5-trisphosphate (IP₃), one of the products of PtdIns(4,5)P₂ hydrolysis, was the 'missing link' in mediation of the release of Ca²⁺ in response to agonist [Berridge and Irvine, 1984]. This proposal was confirmed when IP₃ injection into the photoreceptor cells of Limulus led to a transient rise in the intracellular Ca²⁺ concentration and subsequently a voltage response similar to that normally produced by light activation [Brown and Rubin, 1984]. Berridge described the receptor mediated stimulation of PtdIns(4,5)P₂ hydrolysis as a bifurcating pathway in which the formation of two second messengers, DAG and IP₃, triggered separate signalling pathways within the cell, through Ca²⁺ mobilisation and PKC activation. This division of the signal did not exclude the possibility that the two pathways might subsequently interact synergistically or exert inhibitory effects on each other. The mechanism by which IP₃ mediates Ca²⁺ mobilisation involves the binding of the second messenger to an intracellular receptor, thought to be localised in the membranes of the endoplasmic reticulum [Furuichi et al., 1989]. The receptor is linked to a calcium channel, which opens upon IP₃ binding, releasing the stores of calcium into the cytoplasm.

1.3.4 The PtdIns-PLC isozymes

The stimulation of PtdIns-PLC activity, leading to the hydrolysis of PtdIns(4,5)P₂ has been demonstrated in many diverse cell types in response to numerous agonists [Meldrum et al., 1991b]. These agonists include long distance hormones such as vasopressin and adrenaline, local hormones such as histamine, growth factors such as EGF and PDGF, neurotransmitters such as Ach and neuropeptides such as substance P. The basis for this diversity of response, achieved through a single enzyme activity, is in part due to the variety of PtdIns-PLC isozymes available to cells and their differential expression, regulation and localisation.

The first PtdIns-PLC isozymes to be isolated were purified from ram seminal vesicles [Hofmann and Majerus, 1982]. PLC I had a molecular weight of 65-kDa, whereas PLC II was identified as an 85-kDa protein. Antibodies raised against the two preparations did not cross-react, suggesting that they represented distinct isoforms of PtdIns-PLC.
Subsequently many other preparations of PtdIns-PLC isozymes with varying molecular weights were purified from other tissues. The cloning and sequencing of three PtdIns-PLC isozymes from rat brain with molecular weights of 148-kDa [Suh et al., 1988a], 138-kDa and 85-kDa [Suh et al., 1988b], and one from bovine brain [Katan et al., 1988] indicated that at least 3 different types of PtdIns-PLC were expressed in brain. Since the initial cloning work, many more isozymes have been purified and cloned, however all of them have homology to one of the originally cloned isozymes and have been grouped into 3 classes, \( \beta \), \( \gamma \) and \( \delta \) [Rhee et al., 1989]. Fig 1.3.1 shows the current members of each class of PtdIns-PLC. The \( \alpha \)-like enzymes with sizes similar to the 68-kDa preparation from sheep seminal vesicles have been excluded, as they were subsequently found to be proteolytic products of the other isozymes [Rhee and Choi, 1992], and the '\( \alpha \)' cDNA clone was found to be the gene encoding \textit{E.coli} thioredoxin [Bennett et al., 1988].
<table>
<thead>
<tr>
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**FIGURE 1.3.1 Cloned PtdIns-PLC Isozymes**

The PtdIns-PLC isozymes shown represent those that have been cloned and sequenced and contain regions with homology to the catalytic X and Y domains. Most of these enzymes have also been purified. PtdIns-PLC isozymes which have been purified but not cloned are not included here [see Cockcroft and Thomas, 1992]. Molecular weights are indicated for PtdIns-PLCβ3 as full clones have not been obtained. The *D.melanogaster* enzymes are most closely related to the β class, whereas the *D.discoideum* and *S.cerevisiae* enzymes are δ-like in their structure. The prokaryotic enzymes contain an X like domain, but cannot hydrolyse PtdIns(4)P or PtdIns(4,5)P2, acting instead on PtdIns in the cell wall, as secreted enzymes.
Comparison of the primary structures of these isozymes revealed two regions of homology shared by all 3 isozymes. A schematic representation of the structure of the isozymes in each of these classes is shown overleaf:

The X and Y boxes represent the highly conserved domains, showing 54% and 42% identity between the different sequences, respectively. As these regions were the only ones with significant homology between all of the isozymes, it was proposed that they might be involved in the catalytic action of the enzyme. The requirement of the X and Y domains for catalytic activity was confirmed by mutagenesis and deletion studies [Bristol et al., 1988]. Outside the catalytic X and Y domains, there is little homology between isozymes of different classes, although within each class there are defined regions of homology, which are thought to represent domains responsible for class-specific regulation of the enzyme activity.

Part of the sequence between the X and Y domains of the γ isozymes is homologous to the non-catalytic regions of oncogene Src [Mayer et al., 1988; Stahl et al., 1988]. These src homology domains (SH2 and SH3) have since been identified in many other proteins [Lowenstein et al., 1992]. Removal of these domains [Bristol et al., 1988]
from the \( \gamma \) enzyme did not alter catalytic activity indicating that they might be involved in the regulation of the enzyme. In the \( \beta \) and \( \delta \) isozymes, the region between the X and Y domains contains a high concentration of acidic residues instead of the SH2 and SH3 domains found in the \( \gamma \) enzyme and has been predicted to act like a hinge, allowing the catalytic X and Y domains to come together to form a catalytically active conformation [Kriz et al., 1990]. Another potential regulatory region is the carboxyterminus following the Y domain, which is greatly enlarged (400 amino acids) in the \( \beta \) isozymes and contains a high proportion (40%) of charged residues. The sequences in the amino termini of \( \beta_1 \) and \( \beta_2 \) are highly conserved, suggesting an important role for this region of the protein in the \( \beta \) class isozymes.

Characterisation of the various isozymes has revealed that each class has a different Ca\(^{2+}\) dependency for hydrolysis of substrate [Ryu et al., 1987], with the \( \delta \) isozyme activated at lower concentrations of Ca\(^{2+}\) than the other enzymes. However, all of the isozymes displayed similar substrate preferences, with PtdIns(4,5)P\(_2\) the most favoured substrate and PtdIns the least favoured. However, as indicated by the varied primary structure of the different isozyme classes, a variety of regulation mechanisms are emerging, which control the activation of the PtdIns-PLC enzymes in the cell.

1.3.5 Signalling through PtdIns-PLC\(\gamma\)

The observation that growth factors such as PDGF could stimulate inositol phosphate production in normal cells [Berridge et al., 1984] led to the investigation of the role of receptor tyrosine kinases in PtdIns-PLC regulation. The receptor tyrosine kinases [reviewed in Cadena and Gill 1992] have been shown to consist of several different functional domains, through detailed mutational studies. The cytoplasmic tyrosine kinase domain, which mediates the biological response, is able to autophosphorylate the receptor upon agonist binding and phosphorylate substrate proteins on tyrosine residues. The sites of autophosphorylation are contained in the regulatory domain, which may consist of a carboxy-terminal tail or a 'kinase insert' located between two kinase domains. The extracellular aminoterminal is responsible for ligand binding and is connected to the
cytoplasmic kinase and regulatory domains by the transmembrane domain. The mechanism by which the peptide growth factors bind to their receptors and activate the tyrosine kinase is not fully understood. However, it has become clear that agonist binding causes dimerisation of the receptor molecules, leading to activation of the tyrosine kinase function. This is followed by intermolecular autophosphorylation of several tyrosine residues in the carboxy-terminal tail of the receptor proteins, enabling the receptors to interact with and phosphorylate their effectors on selected tyrosine residues [Ullrich and Schlessinger 1990].

Immunoprecipitation of PtdIns-PLCγ1 from EGF treated cells with anti-phosphotyrosine antisera [Wahl et al., 1988a] demonstrated that PtdIns-PLCγ1 was phosphorylated on tyrosine residues in response to growth factor stimulation. Co-immunoprecipitation of the EGFR with anti PtdIns-PLCγ1 antisera revealed that the receptor and effector formed a tight complex in EGF treated cells [Kumjian et al., 1989] and the use of a kinase defective EGFR mutant [Margolis et al., 1990] demonstrated that this complex formation was dependent upon activation of the receptor tyrosine kinase. This evidence together with a demonstration of PtdIns-PLCγ1 phosphorylation by the EGFR in vitro, [Nishibe et al., 1989], suggested that EGF regulated PtdIns-PLC activation through its association with and phosphorylation by the EGFR. Similar results were obtained with PDGF [Wahl et al., 1989b], but not with Insulin or Bombesin [Meisenhelder et al., 1989], indicating that only certain growth factors mediated the activation of PtdIns(4,5)P2 hydrolysis through a mechanism involving tyrosine phosphorylation of the effector PtdIns-PLC by the activated receptor tyrosine kinases. The limited selection of PtdIns-PLC agonists able to stimulate tyrosine phosphorylation and the absence of growth factor stimulated tyrosine phosphorylation on PtdIns-PLCβ or δ isozymes indicated that more than one mechanism of receptor mediated PtdIns-PLC activation existed.

The tyrosine kinase activity of the EGF receptor (EGFR) was shown to be required for growth factor stimulation of PtdIns(4,5)P2 hydrolysis [Moolenaar et al., 1988] and the time-course of PtdIns-PLCγ1 tyrosine phosphorylation in intact cells mirrored that of EGF-induced IP3 production [Wahl et al., 1988a]. Thus it seemed likely that the tyrosine phosphorylation of PtdIns-PLCγ1 had a direct effect upon its catalytic activity. However,
several studies indicated that the catalytic activity of purified PtdIns-PLCγ1 was not altered by tyrosine phosphorylation [Kim et al., 1990]. The activation of PtdIns-PLCγ1 by tyrosine phosphorylation was successfully demonstrated using an immunoprecipitated preparation in a mixed micellar-two detergent assay [Nishibe et al., 1990]. The discrepancy between these results suggested that another protein, which co-immunoprecipitated with PtdIns-PLCγ1 might facilitate the phosphorylation-inducible activation.

An actin-binding protein, profilin, had previously been shown to bind PtdIns(4,5)P₂ and prevent its hydrolysis by PtdIns-PLC [Goldschmidt-Clermont et al., 1990]. Reconstitution of EGF regulated IP₃ production using synthetic membranes revealed that profilin only inhibited the hydrolysis of PtdIns(4,5)P₂ by unphosphorylated PtdIns-PLCγ1 [Goldschmidt-Clermont et al., 1991]. Thus an activation mechanism was proposed whereby the activated EGFR phosphorylated the PtdIns-PLCγ1, enabling it to overcome the inhibitory effect of profilin and hydrolyse its substrate. Another actin-binding protein, gelsolin, has also been implicated in the regulation of PtdIns-PLCδ and γ isoforms [Banno et al., 1992], indicating that the association of PtdIns-PLC isoforms with the cytoskeleton of the cell may be an important for the regulation of PtdIns(4,5)P₂ hydrolysis. An alternative mechanism for the inhibition of PtdIns(4,5)P₂ hydrolysis in unstimulated cells was suggested by the discovery of a PtdIns-PLC inhibitory region (PCI) adjacent to the second SH2 domain of PtdIns-PLCγ1 and PtdIns-PLCγ2 [Homma et al., 1992]. Fusion proteins containing this domain inhibited PtdIns(4,5)P₂ hydrolysis, by all 3 classes of PtdIns-PLC, non-competitively, implying a direct interaction with the catalytic X and Y domains. As phosphorylation of the fusion proteins destroyed their inhibitory properties, it was suggested that in vivo, receptor mediated phosphorylation of tyrosine residues near to this PCI might overcome the inhibitory interaction, leading to activation of the enzyme.

Three target sites for growth factor receptor-mediated tyrosine phosphorylation of PtdIns-PLCγ1 have been identified :- Y771 and Y783 in the region between the SH2 and SH3 domains and Y1254 in the carboxyterminus [Kim et al., 1990; Wahl et al., 1990]. Expression of mutant PtdIns-PLCγ1 proteins containing phenyalanine in place of these tyrosine residues [Kim et al., 1991] has revealed that Y783 is absolutely required for
PDGF stimulation of the enzyme. However, loss of Y1254 only resulted in a 40% inhibition of PDGF stimulated activation and mutation of Y771 caused a 50% increased PDGF response. All of these mutant proteins retained catalytic activity in vitro and the non-stimulatable F783 mutant was able to associate with the receptor, indicating that the mutations had only altered the ability of the enzyme to act as a substrate for phosphorylation by the PDGFR. These results demonstrated that tyrosine phosphorylation was necessary for PtdIns-PLCγ1 activation, but not for receptor association and translocation.

In addition to the phosphorylation of PtdIns-PLCγ1, EGF treatment of cells was shown to cause the translocation of the enzyme from the cytoplasm, to the cell membrane [Todderud et al., 1990]. Using PtdIns-PLCγ1 specific monoclonal antibodies, it was determined that in unstimulated cells 88% of the enzyme was located in the cytosol, whereas only 1 minute after EGF stimulation, 68% of the enzyme was membrane associated and although the total PtdIns(4,5)P2-hydrolysing activity in the cells was unchanged, it was seen to disappear from the cytosol and redistribute to the membrane fractions. Another activator of PtdIns(4,5)P2 hydrolysis, bradykinin did not stimulate either phosphorylation of PtdIns-PLCγ1, its translocation to the membrane, or a re-distribution of PtdIns-PLC activity, again indicating the existence of more than one PtdIns-PLC activation mechanism in cells. The translocation was shown to be reversible and dependent upon the occupancy of the EGFR.

The link between the tyrosine phosphorylation of PtdIns-PLCγ1 and its translocation to the membrane was made after the role of the SH2 domains in the enzyme had been determined. The tyrosine phosphorylated carboxy-terminus of the activated EGFR was found to be the binding site for PtdIns-PLCγ1 [Margolis et al., 1990]. Association of PtdIns-PLCγ1 with activated EGFR was shown to be mediated by the SH2 domains of the PtdIns-PLC [Anderson et al., 1990] and experiments using GST fusions of SH2 domains demonstrated that the domains lowered the Km for phosphorylation of PtdIns-PLCγ1 by the EGFR, thereby facilitating the activation of the effector [Rotin et al., 1992]. Site-directed mutagenesis and phosphatase protection studies identified the site of
interaction with the PtdIns-PLCy1 SH2 domains as phosphorylated Y992, in the carboxy-
terminus of the EGFR.

The role of the SH3 domain in PtdIns-PLCy1 and other signalling enzymes has not
been fully elucidated, although it is also thought to mediate protein:protein interactions,
with a putative recognition sequence rich in proline [Cicchetti et al., 1992]. It has been
proposed that this domain may facilitate binding to cytoskeletal proteins, such as actin and
in this respect may be important for the localisation of the phosphorylated PtdIns-PLC in
the cell membrane, where it can access its substrate PtdIns(4,5)P2. Many other proteins
have also been found to contain SH2 and SH3 domains, including the 85-kDa subunit of
PtdIns 3-kinase and rasGAP. The diversity of proteins with these domains and the absence
of an associated enzymatic activity implies that these regions act to confer common
recognition properties, hence their ability to act alone as recombinant domains. The
presence of multiple phosphoryrosines in the cytoplasmic tails of activated growth factor
receptors and the existence of multiple SH2 and SH3 regions in a variety of proteins could
potentially enable the formation of large protein complexes at the cell membrane, facilitating
tightly controlled interactions between many different signalling pathways.

1.3.6 Other mechanisms for PtdIns-PLC activation

Many of the known activators of PtdIns-PLC, such as thrombin and vasopressin,
do not activate PtdIns-PLCy1 through the tyrosine kinase receptors and the receptor
tyrosine kinases do not cause phosphorylation of the β and δ PtdIns-PLC isozymes,
suggesting that an alternative mechanism for PtdIns-PLC activation existed in some cells. It
was observed that many receptors activate PtdIns-PLC in a GTP dependent manner
[Litosch et al., 1985; Cockcroft and Gomperts, 1985] and that PtdIns(4,5)P2 hydrolysis
could be stimulated by the non-hydrolysable analogue, GTPγS, in the absence of agonist.
This implied that a G-protein was involved in the activation of some PtdIns-PLC mediated
pathways. It was later observed that G-protein mediated PtdIns-PLC activation was mainly
PTX insensitive, although in some cells such as HL60s [Gierschik et al., 1989], PtdIns-
PLC agonists acted through a PTX sensitive mechanism [Cockcroft 1987; Fain 1990]
This implied that more than one G-protein was responsible for PtdIns-PLC activation. Further evidence for G-protein mediated PtdIns-PLC activation has been extensively reviewed [Fain, 1990; Meldrum et al., 1991b; Cockcroft, 1992].

The search for the PTX insensitive G-protein responsible for PtdIns-PLC activation (initially named Gp) was recently rewarded by the cloning of a new G-protein family, Gq [Strathman and Simon., 1990], which included αq, α11, α14, α15 and α16. At the same time, G-proteins from this family were purified in 3 different laboratories, from bovine liver membranes [Taylor et al., 1990], bovine brain [Pang and Sternweis 1990] and turkey erythrocytes [Waldo et al., 1991]. These G-protein α subunits were demonstrated to activate PtdIns-PLCβ isozymes, but not the γ or δ isozymes [Taylor et al., 1991]. A G-protein responsible for the PTX sensitive pathways has yet to be identified, although Go is a good candidate, as expression of the αo subunit in Xenopus oocytes has been shown to stimulate PtdIns-PLC activity [Moriarty et al., 1990]. Studies of thrombin receptor mediated PtdIns-PLC activation in human platelets have led to the proposal that Gi might be responsible for PTX sensitive coupling [Banno et al., 1987; Crouch et al., 1988].

The lack of SH2 and SH3 domains in these two isozyme classes clearly correlates with an inability to be activated by the tyrosine kinase receptors. It is now apparent that the β class enzymes are regulated by the PTX insensitive Gq α subunits. The presence of an extended carboxyterminus in this class of PtdIns-PLC isozymes and the absence of such a domain in the other isozymes suggests that this region may be responsible for Gqα driven PtdIns-PLC activation. The absence of SH domains and a large carboxy terminus in the δ class isozymes implies that they are regulated through a third mechanism. This third mechanism for PtdIns-PLCδ activation may account for the PTX sensitive pathway, therefore the identification of the G-proteins involved in this pathway will be important in the study of the regulation of this class of isozymes.
1.3.7 Control of PtdIns-PLC activation

As for the adenylate cyclase pathway, there is evidence for the negative regulation of PtdIns(4,5)P$_2$ hydrolysis, although the precise mechanisms through which this control is achieved are not understood. The coupling between the receptor and PtdIns-PLC is the most obvious point for regulation. Phosphorylation of PtdIns-PLC$_{\gamma 1}$ on serine as well as tyrosine residues has been observed in EGF treated cells [Meisenhelder et al., 1989]. The catalytic activity of the enzyme as measured in vitro is not changed by this modification, however it may represent part of a regulatory mechanism in vivo. Studies on C$_6$Bu1 cells suggest that this regulation could be exerted through PKA [Kim et al., 1989], allowing cross talk between pathways. In Jurkat cells, PKC and PKA have been implicated in the phosphorylation of PtdIns-PLC$_{\gamma 1}$ S1248. This modification is accompanied by a lack of PtdIns-PLC$_{\gamma 1}$ phosphorylation on tyrosine residues, causing inhibition of its activation by the T-cell receptor [Taylor et al., 1991]. Feedback regulation of PtdIns-PLC activity by PKC has been observed in many other systems [Gallo-Payet et al., 1991; Bazan et al., 1993] acting on different targets, including the adrenergic receptor [Leeb-Lundberg et al., 1985], the EGFR [Downward et al., 1985] and PtdIns-PLC$\beta 1$ [Ryu et al., 1990]. Recently it was demonstrated that PKC feedback was mediated through specific isozymes [Ozawa et al., 1993]. PKC mediated negative feedback on IP$_3$ production is apparently involved in regulating intracellular calcium oscillations [Bird et al., 1993] although PKC can also phosphorylate the IP$_3$ receptor [Matter et al., 1993], positively regulating Ca$^{2+}$ flux into the cytoplasm. Evidence for negative regulation of PtdIns-PLC activity through a calcium/calmodulin dependent kinases has also been presented [Hedeskov et al., 1991]. Clearly the activation of PtdIns-PLC is carefully regulated and integrated with the many other intracellular signalling pathways.
The aim of the work described in this thesis was to establish complementary *in vitro* and *in vivo* systems to try to understand more fully the positive and negative regulation mechanisms of the PtdIns-PLC enzymes, in particular those of the β and δ families.
CHAPTER 2 - MATERIALS AND METHODS

2.1 Materials

Restriction enzymes were obtained from Boehringer, Northumbrian Biologicals and New England Biolabs. Vent polymerase was also obtained from New England Biolabs and Mung bean nuclease from Stratagene. T4 DNA ligase, Klenow fragment and calf intestinal phosphatase were obtained from Boehringer. Sequencing was performed using USB Sequenase kits. Oligonucleotides for PCR, sequencing and subcloning were made at the ICRF. The pET vector system was obtained from Novagen. The pTZ18R vector, the pGEX-2T vector and glutathione sepharose beads were obtained from Pharmacia, as were the pre-packed heparin-Sepharose, Mono-Q, Mono-S, Superose 6, Q-Sepharose and S-Sepharose columns and phast gel materials and apparatus. Ultra-pure agarose was obtained from Gibco. Acrylamide and bis-methyl acrylamide were from National Diagnostics and Urea from Fisons. Radiolabelling kits, Hyperfilm, the ECL Western blotting kit and rainbow markers were obtained from Amersham International. Standard SDS-PAGE markers and protein determination assay reagent were from Bio-Rad. Nitro-cellulose membrane was from Schleicher and Schuell and skimmed powdered milk was obtained from Boots Company Ltd. Ethanol, methanol and Triton X-100 were from BDH. Trypsin, soybean trypsin inhibitor and IPTG were all obtained from Worthington Chemicals. NZ-amine was obtained from ICN Biochemicals. LB medium, PBS, DMEM and trypsin-versine were all obtained from ICRF central services as were the COS-1 cells. Ultima Gold scintillant was from Packard. Sterile plasticware for tissue culture was obtained from Falcon and foetal calf serum from Gibco.

Phosphatases were generously provided by Dr.J. Goris. Purified calcium calmodulin kinase was given by Dr. J. Woodgett. PKC and MAPKinase preparations were purified from recombinant baculovirus infected SF9 cells and U937 cells respectively, by Dr.P.Parker. PtdIns-PLCβ2 was purified from bovine brain by Dr.E.Meldrum and bovine brain PtdIns-PLCβ1 and Hela cell PtdIns-PLCβ3 were purified by Dr.A.Carozzi. Purified βγt was kindly given by Dr.P.Gierschik. Anti-GST antisera was provided by
Dr. J. Downward and monoclonal PtdIns-PLCδ1 antisera by Dr. S. G. Rhee. Antisera directed against the G-protein β subunit were provided by Dr. G. Milligan. All other antisera were made for the Parker laboratory at the ICRF (unpublished).

All PtdIns-PLC cDNA clones employed here were kindly given by Dr. J. Knopf and Dr. R. Kriz; the cDNA Gαq clone was given by Dr. M. Simon; the cDNA clones for muscarinic acetylcholine receptors M1 and M2 were given by Dr. N. Buckley. The rac-PH-pGEX-2T construct was given by Dr. B. Hemmings and the PtdIns-PLCβ2-PH-pGEX construct was made by Catherine Webster. The pKS I vector was obtained from Dr. P. McIntyre and was constructed from a pUC18 backbone and CDM8 vectors [Dekker et al., 1992]. All other chemicals and biochemicals were obtained from Sigma Chemical Company or BDH.

2.2 Methods

2.2.1 Molecular Biology

Standard methods such as restriction enzyme digests, ethanol precipitation, phenol-chloroform extraction and electrophoresis (using 0.5X TBE for running buffer and gels) were carried out in accordance with the enzyme manufacturers instructions and as described by Maniatis et al., [1989].

2.2.1.1 Klenow blunt ending

The DNA to be blunt ended was incubated at 37°C for 45 minutes with 1X Klenow lbuffer, a mixture of dATP, dTTP, dGTP and dCTP each at 0.04mM and 0.2U/μl Klenow lfragment. The final volume of the incubation was varied according to the amount of DNA to be treated, (in a small reaction 500ng of DNA was treated in a 25μl reaction with 1U of lKlenow fragment).
2.2.1.2 Mung-bean nuclease blunt ending

The digested DNA was incubated at 30°C for 30 minutes with 1X Mung bean buffer (supplied with enzyme) and Mung bean nuclease at 0.2U/µl, diluted in the supplied Mung bean dilution buffer. In a standard 60µl reaction 5µg of digested DNA would be treated with 10 U of Mung-bean nuclease.

2.2.1.3 Phosphatase treating digested vector DNA prior to ligation

The digested vector DNA was treated with alkaline phosphatase to reduce the background of vector self ligation and recircularisation, during symmetrical sticky ended ligations or in double blunt-ended ligations. 50µl of digested vector containing 1µg of DNA was incubated with 0.04U of calf intestinal phosphatase (Boehringer) in 1X CAP buffer (provided) for 30 minutes at 37°C. The phosphatase treated DNA was then purified either by agarose gel electrophoresis or using phenol-chloroform extraction followed by spun column chromatography (see Maniatis).

2.2.1.4 Gel purification of treated DNA

The sample containing the DNA fragment to be purified was separated by agarose gel electrophoresis as described by Maniatis. The ethidium bromide stained bands were visualised by placing the gel on a U.V. illuminator. The band of interest was cut out of the gel and placed in dialysis tubing that had been boiled in 2mM EDTA. One end of the tubing was sealed with a clip and 500µl of 0.5X TBE were added to the tubing, before sealing the other end. The tubing was then placed in the gel electrophoresis tank and the current switched on as for a normal gel. The length of time taken to elute the band out of the gel slice varied according to the band size. Once the band had eluted from the gel, the TBE was taken from the tubing bag and the DNA precipitated using ethanol precipitation (0.3M sodium acetate) and centrifuged to collect the DNA as a pellet. The pellet obtained was washed in 70% ethanol, dried under vacuum and resuspended in water or TE.
2.2.1.5 Ligation

In a simple two fragment ligation a molar ratio of 2 insert : 1 vector was employed. As the concentrations of prepared fragments were usually too low to be measured accurately, they were estimated on the basis of the intensity of the bands seen by ethidium bromide staining of the agarose gels used to separate and purify the fragments. This estimation meant that frequently several ligation ratios had to be tested to obtain efficient ligation. The reactions were performed overnight at 4°C in 1X ligation buffer (supplied), with 1U/ml T4 DNA ligase. Generally, a 20μl ligation reaction contained a total of 500ng of DNA with 1U of T4 DNA ligase. Successfully ligated constructs were screened for by transformation of *E.coli* with the ligation reactions, picking colonies, extracting the DNA by the mini-prep method and checking the constructs by restriction enzyme analysis. The number of colonies picked was based on the ratio of colonies on the control (vector alone ligation) plate to the number of colonies growing on the full ligation plate. For example if there were 5 colonies on the control plate and 15 on the test plate, in a random selection of 12 colonies from the test plate, at least 4 of them would be expected to contain re-circularised vector. Such a selection would give a reasonable chance of picking a clone containing the desired construct. With a symmetrical or blunt-ended ligation, the chances are reduced because the insert could insert in two possible orientations.

2.2.1.6 Using oligonucleotides as a linker in a 3-way ligation

The two single-stranded linker oligonucleotides were annealed by mixing 0.1μg of each purified oligonucleotide in a volume of 20μl of 1X ligation buffer (provided with T4 DNA ligase), for 1 hour at 60°C. The linker was then ready for use in the 3-way ligation. A selection of molar ratios for oligo to vector were tested; the recommended ratios were 50 : 1 or 100 : 1, although a ratio 40 oligo : 2 insert : 1 vector produced the best ligation efficiency in the scheme described in 8.2.1.
2.2.1.7 DNA Purification

Small scale purification (mini prep) of DNA from colonies of transformed \textit{E.coli} were performed by alkaline lysis as described by Maniatis. Large scale purification (maxi prep) was again performed by alkaline lysis, followed by double banding on a CsCl gradient as described by Maniatis.

2.2.1.8 Double-stranded DNA sequencing

Double-stranded CsCl banded plasmids were prepared for sequencing by alkaline denaturation. The DNA was incubated with 0.2M NaOH and 0.2mM EDTA for 5 minutes at room temperature. The denatured DNA was then recovered using an ethanol precipitation with 0.3M sodium acetate. The dried pellet of DNA was then resuspended in water for the annealing step. The template DNA was incubated with the primer and 1X reaction buffer at 37°C for 15 minutes and the sequencing reactions performed using the method described in the kit. The sequencing reactions were separated on a 5\% acrylamide gel (1X TBE) using the BioRad gel apparatus at a constant power of 50W. To read sequence near to the primer the reactions were performed using the manganese buffer provided in the kit and the gel run was terminated when the first dye front had moved off the bottom of the gel. This generally took about 2 hours. To read sequence further away from the primer, longer gel runs of up to 5 hours were used and the manganese buffer was omitted from the reactions.

2.2.1.9 Transformation of \textit{E.coli}

For the early subcloning work the heat-shock method (described by Maniatis) was used to transform both ligations and double-banded plasmids into the different strains of \textit{iE.coli}. However the preparation of highly competent \textit{E.coli} for this method of transformation was both long and difficult, generally only yielding bacteria with a competence of $10^6$ to $10^7$ colonies per µg of DNA. Therefore the Biorad electroporator was used for all subsequent bacterial transfections. Electrocompetent cells were prepared as suggested by the manufacturer and using the method described below, various \textit{E.coli}
strains prepared in this way could easily be transformed with an efficiency of $10^8$ cells/µg of DNA. This high efficiency made it much easier to obtain clones from inefficient ligations, than with the heat-shock method of transformation.

The electrocompetent bacteria (prepared as described in the manual) were thawed at room temperature and 40µl mixed on ice with 1µl of DNA (ligation mix or pure plasmid). The mix was pipetted into a pre-cooled 0.1cm cuvette which was loaded into the pre-cooled cuvette holder and electroporated with a pulse of 1.7 kV at a capacitance of 25µF. The time constant for the pulse was generally between 4 and 5 ms. The electroporated bacteria were then mixed with 1ml of SOC medium (pre-warmed to 37°C) and incubated for 1 hour at 37°C with light shaking (225rpm). 100µl of the transformed bacterial cells were plated into 9cm LB plates (100µg/ml ampicillin) and grown at 37°C overnight.

2.2.1.10 Polymerase Chain Reaction (PCR)

PCR reactions were performed using Vent polymerase with the supplied buffer at 1X. A stock mixture of dATP, dTTP, dCTP and dGTP was made at 10mM with respect to each deoxy nucleotide phosphate (dNTP). The dNTP was diluted to a final concentration of 800µM in the PCR reactions. The purified sense and antisense primers were used at a final concentration of 0.02mg/ml and 1ng of template was incubated with 1 unit of Vent polymerase in a 50µl reaction. The same programme of 30 cycles was used for all of the PCR reactions described, each cycle including 1min pre-annealing denaturation at 94°C, followed by 1 minute annealing at 60°C and 2 mins extension at 72°C.

2.2.2 Assaying PtdIns-PLC activity

Three in vitro assays were used to measure PtdIns(4,5)P$_2$ hydrolysis.

2.2.2.1 Standard cholate assay

The standard cholate assay measures total PtdIns-PLC activity in the absence of activators, and was used in PtdIns-PLC purifications or to normalise the different isozyme
preparations for comparison in the other assays. This assay was based on that described by Katan and Parker [1987].

The sample to be assayed was incubated with 1X reaction mix and a sonicated lipid mixture containing PtdIns(4,5)P$_2$ sonicated with $[^{3}$H]$PtdIns(4,5)P_2$ to give a final concentration in the assay of 180µM, with a specific activity of approximately 2500 cpm/nmol. The incubation was performed in 50µl for 10 minutes at 37°C and stopped by vortexing with 260µl of CHCl$_3$ : CH$_3$OH : conc HCl (100 : 100 : 0.6). A standard 50µl reaction would contain 10µl of sample, 30µl of 1X reaction buffer and 10µl of lipid mix made up in 2X Reaction buffer. The lipid mix was made using 2µl of cold PtdIns(4,5)P$_2$ (1:1 chloroform : methanol) and 2µl of $[^{3}$H]$PtdIns(4,5)P_2$ (10nCi/µl), mixed dried down and sonicated in 10µl 2X Reaction mix, per assay tube. The mixture was further acidified with 75µl of 1M HCl and the phases separated by centrifugation for 8 minutes in a minifuge. The upper phase was sampled (150µl) and counted by liquid scintillation counting. 1U was defined as the amount of enzyme that would hydrolyze 1nmol of substrate/minute under the standard assay conditions (10µM calcium).

When PtdIns was used as a substrate, the calcium concentration was maintained at 1mM. For the calcium titrations, EGTA buffers were used, based upon calculations performed with the Chelate programme.

2.2.2.2 Gxα reconstitution assay

This assay was used to measure the activation of PtdIns-PLC isozymes by activated G-protein α subunits partially purified from the membranes of COS-1 cells in which they had been overexpressed. The preparation of the membranes is described in 4.2. The α subunit membrane preparations were activated by either 10µM AlF$_4^-$ or 100µM GTPγS.

The assay was adapted from that described by Wu et al., [1992a] and was performed in 60µl. The sample (5µl) was mixed on ice with 30µl reaction mix, 5µl of membranes, 5µl of inhibitor or buffer and 5µl of activator (AlF$_4^-$ or GTPγS) or water. The reaction was started by addition of the sonicated lipid mixture (containing PhosphatidylEthanolamine (PtdEtn), PhosphatidylSerine (PtdS), PtdIns(4,5)P$_2$ and
[\textsuperscript{3}H]PtdIns(4,5)P\textsubscript{2} dried down under N\textsubscript{2} and sonicated into a buffer containing 50mM Hepes (pH 7.0) and 100mM NaCl. The final lipid concentrations in the assay were 200\mu M, 200\mu M and 20\mu M respectively, with a specific activity of 20,000 cpm/nmol of PtdIns(4,5)P\textsubscript{2}. The mixture was incubated at 37\textdegree C for 30 minutes and stopped and processed as for the standard cholate assay. The activity was calculated as nmol of PtdIns(4,5)P\textsubscript{2} hydrolysed per minute and each enzyme sample (unless otherwise stated) was used at a dilution of 6 standard cholate U/ml to give 0.5 units per 60\mu l assay.

2.2.2.3 \(\beta\gamma\) reconstitution assay

This assay was performed using the method of Camps et al., [1992a]. The assay was performed in a final volume of 70\mu l, using \(\beta\gamma\) subunits at a final concentration in the assay of 1.5\mu M at a final free calcium concentration of 100nM. The lipids PtdEtn, PtdIns(4,5)P\textsubscript{2} and [\textsuperscript{3}H]PtdIns(4,5)P\textsubscript{2} were mixed, dried down and sonicated into the reaction mix to give final concentrations in the assay of 280\mu M and 28\mu M respectively at a specific activity of 12,000 cpm/nmol of PtdIns(4,5)P\textsubscript{2}. The assay was started by the addition of the lipid mixture, incubated at 25\textdegree C for 10 minutes and stopped in the same way as for the cholate assay. 175\mu l of the upper phase were sampled for scintillation counting and the activity was calculated as nmoles of PtdIns(4,5)P\textsubscript{2} hydrolysed per minute.

2.2.3 Preparation of Triton X-100 soluble supernatants - Lysozyme method

The following protocol describes the extraction of 10ml of supernatant from a growing 500ml culture of \textit{E.coli}.

The bacteria were harvested by centrifugation at 4000 x g for 5 minutes and the pellet washed in 50ml of buffer containing 20mM Tris-HCl (pH 7.5), 50mM NaCl and 10mM benzamidine. The cells were then re-suspended in 7.5ml of buffer containing 10% (w/v) sucrose, 100mM Tris-HCl (pH 7.5), 1.5mM EDTA and 10mM benzamidine. This suspension was treated with 100\mu g/ml lysozyme for 20 minutes on ice, before extraction with 2.5ml of a buffer containing 20mM Tris-HCl (pH 7.5), 2mM EDTA, 10mM benzamidine, 1.2% (v/v) \(\beta\)-mercaptoethanol, 40\mu g/ml aprotinin, 40\mu g/ml leupeptin, 1mM
PMSF and 4% (v/v) Triton X 100. The DNA was sheared by homogenisation in a 15ml glass homogeniser with dounce B for 30 strokes. The extract was then cleared by centrifugation at 43,700 g for 50 minutes.

2.2.4 Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli [1970], using Høeffer Sturdier gel apparatus. Generally, full length PtdIns-PLCδ or φ proteins were separated on gels consisting of 7% running gels with 3% stacking gels, the GST-β1COOH fusion protein was analysed on 8% running gels with 5% stacks, the GST-PH fusion proteins were visualised on 10% running gels with 6% stacks and the G-protein β subunits and COS-1 expressed PtdIns-PLCβ truncated proteins were separated on 12.5% running gels with 8% stacks. The samples were mixed 3:1 with 4X Laemmli sample buffer (250mM Tris-HCl (pH 6.8), 8% (w/v) SDS, 10% (v/v) glycerol, 100mM DTT, 0.01% (w/v) bromophenol blue) and boiled for 10 minutes. Standard molecular weight markers or rainbow markers were run alongside the samples to enable molecular weight determination. The proteins were visualised by Coomassie blue or silver staining [Oakley et al., 1980]. In many cases the loadings of samples were normalised on the basis of their protein concentration determined by the method of Bradford [1976], with up to 100μg of total protein being loaded per lane of a 10 well gel.

2.2.5 Western Blotting

Non-radioactive proteins were transferred to nitrocellulose membrane using the Høeffer semi-dry blotting apparatus. For full size 11x14 cm gels this was achieved at 250V for 1 hour at 4°C, with membrane and 3MM paper soaked in Towbin [Towbin et al., 1979] buffer containing SDS (0.04%). When transferring large proteins such as the full length PtdIns-PLCβ isozymes of 150-kDa the transfer was continued for an extra 30 minutes to ensure full transfer.
The transferred proteins were visualised by staining with 2% ponceau S in 5% acetic acid, allowing the standard markers to be visualised. The membranes were subsequently de-stained in PBSA and incubated in blocking solution (PBS containing 0.1% Triton X 100 and 10% (w/v) powdered milk) for 1 hour with shaking. The blots were then sealed in a bag containing 10ml of the blocking solution and the relevant antisera and incubated on a wheel for up to 16 hours at 4°C. All the antisera used, apart from the Pantopic PtdIns-PLC antisera, were found to be effective enough to give a good reaction after only 2 hours incubation with the blots on a wheel at room temperature.

After incubation with the primary antisera, the blots were washed once in PBS containing 0.1% Triton X 100 for 15 minutes, once in PBS containing 0.1% Triton X 100 and 0.5M NaCl for 15 minutes and finally in the PBS containing 0.1% Triton X 100 for 15 minutes. The washed blots were then incubated in a bag with 10ml of PBS containing 0.1% Triton X 100 and 5% (w/v) powdered milk, and a 1/5000 dilution of a donkey antirabbit IgG coupled to horseradish peroxidase (or in the case of the mouse monoclonal PtdIns-PLC antiserum blots, a donkey anti-mouse IgG coupled to horseradish peroxidase) for 1 hour. The blots were washed as before and developed according to the manufacturers instructions, before exposing to film for approximately 1 minute.

2.2.6 Standard purification of GST fusion proteins

A 500ml culture of E.coli transformed with the PGEX-2T construct was set up at an A600 of approximately 0.05 to grow in LB medium (ampicillin at 100μg/ml) at 37°C. The bacteria were grown to an A600 of between 0.6 and 1.0 before induction with 0.5mM IPTG for 1-2 hours. The culture was harvested and 10ml of supernatant were prepared from it as in 2.2.3. This supernatant containing the GST fusion protein was mixed with 1ml of glutathione-Sepharose beads which had been washed twice in PBSA containing 0.02% Triton X 100 and resuspended in a 1:1 slurry in the washing buffer. This mixture was incubated at 4°C for 2-16 hours, after which time the beads were pelleted by low speed centrifugation (500 x g) for 2 minutes. The supernatant was removed and the beads washed twice in PBSA-0.02% Triton X 100. 2ml of elution buffer I were mixed with the
beads and incubated by tumbling on a wheel at 4°C for 1 hour. The supernatant was then collected, diluted in buffer containing 20mM Tris-HCl (pH 7.5), 0.02% Triton X 100, 0.3% β-mercaptoethanol, 10mM benzamidine, 10μg/ml aprotinin and 10μg/ml leupeptin and concentrated using the Amicon centricron-30 concentrators.

2.2.7 Preparation of trypsinised PKC

The PKC αβγ mix and PKC ε used in the phosphorylation experiments outlined in 3.5.1 and 6 were trypsinised to avoid having to use TPA and PtdSer as activators in the phosphorylation reactions.

2.2.7.1 Trypsinisation of PKC

As the proteolytic activation of the PKC preparations to be used had previously been characterised it was not necessary to do a time course of trypsin treatment. The reaction was performed at 30°C for 3 minutes. The PKC (100-1000 U) was incubated with 4μg/ml of trypsin in a 40μl reaction containing 10mM Tris (pH 8.0), 0.5μg/ml BSA and 20mM β-mercaptoethanol. The reaction was stopped with 20μl of 400μg/ml trypsin inhibitor and a sample assayed as described below. The remainder of the activated PKC was stored in small aliquots at -20°C for up to 3 days.

2.2.7.2 PKM activity assay

Trypsinised PKC was incubated at 30°C for 5 minutes with 5mg/ml Histone as the substrate. The final concentration of MgCl₂ was 12.5mM, with [³²P] labelled ATP at 0.125mM and a specific activity of approximately 200cpm/pmole. The reaction was carried out in a volume of 40μl and stopped by removing 25μl, spotting it onto P81 paper and washing twice for 10 minutes in 30% acetic acid. The papers were then counted by scintillation counting and the activity calculated as nmoles of phosphate incorporated per minute.
2.2.8 Phosphorylation of PtdIns-PLC preparations

In the standard phosphorylation reaction with non trypsinised PKC a mixture of PtdSer and TPA was used to activate the PKC preparations. The lipid mixture was made by drying down TPA and PtdSer under N\textsubscript{2} and sonicating the lipid film into 20mM Hepes (pH 7.0). The final concentrations of TPA and PtdSer in the 40\mu l reactions were 1\mu g/ml and 1mg/ml respectively. As for the PKC activity assay, the MgCl\textsubscript{2} was at a final concentration of 12.5mM and Ca\textsuperscript{2+} was at 750\mu M in the assay. The final ATP concentration was 20\mu M, with a variable specific activity (see individual experiments). The MAPKinase and trypsinised PKC phosphorylations were performed in the same assay, but without the lipid mixture. Unless otherwise stated, the reactions were incubated at 30\textdegree C for 5 minutes and stopped by addition of 4x Laemmli sample buffer or quenched with a stopping buffer (see individual experiments). If the samples were separated by SDS-PAGE for autoradiography, the resultant gel was dried down and exposed to film at -70\textdegree C using a double intensifying screen.

2.2.9 Phosphatase treatment of PtdIns-PLC preparations

The PtdIns-PLC preparations and phosphatases were diluted to the desired concentrations in buffer K. Unless otherwise stated, 10\mu l of each phosphatase (5U/ml) were mixed with 10\mu l of the PtdIns-PLC (500U/ml) and incubated for 20 minutes at 30\textdegree C. The reaction was stopped by the addition of 9 volumes of dilution buffer K containing microcystin to give a final concentration of 10\mu M (more than enough to inhibit the 0.05U of phosphatase in each reaction). This phosphatase treated PtdIns-PLC was then directly assayed in the standard cholate assay.

2.2.10 Growing and transfecting COS-1 cells

COS-1 cells were routinely grown in a growth medium of DMEM supplemented with 10% (v/v) foetal calf serum and penicillin streptomycin (50\mu g/ml) at 37\textdegree C in a humidified atmosphere of air/CO\textsubscript{2} (9:1). The cells were subbed every 2-3 days when they
reached 80-100% confluence, by removal of the old medium, treatment with 0.25% trypsin-versine for 5 minutes, resuspension in fresh growth medium and centrifugation for 4 minutes at 1000 rpm. The pellet was then resuspended in more fresh growth medium to allow the cells to be plated at 1/4 of the density they had been growing at. To obtain cells at approximately 70% confluence, for electroporation, they were counted using a haemocytometer, plated at a density of 3x10⁶ cells per ml and grown for 24 hours. Generally, for one electroporation two 150mm dishes containing 20ml of medium were required.

2.2.10.1 Electroporation of COS-1 cells

Plates of actively growing COS-1 cells at approximately 70% confluence were treated with trypsin, resuspended in fresh growth medium and centrifuged as normal. The cells were then washed 3 times in PBS and counted using a haemocytometer. The cells were resuspended in PBS at a density of 6.25 x 10⁶ cells/ml ready for the electroporation. Under sterile conditions the DNA to be transfected into the cells was aliquoted into the pre-cooled cuvettes. 800μl of the cell suspension was mixed in each cuvette with the plasmid (40μg of plasmid unless otherwise stated) before placing the cuvette on ice for 10 minutes. The cooled cuvettes were individually loaded into the pre-cooled cuvette holder at room temperature and electroporated with a pulse of 450 volts at a capacitance of 250μF. A time constant of between 7 and 8 ms was usually recorded for the pulse. The cuvette was then placed back on ice for 10 minutes, before plating the entire contents of the cuvette onto a 150mm dish containing 20ml of fresh growth medium. After approximately 4 hours incubation at 37°C the living cells had adhered to the dish, therefore the growth medium was replaced to remove the mass of floating dead cells and debris. Depending on the promoter directing the expression of the transfected construct these cells were grown for 48 to 72 hours under normal conditions.
2.2.11 Reconstructing the M1/M2 pathways in COS-1 cells and measuring PtdIns-PLC activity

The COS-1 cells were transfected with different combinations of plasmids by electroporation. After resting on ice for 10 minutes post electroporation, the contents of each cuvette were re-suspended in 2ml of standard growth medium (see 2.2.10). A 24-well plate was prepared to contain 500μl of growth medium per well. From each electroporation, 6 wells were seeded each with 200μl of the cell suspension. The plates were incubated as normal for 4 hours and the growth medium replaced with fresh medium. The cells were then grown for a further 40 hours, after which time the growth medium was carefully removed and 300μl of the [3H] labelled inositol medium added to each well. Great care had to be taken not to disturb the cells in the well as they were not very tightly attached. The cells were then grown for a further 16 hours before stimulation and harvesting. To stimulate, half of the wells were treated with 20μl of a LiCl chloride solution to give a final concentration of 20mM in the wells. The other samples were stimulated with 20μl of a solution containing LiCl and carbachol, to give final concentrations in the wells of 20mM and 10μM respectively. The cells were incubated for 25 minutes at 37°C before harvesting. The medium was then removed and 300μl of ice-cold 3% perchloric acid were added to each well. After 30 minutes at 4°C the solution in each well was transferred to an eppendorf tube and neutralised with a buffer containing 1M KOH, 50mM Hepes and 0.005% (w/v) phenol red. Approximately 145μl of this solution was enough to cause a colour change from yellow to a pinky red colour, indicating neutrality. The samples were briefly (1 minute) centrifuged in a minifuge to bring down the sediment that had formed. The supernatant from each sample was diluted into 5ml of water and loaded onto an individual pre-prepared 0.5ml Dowex column. The columns were prepared using 1x8, 200-400 mesh formate form columns from BioRad and 1ml of a 50%(w/v) slurry of dowex in water. After the applied samples had drained through the columns, they were washed with 5ml of water followed by 5ml of 1X Borax solution. The columns were then placed over 20ml scintillation vials and the bound inositol phosphates...
were eluted with 3ml of 1M formate elution buffer. Each eluate was mixed with 10ml of liquid scintillant and counted for 10 minutes to determine the $^3$H content. The remaining 'fixed' cells in the plates were treated with 200μl of 0.2M NaOH per well for 5 minutes and 20μl of this solution was counted by scintillation counting for 1 minute. The PtdIns-PLC activity was represented as cpm of inositol phosphates released as a % of the total cpm retained in the cells after 16 hours labelling; this 'normalisation' accounted for cell losses after plating out, as variable numbers of cells died as a result of the electroporation and during the final 16 hours in the medium lacking inositol.

2.2.12 Cell fractionation

COS-1 cells transfected with the pKS I constructs were grown for 72 hours on 90mm plates with two medium changes. The plates were placed on ice and washed in buffer containing 20mM Tris-HCl (pH 7.5), 1mM EGTA and 0.1mM DTT. Each plate was then treated with 0.5ml of the above buffer containing 20mM NaF, 1mM PMSF, 10μg/ml leupeptin and 10μg/ml aprotinin - denoted homogenisation buffer. The cells from 3 plates were scraped into a 15ml homogeniser and homogenised with dounce B for 30 strokes. The homogenate was centrifuged at 90,000rpm for 10 minutes in a Beckman TL 100 centrifuge at 4°C. The supernatant was mixed with Laemmli sample buffer and boiled ready for protein determination and SDS-PAGE. The pellet was mixed with 1ml of the homogenisation buffer containing 1% Triton X 100 and tumbled on a wheel at 4°C for 20 minutes. The mixture was centrifuged as before and the supernatant mixed with Laemmli sample buffer in the same way as the previous supernatant. The pellet was mixed with 500μl of Laemmli sample buffer, sonicated briefly and boiled. The protein content of each sample was determined by the method of Bradford [1976] to enable equal amounts of total protein to be loaded into each lane of the polyacrylamide gel, for comparison between lanes.
### 2.3 Commonly used buffers

**Buffer A**

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>20mM</td>
<td>Tris-HCl (pH 7.5)</td>
</tr>
<tr>
<td>2mM</td>
<td>EDTA</td>
</tr>
<tr>
<td>0.02% (w/v)</td>
<td>Triton X 100</td>
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<tr>
<td>0.3% (v/v)</td>
<td>β-mercaptoethanol</td>
</tr>
<tr>
<td>10mM</td>
<td>Benzamidine</td>
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</table>

**Buffer B**

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<td>Hepes (pH 7.0)</td>
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<tr>
<td>2mM</td>
<td>EDTA</td>
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<tr>
<td>0.02% (w/v)</td>
<td>Triton X 100</td>
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<tr>
<td>0.3% (v/v)</td>
<td>β-mercaptoethanol</td>
</tr>
<tr>
<td>10mM</td>
<td>Benzamidine</td>
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**Buffer C**

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<th>Component</th>
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</thead>
<tbody>
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<td>EGTA</td>
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<tr>
<td>0.1mM</td>
<td>DTT</td>
</tr>
<tr>
<td>20mM</td>
<td>NaF</td>
</tr>
<tr>
<td>10μM</td>
<td>microcystin</td>
</tr>
<tr>
<td>50μg/ml</td>
<td>PMSF</td>
</tr>
<tr>
<td>100μg/ml</td>
<td>Leupeptin</td>
</tr>
<tr>
<td>10mM</td>
<td>Benzamidine</td>
</tr>
</tbody>
</table>
Buffer D
20mM Tris-Hcl (pH 7.5)
1mM EGTA
0.1mM DTT
10mM Benzamidine
20mM NaF

Buffer E
50mM MES (pH 5.5)
2mM EDTA
10mM Benzamidine
0.3% (v/v) β-mercaptoethanol
0.05% (w/v) Triton X 100

Buffer F
50mM Hepes (pH 7.0)
0.2mM EGTA
0.01%(w/v) Soybean trypsin inhibitor
1mM PMSF
40µg/ml Leupeptin
40µg/ml Aprotinin
1mM DTT

Buffer G
1:1 mixture of 2M KCl : Buffer F
Buffer H

0.5% Nonidet P-40
20mM Tris-HCl (pH 8.0)
100mM NaCl
1mM EDTA

Buffer I

100mM Tris-HCl (pH 8.0)
120mM NaCl

Kept as a stock and made up with reduced glutathione to 20mM just before to use

Buffer J

20mM Tris-HCl (pH 7.5)
0.02% (w/v) Triton X 100
0.3% (v/v) β-mercaptoethanol
40μg/ml Leupeptin
40μg/ml Aprotinin
1mM PMSF

Buffer K

20mM Tris-HCl (pH 7.5)
100μM DTT
100μg/ml BSA

TBE 5X

5.4% (w/v) Tris-base
2.75% (w/v) Boric acid
10mM EDTA (pH 8.0)
TE (pH 8.0)
10mM  Tris-HCl (pH 8.0)
1mM    EDTA (pH 8.0)

Cholate Assay 2X Reaction Mix
40mM  Tris-maleate (pH 7.0)
200mM NaCl
0.8mg/ml BSA
1/5    10μM free Calcium buffer
1.2% (w/v) Sodium cholate
0.7% (v/v) β-mercaptoethanol

10μM free Calcium buffer
21mM  CaCl2
25mM  HEDTA

Gαα reconstitution assay mix
50mM  Hepes (pH 7.0)
100mM KCl
6mM   MgCl2
0.6mM CaCl2
2mM   EGTA

10μM AlF4-
10mM  NaF
10μM  AlCl3
**βγ reconstitution assay mix**

<table>
<thead>
<tr>
<th>Concentration (mM)</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Tris-maleate (pH 7.5)</td>
</tr>
<tr>
<td>80</td>
<td>KCl</td>
</tr>
<tr>
<td>10</td>
<td>β-glycerophosphate</td>
</tr>
<tr>
<td>10</td>
<td>LiCl</td>
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<tr>
<td>0.04% (w/v)</td>
<td>Sodium deoxycholate</td>
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<tr>
<td>10</td>
<td>EGTA</td>
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**βγ reconstitution assay calcium buffer**

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<tr>
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<td>CaCl₂</td>
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**1X Borax solution**

<table>
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<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>Sodium borate</td>
</tr>
<tr>
<td>6</td>
<td>Sodium formate</td>
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</table>

**1X Formate elution buffer (1M)**

<table>
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<th>Concentration (M)</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ammonium formate</td>
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<tr>
<td>0.43% (v/v)</td>
<td>Formic acid</td>
</tr>
</tbody>
</table>

**Luria-Bertani medium**

<table>
<thead>
<tr>
<th>Concentration (w/v)</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>1%</td>
<td>bacto-tryptone</td>
</tr>
<tr>
<td>0.5%</td>
<td>bacto-yeast extract</td>
</tr>
<tr>
<td>1%</td>
<td>NaCl</td>
</tr>
</tbody>
</table>

**M9ZB medium**

<table>
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<th>Concentration (w/v)</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>1%</td>
<td>NZ-amine</td>
</tr>
<tr>
<td>0.5%</td>
<td>NaCl</td>
</tr>
<tr>
<td>0.4%</td>
<td>glucose</td>
</tr>
<tr>
<td>1X</td>
<td>M9 salts</td>
</tr>
</tbody>
</table>
**M9 salts 5X**

6.4% (w/v) \( \text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O} \)

1.5% (w/v) \( \text{KH}_2\text{PO}_4 \)

0.25% (w/v) \( \text{NaCl} \)

0.5% (w/v) \( \text{NH}_4\text{Cl} \)

**Standard COS-1 cell growth medium**

Dulbecco's MEM (DMEM)

10% foetal calf serum

50µg/ml penicillin

50µg/ml streptomycin

**[3H] inositol labelled medium**

DMEM - inositol

10% dialysed foetal calf serum

xµg/ml penicillin

xµg/ml streptomycin

10µCi/ml [3H] myo-inositol

**1X Towbin semi-dry transfer buffer**

200mM glycine

0.04% (w/v) SDS

25mM Tris-HCl (pH 8.3)

20% (v/v) methanol
CHAPTER 3 - ESTABLISHING AN EXPRESSION SYSTEM

Introduction

To study the regulation mechanisms of the PtdIns-PLC enzymes in vitro it was necessary to find a convenient source of pure, full length PtdIns-PLC isozymes. The ability to produce their component sub-domains, with scope for generating mutated and chimeric proteins was also a requirement. Bacterial expression was initially chosen because of the apparent speed and simplicity of this approach in comparison with the insect cell baculovirus system in use in the laboratory. Bacterial expression was also advantageous because there are no endogenous PtdIns-PLC enzymes which act on PtdIns(4,5)P2 in E.coli, making purification of individual isozymes much easier than from eukaryotic expression systems such as baculo-virus or COS-1 cells, where several different isozymes are endogenously expressed. Portions of PtdIns-PLCγ isozymes had been produced in this manner previously [Emori et al., 1988] for the purpose of raising antibodies against those proteins.

One concern with using bacterial expression to produce PtdIns-PLC isozymes was that the resultant proteins would not have been post-translationally modified. It was uncertain whether these proteins would be catalytically active in the absence of modifications such as phosphorylation - Cazaubon et al., [1990] had expressed PKC α in E.coli and found it to be inactive in the absence of phosphorylation. However, Emori et al., [1989] successfully expressed full-length active PtdIns-PLCγ2 in their bacterial expression system, indicating that bacterial expression could be used to produce active PtdIns-PLC isozymes. Even if phosphorylation turned out to be necessary for PtdIns-PLC activity in some isozymes, production of such unmodified proteins would be very useful for studying the effects of such modifications.

PtdIns-PLCδ1 was chosen for the initial expression studies because the δ class isozymes have the lowest molecular weights of all the PtdIns-PLC enzymes. This is an important factor when expressing proteins in bacteria, as larger proteins are less likely to be expressed in a soluble form, making their extraction and purification difficult. As there was
no known mechanism of regulation for this class of PtdIns-PLC enzymes, it was hoped that expression of this isozyme in large quantities would facilitate a screen for its regulators.

Results and Discussion

3.1 Sub-cloning PtdIns-PLCδ1 into pTZ18R

A 2.5 kb Klenow-blunted Xba I fragment (see 2.2.1.1) of the human cDNA clone of PtdIns-PLCδ1 was inserted into the Xba I site of the plasmid pTZ18R using a blunt-ended ligation (see 2.2.1.3 - 2.2.1.5) to create pTZ18R-PLCδ1. Dideoxy-sequencing of this new clone confirmed that it contained an open reading frame for a β-galactosidase-PtdIns-PLCδ1 fusion protein. This consisted of 22 amino acids of β-galactosidase fused to the entire PtdIns-PLCδ1 sequence, minus the first methionine residue, under the control of the lac z promoter.

3.2 Expression and extraction of PtdIns-PLCδ1 fusion protein

The TG-1 strain of E.coli was transformed with pTZ18R-PLCδ1 and transformants grown at 37°C overnight in Luria - Bertani medium and ampicillin (100µg/ml). The culture was harvested (as described in 2.2.3) and both Triton-soluble and insoluble fractions were resuspended in Laemmli sample buffer containing 8% SDS. These samples were separated on an 8% polyacrylamide gel, which was subsequently stained with Coomassie blue (fig 3.2). A protein of 88-kDa, absent from untransformed TG-1 extracts, was clearly visible in the Triton-insoluble fraction of the pTZ18R-PLCδ1 transformed TG-1 extracts.
FIGURE 3.2 Expression of the PtdIns-PLCδ₁-lac z fusion protein.

TG-1 bacteria were transformed with the pTZ18R-PLCδ₁ clone (1 and 2) or pTZ18R alone (3 and 4). The transformed bacteria were grown in a 2ml culture overnight and harvested using the lysozyme procedure (2.2.3). The soluble (1 and 3) and particulate (2 and 4) fractions from each extract were separated by SDS-PAGE on an 8% polyacrylamide gel and the proteins visualised by coomassie staining. The molecular weight markers on the left represent (from top to bottom), 200, 116, 97, 66 and 45 kDa. The arrow indicates the overexpressed 88-kDa β-galactosidase-PtdIns-PLCδ₁ fusion protein in lane 2.
To improve the yield of Triton-soluble fusion protein, several growth conditions were tested. It became clear that, despite being controlled by the lac z promoter of pTZ18R, the expression of the β-galactosidase-PtdIns-PLCδ1 fusion protein did not require the addition of IPTG. Cultures of pTZ18R-PLCδ1 transformed TG-1 bacteria were grown to different optical densities and the yield of Triton-soluble fusion protein assessed by Western blotting (see 2.2.5). The best yield was obtained when cells were grown to an A600 of 1.0 before harvesting. As the majority of the fusion protein produced was still Triton-insoluble, pTZ18R-PLCδ1 was transformed into several different strains of E.coli. When the HB101 strain was used, almost 50% of the expressed fusion protein was Triton X-100-soluble, compared with less than 10% in the TG-1 strain (fig 3.2.1). Using the PtdIns-PLC assay (2.2.2.1), activity was detected in the Triton-soluble extracts of HB101 bacteria which had been transformed with pTZ18R-PLCδ1 and grown to an A600 of 1.0.

3.3 Purification of the β-galactosidase-PtdIns-PLCδ1 fusion protein

The expression and extraction procedure was scaled up for preparation of purified fusion protein. Transformants from a 9cm plate were used to seed a 500ml culture, which was grown to an A600 of 1.0. The bacteria were harvested and extracted (see 2.2.3) to produce 10ml of cleared Triton soluble lysate.

3.3.1 8ml Mono Q

The above extract was loaded onto an 8ml Mono Q 10/10 column which had previously been equilibrated in buffer A at a flow rate of 0.3 ml/min. The chosen flow rate was low to avoid the generation of a back-pressure, which can be a problem with viscous bacterial extracts. Recently Pharmacia have produced 'disposable' 1 and 5ml Q-Sepharose pre-packed columns, which avoid many of the problems experienced in cleaning the 10/10 columns, after use with bacterial extracts. They also allow viscous extracts to be loaded at a higher flow rate, although some resolution may be lost.
FIGURE 3.2.1 Extraction of Triton X 100-soluble fusion protein.

HB101 bacteria were transformed with PTZ18R-PLCδ1 and grown to an A₆₀₀ of 1.0 and extracts were prepared in 1% Triton X-100 to yield soluble (S) and particulate (P) fractions (see 2.2.3). These were separated by SDS-PAGE on an 8% polyacrylamide gel and the proteins transferred to nitrocellulose. PtdIns-PLCδ1 was visualised with a polyclonal antiserum raised against a unique carboxyterminal epitope. The specific immunoreactive protein is indicated by the arrow. Molecular mass markers (kDa) are shown on the left. The same number of cell equivalents were loaded on the two lanes; the poor resolution of the particulate fraction appears to be due to the high DNA content.
The loaded column was washed with 20ml of buffer A and the protein eluted using a linear 0-1.0M NaCl gradient in buffer A (24ml). Fractions of 0.4 ml were collected and assayed for PtdIns-PLC activity (fig 3.3). The activity eluted as a broad peak and fractions 30-40 were pooled and dialysed into buffer A in preparation for the next step in the purification.

3.3.2 1ml heparin-Sepharose

The active pool from the Mono Q column was loaded onto a pre-packed 1ml heparin-Sepharose column (pre-equilibrated in buffer A) at a flow rate of 0.5ml/min. The loaded column was washed with 10ml of buffer A and the protein eluted with a 0-1.0M NaCl gradient (10ml), collecting 0.5ml fractions (fig 3.3.1). Much of the contaminating protein did not bind to the column (fractions 0-6) and the majority of the protein that did bind was eluted by 200-400mM NaCl, which preceded the elution of PtdIns-PLC activity at 400-800mM NaCl. The leading shoulder on the activity peak (fractions 21-25) was present to varying degrees in different preparations and may represent a degraded fragment of the fusion protein. Fractions 25 and 26 were pooled and dialysed into buffer B before loading onto the final column.

3.3.3 1ml Mono Q

The active pool from the heparin-Sepharose column was loaded onto a 1ml Mono Q 5/5 column (pre-equilibrated in buffer B) at 0.4ml/min. The loaded column was washed with 8ml of buffer B and a 0-1.0M NaCl gradient was applied (10ml) (fig 3.3.2). The major peak of activity eluted at 0.3M NaCl. Fractions through this major peak were separated on an 8% polyacrylamide gel and the proteins visualised by silver staining (fig 3.3.3). A polypeptide of 88-kDa was visible in the fractions of peak activity (14 and 15).
FIGURE 3.3 8ml Mono Q.

6ml of Triton X 100-soluble extract were separated on an 8ml Mono Q column using fplc. Much of the protein does not bind, appearing in the flow-through (not shown). The bound protein (filled circles) elutes on the salt gradient (-) either side of the two peaks of PtdIns-PLC activity (open boxes) at about 550mM NaCl.
FIGURE 3.3.1 1ml Heparin-Sepharose.

Activity from the first Mono Q column was pooled, dialysed and re-chromatographed on a 1ml heparin-Sepharose column. The bulk of the protein (filled circles) did not bind to the column matrix, appearing in the flow through (fractions 1-5). The PtdIns-PLC activity (open boxes) eluted on the salt gradient (-) at ~600mM NaCl, well separated from much of the bound protein.
FIGURE 3.2.2 1ml Mono Q.

Pooled activity from the heparin-Sepharose column was dialysed and loaded onto a Mono Q (5/5) column. Activity (open boxes) eluted as major and minor peaks on a linear salt gradient from 0 to 800 mM NaCl (-). Protein (filled circles) is also shown.
FIGURE 3.3.3 Silver Stain of 1ml Mono Q profile.

Fractions through the major activity peak from the second Mono Q separation were analysed by SDS-PAGE (8% acrylamide). A polypeptide (arrow) of 88 kDa was coincident with this activity peak. The molecular weight markers shown are in kDa.
The two fractions containing peak activity were pooled and a sample of the pool analysed by Western blotting, after separation on an 8% polyacrylamide gel. Probing the nitrocellulose blot with polyclonal antisera specific to a C-terminal epitope of PtdIns-PLCβ₁, in the absence or presence of competing peptide (fig 3.3.4) confirmed that this purified protein of 88-kDa was the PtdIns-PLCβ₁ fusion protein.

The data from the purification are shown in the table below:

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Protein (mg)</th>
<th>Activity (Units)</th>
<th>Specific Activity (U/mg)</th>
<th>Purification (fold)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.0</td>
<td>72</td>
<td>24,000</td>
<td>330</td>
<td>1.0</td>
<td>100</td>
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<tr>
<td>2</td>
<td>6.6</td>
<td>26</td>
<td>13,000</td>
<td>500</td>
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<td>54</td>
</tr>
<tr>
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<td>8,500</td>
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<tr>
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<td>600</td>
<td>200,000</td>
<td>606</td>
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</tbody>
</table>

The steps were Extraction (1), 8ml Mono Q 10/10 (2), 1ml Heparin-Sepharose (3) and 1ml Mono Q 5/5 (4). Units of activity (U) are nmol/min. The protein concentration of the purified protein in step 4 was determined by amino acid analysis (in all other samples it was determined by the method of Bradford [1976]).

These 3 chromatographic steps achieved a 600 fold purification, yielding a highly purified preparation of PtdIns-PLCβ₁ with a specific activity of 200 μmole/min/mg under standard assay conditions. There were large losses during the purification after each column step, largely due to the selection of peak fractions only for subsequent purification. If all fractions containing PtdIns-PLC activity had been used at each stage, the final preparation would have been less pure.
FIGURE 3.3.4 Immunorectivity of the purified protein.

The fractions containing the purified protein were pooled and samples separated by SDS-PAGE on an 8% polyacrylamide gel. The proteins were transferred to nitrocellulose and probed with the polyclonal antisera raised against the PtdIns-PLCδ1 carboxy-terminal epitope, in the absence (-) or presence (+) of competing peptide. The arrow indicates an 88-kDa immunoreactive band which is competed out by the peptide.
3.4 Characterisation

Having successfully expressed PtdIns-PLCδ1 as an active fusion protein in E.coli, extracted it in 1% Triton X 100 and purified it using 3 chromatographic steps, it was essential to characterise the fusion protein to assess the validity of using such a bacterial expression system to study the mechanisms of PtdIns-PLC regulation in eukaryotic cells.

3.4.1 Storage conditions

Activity could be maintained for several months by storing the final preparation at -80°C 1:1 in glycerol or at -20°C 1:1 in ethanediol.

3.4.2 Substrate specificity

The PtdIns-PLCδ1 preparation was assayed for its ability to hydrolyse varying concentrations of PtdIns, PtdIns4P or PtdIns(4,5)P₂ at calcium concentrations of 1mM, 10μM and 10μM respectively. The results from each titration are shown in figs 3.4, 3.4.1 and 3.4.2. The apparent Km and Vmax values were determined from simple linear regression analyses of the reciprocal plots of 1/activity against 1/ substrate concentration and are shown in the table below:

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Apparent Km (μM)</th>
<th>Vmax (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PtdIns</td>
<td>130</td>
<td>140</td>
</tr>
<tr>
<td>PtdIns4P</td>
<td>310</td>
<td>680</td>
</tr>
<tr>
<td>PtdIns(4,5)P₂</td>
<td>170</td>
<td>2200</td>
</tr>
</tbody>
</table>

The apparent Km values fall within the expected range when compared with values previously quoted in the literature for purified δ enzymes, e.g. for PtdIns, PtdIns4P and PtdIns(4,5)P₂ respectively, Km values of 143μM, 31μM, 31μM [Fukui et al., 1988] and 350μM, >500μM and 250μM [Rebecchi and Rosen, 1987] have been reported.
FIGURE 3.4 PtdIns(4,5)P$_2$ Titration.

Graph a shows purified recombinant PtdIns-PLC$_{\delta1}$ assayed under standard conditions (see 2.2.2.1), but with varying concentrations of PtdIns(4,5)P$_2$. The activity (filled circles) approached saturation at ~1mM substrate. Graph b shows the reciprocal plot used to determine the $K_m$ and $V_{max}$ values.
FIGURE 3.4.1 PtdIns4P Titration.

Graph a shows purified recombinant PtdIns-PLCδ₁ assayed under standard conditions (see 2.2.2.1), but with varying concentrations of PtdIns-4P. The activity (filled circles) approached saturation at ~1mM substrate. Graph b shows the reciprocal plot used to determine the Kᵣ and Vₑᵣ values.
FIGURE 3.4.2 PtdIns Titration.

Graph a shows purified recombinant PtdIns-PLCδ₁ assayed under standard conditions (see 2.2.2.1), but at 1mM Ca²⁺, with varying concentrations of PtdIns. The activity (filled circles) approached saturation at ~200μM substrate. Graph b shows the reciprocal plot used to determine the $K_m$ and $V_{max}$ values.
The relative $V_{\text{max}}$ values for the phosphatidylinositol phosphates relative to PtdIns are 15:1 for PtdIns(4,5)P$_2$ and 5:1 for PtdIns4P. These ratios fall within the broad range observed for the δ class of mammalian enzymes (e.g. 12:1 and 5:1 for PLC-III [Homma et al., 1988] 3:1 and 14:1 [Rebecchi and Rosen, 1987]).

The wide variations in the values quoted in the literature are probably due to differences in assay conditions, especially detergent concentrations which affect the true micellar concentration of the substrate. These differences between laboratories mean that to perform a reliable comparison between two enzyme preparations it would be necessary to characterise them side by side in the same assay. However, allowing for variable experimental conditions between laboratories, the above results suggest that PtdIns-PLCδ1 expressed in *E.coli* has the same substrate specificity as its eukaryotic counterparts.

### 3.4.3 Calcium dependence

It had been reported that although the eukaryotic PtdIns-PLC enzymes were able to utilise PtdIns as a substrate, this was only possible at much higher Ca$^{2+}$ concentrations than were required when PtdIns(4,5)P$_2$ was the substrate. To determine whether this was also the case for the bacterially expressed PtdIns-PLCδ1 fusion protein, PtdIns-PLC activity was measured at varying concentrations of Ca$^{2+}$ with PtdIns, PtdIns4P or PtdIns(4,5)P$_2$ as the substrate (fig 3.4.3). Clearly PtdIns could not be hydrolysed by the bacterially expressed PtdIns-PLCδ1 unless the free Ca$^{2+}$ concentration was $\geq$ 1mM. When PtdIns(4,5)P$_2$ was used as a substrate, the bacterially expressed PtdIns-PLCδ1 displayed a biphasic Ca$^{2+}$ dependence which was significantly shifted to a higher Ca$^{2+}$ concentration if PtdIns4P was used as a substrate instead. These effects of Ca$^{2+}$ concentration are similar to those previously reported for mammalian δ enzymes [Homma et al., 1988; Fukui et al., 1988] although the difference between the Ca$^{2+}$-dependence for the three substrates appears to be accentuated with the bacterially expressed PtdIns-PLCδ1.
FIGURE 3.4.3 Calcium Dependence of PtdIns-PLCδ1.

Purified recombinant PtdIns-PLCδ1 was assayed with PtdIns-4,5P2 (filled circles), PtdIns-4P (open boxes) or PtdIns (open circles) as a substrate at varying Ca^{2+} concentrations, employing a Ca^{2+}-EGTA buffer as previously described [Katan and Parker, 1987].
3.4.4 pH dependence

The effect of pH on bacterially expressed PtdIns-PLCδ1-catalysed PtdIns(4,5)P₂ hydrolysis was determined using the standard assay for PtdIns-PLC activity, with a range of Tris/maleate buffers from pH 5.1 to pH 8.1. The pH optimum was in the neutral range (fig 3.4.4), thus agreeing with the values observed for the mammalian enzyme (between pH 5.5 and pH 7.5) [Homma et al., 1988; Ryu et al., 1987].

3.4.5 Activation by bovine serum albumin (BSA)

Previously it had been shown that bovine serum albumin had an activatory effect on PtdIns-PLCδ1 [Ryu et al., 1987]. Therefore the effect of BSA on the bacterially expressed PtdIns-PLCδ1 enzyme was tested (fig 3.4.5). The fusion protein was activated by BSA, with half-maximal activation at 0.1 mg/ml. This effect of BSA on PtdIns(4,5)P₂ hydrolysis is unique to the δ class of PtdIns-PLC isozymes.

When the bacterially expressed PtdIns-PLCδ1 was assayed under optimal conditions with BSA at 1mg/ml (in the standard assay BSA is used at a concentration of 0.4 mg/ml), the specific activity was calculated at 320 μmol/min/mg. This figure for the specific activity seemed very high when compared with the broad range of values quoted in the literature for purified mammalian δ enzymes (10-400 μmol/min/mg) [Homma et al., 1988; Fukui et al., 1988]. It was proposed that the high specific activity of the bacterially expressed PtdIns-PLCδ1 might be due to the absence of post-translational modifications, such as phosphorylation, in proteins expressed in E.coli.
FIGURE 3.4.4 pH Dependence of PtdIns-PLCδ₁.

Activity of the bacterial PtdIns-PLCδ₁ towards PtdIns-4,5P₂ was determined at a range of pH values employing Tris-maleate buffers under otherwise standard reaction conditions [Katan and Parker, 1987].
With PtdIns-4,5P2 as substrate and under standard assay conditions (see 2.2.2.1) purified PtdIns-PLCδ1 activity was determined at increasing concentrations of bovine serum albumin (BSA). At optimal concentrations of bovine serum albumin, activity is increased ~2-fold. It should be noted that in the standard assay 400 μg/ml BSA is normally present.
3.5 Investigating the effect of phosphorylation on PtdIns-PLCδ1 activity

It had been reported [Ryu et al., 1990] that PtdIns-PLC isozymes δ₁ β₁ and γ₁ were all basally phosphorylated on serine and threonine residues when immunoprecipitated from [³²P] phosphate-labelled cells. It therefore seemed likely that unmodified bacterially expressed enzymes might behave differently in some way from their phosphorylated mammalian counterparts. To assess whether phosphorylation of PtdIns-PLCδ₁ could change its specific activity, two approaches were adopted, testing possible feedback regulators for their ability to phosphorylate the bacterially expressed PtdIns-PLCδ₁ and measuring the effects of dephosphorylation on fully modified eukaryotic PtdIns-PLCδ₁.

3.5.1 Screen for a kinase that phosphorylates PtdIns-PLCδ₁

Several serine-threonine protein kinases were tested for their ability to phosphorylate the 'under-phosphorylated' PtdIns-PLCδ₁ fusion protein in vitro. Protein kinase C was an obvious candidate because it is activated down-stream of the PtdIns-PLC enzymes by DAG and is therefore a potential feedback regulator. Two different PKC classes were tested; a preparation of trypsinised (see 2.2.7.1) PKC αβγ representing the cPKC class and a preparation of trypsinised PKC ε, representing the calcium independent (nPKC) class. Due to the presence of a putative 'EF hand [Bairoch and Cox, 1990] in the PtdIns-PLCδ₁ sequence and its increased sensitivity to calcium concentration, compared with the other PtdIns-PLC isotypes, calcium calmodulin kinase was tested as a potential regulator.

1.26 pmoles PtdIns-PLCδ₁ were incubated with each of the different kinases for 20 minutes at 30°C in the presence of [³²P] labelled ATP at a specific activity of 420 cpm/pmol (see section 2.2.8). The reactions were terminated by the addition of Laemmlli sample buffer and boiling for 10 minutes. The samples were then separated on 10% polyacrylamide gels which were subsequently dried down and exposed to film for 16 hours (fig 3.5 and fig 3.5.1).
FIGURE 3.5 PKC treatment of PtdIns-PLCδ1.

Phosphorylation reactions were carried out as described in 2.2.8 and the reactions were analysed by autoradiography. Lanes 1 and 2 represent samples treated with trypsinised PKC αβγ mix (0.8U/ml) and lanes 3 and 4 contain samples treated with trypsinised PKC ε (1U/ml). Lanes 1, 3 and 5 represent samples from reactions including PtdIns-PLCδ1.
FIGURE 3.5.1 Ca^{2+}/Calmodulin/Kinase treatment of PtdIns-PLC{delta}1.

Phosphorylation reactions were carried out as described in 2.2.8 and 3.5.1. The samples were analysed by autoradiography; lane 1 contained PtdIns-PLC{delta}1 and Ca^{2+}/Calmodulin/Kinase (1U/ml), with 100μM Ca^{2+} and 0.01mg/ml calmodulin. Lane 2 contained PtdIns-PLC{delta}1 alone and lane 3 contained the kinase alone.
Comparison of the lanes containing kinase alone with those containing kinase plus PtdIns-PLCδ1 revealed that the only phosphorylated proteins originate from the kinase preparations. Clearly, none of the kinases tested were able to phosphorylate the bacterially expressed PtdIns-PLCδ1. It is possible that the bacterially expressed enzyme is incorrectly folded and therefore unable to be phosphorylated, although this seems unlikely considering that the thorough characterisation of this enzyme has shown it to behave almost identically to its eukaryotic counterparts. The failure of these kinases to phosphorylate PtdIns-PLCδ1 does not exclude the possibility of another kinase performing such a task in vivo, therefore to avoid having to test every possible kinase, an alternative approach was taken.

3.5.2 The effect of dephosphorylation on specific activity

The postulate that phosphorylation of PtdIns-PLCδ1 might lower its specific activity implies that dephosphorylation of the phosphorylated enzyme would cause an increase in its specific activity. To test this idea it was necessary to compare the effects of a selection of phosphatases on the specific activities of a 'phosphorylated' PtdIns-PLCδ1 preparation with the 'under-phosphorylated' PtdIns-PLCδ1 bacterial fusion protein. For this purpose, PtdIns-PLCδ1 was expressed in and purified from the COS-1 eukaryotic cell line.

3.5.2.1 Purification of PtdIns-PLCδ1 expressed in COS-1 cells

COS-1 cells were transfected (see 2.2.10.1) with PMT-2-PtdIns-PLCδ1 and grown for 48 hours to allow expression of PtdIns-PLCδ1 from the adenovirus major late promoter. Six 9cm plates were harvested in Buffer C containing 1% Triton X 100, the phosphatase inhibitors sodium fluoride and microcystin and protease inhibitors; this extract was cleared by ultra-centrifugation at 90,000 rpm for 10 minutes.

The supernatant (8ml) was loaded onto a 2ml DEAE column, which had been pre-equilibrated in buffer D, at a flow rate of 1ml/min. After washing the column with 10 ml of buffer D, the protein was eluted with a stepped 1M NaCl gradient (in buffer A) (fig 3.5.2). The activity eluted in the first step of the gradient (0-300 mM NaCl) as a broad peak.
FIGURE 3.5.2 2ml DEAE Column.

COS-1 cells over-expressing PtdIns-PLCδ1 were harvested and extracted as described in the text. 8ml of the cytosolic fraction of the extract were chromatographed on a 2ml DEAE column. The bulk of the protein (filled circles) did not bind to the column (not shown). Most of the bound protein was eluted in the first NaCl gradient (0-300mM) (-), with the PtdIns-PLC activity (open boxes).
The 1ml fractions were pooled, diluted 1:1 in buffer A and loaded onto a 1ml heparin-Sepharose column (pre-equilibrated in buffer A at 100 mM NaCl) at 0.5 ml/min. Again a stepped NaCl gradient was used (100-700mM in 20 ml, followed by 700-1000mM in 5ml). This time the PtdIns-PLC activity eluted as a tighter peak between 400 and 600mM NaCl (fig 3.5.3).

Fractions of 0.5ml were collected and 20μl samples of each fraction were analysed by 7% polyacrylamide gels which were either Western blotted and probed with polyclonal PtdIns-PLCδ1 antisera or silver stained (fig 3.5.4). A protein of 85-kDa eluted with the same profile as the PtdIns-PLC activity, peaking in fraction 25. The protein concentration of PtdIns-PLCδ1 in this peak fraction was estimated by the method of Bradford [1976] and the specific activity was calculated to be 134 μM/min/mg - not in fact much lower than the specific activity of the bacterially expressed PtdIns-PLCδ1.

3.5.2.2 Comparing the Ca²⁺ titrations of PtdIns-PLCδ preparations

The 3 preparations of PtdIns-PLCδ used in the following studies were the bacterially expressed PtdIns-PLCδ1 (200 U/mg), the COS-1 cell expressed PtdIns-PLCδ1 (134 U/mg) and a preparation of PtdIns-PLCδ2 purified from bovine brain by Eric Meldrum [Meldrum et al., 1989] (24.2U/mg). To establish whether the different specific activities were due to a difference in Ca²⁺ sensitivity, the Ca²⁺ titrations of these 3 preparations of PtdIns-PLCδ were compared (fig 3.5.5). The titrations were almost identical for each preparation, indicating that there was no difference in sensitivity to Ca²⁺ concentration between these three enzymes of different specific activity.
FIGURE 3.5.3 1ml Heparin-Sepharose.

The peak of active fractions from the DEAE column were pooled and chromatographed on a 1ml heparin-Sepharose column. The protein (closed circles) eluted on the 100mM-700mM NaCl gradient (-) in two peaks, just before the PtdIns-PLC activity (open boxes) eluted at 500mM NaCl.
FIGURE 3.5.4 Silver Stain of 1ml Heparin-Sepharose profile.

Samples of fractions 20-31 were separated by SDS-PAGE on a 7% polyacrylamide gel and the proteins were visualised by silver staining. Molecular weight markers on the left indicate 97, 69 and 45 kDa. The arrow indicates the partly purified PtdIns-PLCδ1 (approximately 85-kDa) which coelutes with the activity peak (fractions 20-30) in the previous figure.
FIGURE 3.5.5 Comparison of Ca\(^{2+}\) dependence.

The Ca\(^{2+}\) dependences of 3 preparations of PtdIns-PLC\(\delta\) were compared: COS-1 \(\delta1\) (filled circles), Bovine \(\delta2\) (open circles) and Bacterial \(\delta1\) (open boxes). The activity of each preparation was measured using the standard cholate assay (see 2.2.2.1) at different Ca\(^{2+}\) concentrations, employing a Ca\(^{2+}\)-EGTA buffer system as previously described [Katan and Parker, 1987].
3.5.2.3 The effect of phosphatase treatment on PtdIns-PLCδ activity

Each of these PtdIns-PLC preparations was incubated with two different classes of phosphatase, including two different subunits of PP2A (PCK and PCSM) and PP1C. The phosphatases were diluted to give 0.05 U per incubation) for 20 minutes at 30°C, having been diluted in a buffer containing BSA (100μg/ml), DTT (100μM) and 20mM Tris pH 7.5. The reactions were terminated by the addition of 9 volumes of the dilution buffer, containing 10μM microcystin and then assayed for PtdIns-PLC activity (fig 3.5.6). The final concentrations of the PtdIns-PLCδ preparations were 23.15 U/ml (bacterial δ1), 14.5 U/ml (COS-1 δ1) and 14.4 U/ml (bovine δ2). The no phosphatase control samples either contained buffer instead of phosphatase or phosphatase treated with 10μM microcystin, before the 20 minute incubation with the PtdIns-PLC preparations.

No significant change in activity was observed after phosphatase treatment for either the COS-1 δ1 preparation or the bovine δ2 preparation (no change was expected with the bacterial δ1). It is possible that the PtdIns-PLCδ2 purified from bovine brain did not represent a fully 'phosphorylated' sample, as it had neither been purified nor stored in the presence of phosphatase inhibitors. The COS-1 expressed enzyme should have been a fully basally modified enzyme, however, as the cells were growing in 10% foetal calf serum when they were harvested it is also possible that the over-expressed PtdIns-PLCδ1, although initially basally modified, had been partially or completely activated in response to serum. Despite this, it would be expected that some of the enzyme would still be in a non-activated state and therefore potentially phosphorylated. When purified, the specific activity of the COS-1 enzyme (134U/mg in the standard assay) was not that much lower than that of the bacterially expressed enzyme (200U/mg in the standard assay), suggesting that it was already fairly well activated. This may explain why the phosphatase treatment had no observable effect on the activity of the COS-1 enzyme.
FIGURE 3.5.6 Phosphatase treatment of PtdIns-PLCδ preparations.

The 3 PtdIns-PLCδ preparations (COS-1 δ1, filled bars; Bacterial δ1, striped bars; Bovine δ2 spotted bars) were treated with three different phosphatases as described in the text. Lane 1 represents the non phosphatase treated samples. Lanes 5 to 7 represent samples that contained microcystin at 10μM to inhibit the phosphatase activity. Lanes 2 and 5 were treated with PP2A (PCSH); lanes 3 and 6 were treated with PP2A (PCSM); lanes 4 and 7 were treated with PP1C. All of the samples were assayed under standard conditions in the cholate assay.
Therefore, although no effect of phosphatase treatment on PtdIns-PLC\(\delta_1\) activity could be demonstrated in these experiments, it is still possible that dephosphorylation does play a role in the activation of this enzyme.

### 3.6 Expression of other PtdIns-PLC isozymes

Having successfully expressed PtdIns-PLC\(\delta_1\) in pTZ18R, expression of PtdIns-PLC\(\beta_1\) and \(\gamma_1\) was attempted in the same manner. The vector pTZ18R was prepared for ligation by a restriction digest with \(\text{EcoR I}\) followed by blunt-ending, using Klenow enzyme (see 2.2.1.1). A 4.8 kb fragment of the human cDNA clone of PtdIns-PLC\(\gamma_1\) was prepared for sub-cloning using an \(\text{Xho I}\) restriction digest followed by blunt-ending. The bovine cDNA clone of PtdIns-PLC\(\beta_1\) was digested with \(\text{ApaI}\) (N-terminus) and \(\text{EcoR I}\) and blunt-ended to generate a 4.5 kb fragment for subcloning. The two fragments were ligated into the prepared vector using a blunt-ended ligation (see 2.2.1.5), creating clones encoding a \(\beta\)-galactosidase-PtdIns-PLC\(\gamma_1\) fusion lacking the first 28 amino acids and a \(\beta\)-galactosidase-PtdIns-PLC\(\beta_1\) fusion lacking the first 8 amino acids.

Expression of these clones in a variety of \(E.\text{coli}\) strains and under many different growth conditions only produced insoluble proteins (fig 3.6 and fig 3.6.1). This was probably due in part to the high molecular weights of these fusion proteins (145-kDa and 150-kDa, compared with 88-kDa for the PtdIns-PLC\(\delta_1\) fusion protein). The problem of insolubility was probably magnified by the lack of IPTG inducibility of the fusion protein expression in this vector. For this reason a different bacterial expression system was used for the expression PtdIns-PLC\(\beta_1\), which is discussed in the following chapter.
FIGURE 3.6 Expression of PtdIns-PLCβ₁ in E.coli.

TG-1 bacteria transformed with pTZ18R-PtdIns-PLCβ₁, were grown in 50ml of culture at 37°C for up to 24 hours, harvested and extracted as described in 2.2.3. The samples were separated by SDS-PAGE on a 7% polyacrylamide gel and the proteins transferred to nitrocellulose. The Western blot was probed with polyclonal antisera directed against a unique C-terminal epitope of PtdIns-PLCβ₁. Soluble and particulate samples from the 1 hour time-point are shown in lanes 4 and 5 (all other time points gave similar results) and control soluble and particulate samples of pTZ18R transformed cells are shown in lanes 2 and 3. Lane 1 contains a sample of whole rat brain homogenate. The molecular weight markers on the left indicate 200 and 97 kDa and the arrow indicates the immunoreactive 150-kDa PtdIns-PLCβ₁ proteins.
FIGURE 3.6.1 Expression of PtdIns-PLCγ1 in E.coli.

TG-1 bacteria transformed with pTZ18R-PtdIns-PLCγ1 were grown for up to 24 hours in 10ml cultures at 37°C. The cells were harvested and extracted as described in 2.2.3. The samples were separated by SDS-PAGE on a 7% polyacrylamide gel and the proteins transferred to nitrocellulose. The Western blot was probed with polyclonal antisera directed against a unique C-terminal epitope of PtdIns-PLCγ1. Soluble and particulate samples from the 6 hour time-point (all other time-points were similar) are shown in lanes 2 and 3. Lane 1 contains a sample of whole rat brain homogenate. The molecular weight markers on the left indicate 200 and 97 kDa. The arrow indicates the expressed PtdIns-PLCγ1 in the particulate fraction. The rat brain sample apparently contains two immunoreactive proteins. The upper faint band is thought to be the full length protein, indicating that the E.coli expressed fusion protein is smaller than the native protein, as expected from the amino-terminal truncation of 28 amino acids in the construct (see text).
General Discussion

The studies discussed in this chapter have demonstrated that PtdIns-PLCδ1 can be expressed as a fusion protein in *E. coli*. The protein can be extracted from expressing cultures in 1% Triton X-100 and is catalytically active in the standard PtdIns-PLC activity assay. However, the large quantities of expression originally hoped for were not achieved, ruling out the possibility of making an affinity column of PtdIns-PLCδ1 to screen for regulators of this isozyme. Nevertheless the fusion protein can be purified to near homogeneity using a 3-step chromatographic procedure and characterisation has demonstrated that its properties resemble those of its eukaryotic counterparts. These results suggest that bacterial expression is a valid approach for the preparation of PtdIns-PLC enzymes for use in studies of their regulation.

The vast range of specific activities quoted in the literature for PtdIns-PLCδ enzymes is puzzling, as the variability seems to be too great to be explained on the basis of different assay conditions. The relatively high specific activity of the PtdIns-PLCδ1 fusion protein does not, in the above studies, appear to be due to its 'underphosphorylation'. However, only a limited selection of protein kinases were tested for their ability to phosphorylate the bacterially expressed enzyme and possibly a wider screen for such a kinase is needed to resolve this issue. In the dephosphorylation experiments, it was never certain whether the bovine PtdIns-PLCδ2 and COS-1 expressed PtdIns-PLCδ1 enzymes were actually phosphorylated, although work by Cazoubon and Parker [1993] demonstrated that the PKC α that was un-phosphorylated and inactive when expressed in *E. coli*, was phosphorylated and catalytically active when expressed in COS-1 cells. If such studies were to be followed up, it would be worth comparing the specific activities of PtdIns-PLCδ1 purified from serum starved and serum stimulated cells, as the enzyme preparation purified from actively growing COS-1 cells had a similar specific activity to that purified from bacteria and was probably not the best candidate to use to study the effects of phosphatase treatment on activity.
Having established that active PtdIns-PLCδ1 could be expressed and purified for use in *in vitro* studies, although not in great enough quantities to enable an extensive search for a regulation mechanism for the δ enzymes, the bacterial expression system was deemed a useful tool to study the regulation of the ever expanding family of β PtdIns-PLC isozymes.
CHAPTER 4 - RECONSTITUTION OF G-PROTEIN COUPLED ACTIVATION

Introduction

The discovery of a family of G-proteins - Gq which were able to activate the PtdIns-PLCβ isozymes (see 1.3.3), raised many questions about the regulation of this class of PtdIns-PLCs. The inability of the δ and γ isozymes to be activated by Gqα [Taylor et al., 1991] and the short C-termini in these isozymes implied that the long C-terminal extension which is characteristic of the PtdIns-PLCβ family isozymes might be necessary for activation by the hererotrimeric G-protein α subunits of the Gq family. The precise nature and location of this 'domain of activation' in PtdIns-PLCβ1 was unknown when these studies began.

Previously, Ryu et al., [1990] had observed increased levels of phosphorylation in PtdIns-PLCβ1 immunoprecipitated from TPA treated cells and had presented this as evidence of negative feedback from PKC to PtdIns-PLC. They demonstrated phosphorylation of PtdIns-PLCβ1 by PKC in vitro, identifying a serine residue in the carboxy-terminus as the target for this phosphorylation. However, in these studies they were unable to detect any change in activity of the PtdIns-PLC after phosphorylation and it seemed plausible that such regulation might act at the level of the G-protein interactions with the PtdIns-PLC.

To identify the regions of the PtdIns-PLCβ carboxy-terminus important for activation by G-proteins, it was proposed to reconstitute this activation in vitro, using bacterially expressed PtdIns-PLCβ proteins. Such proteins would be un-modified and hence un-phosphorylated, ideal for studying the effects of phosphorylation on G-protein directed activation of these enzymes. To reconstitute the G-protein activation of PtdIns-PLCβ1, a source of both the PLC and the G-proteins had to be established.
Results and Discussion

4.1 Production of pure, full length PtdIns-PLCβ₁

The failure to express either PtdIns-PLCβ₁ or γ₁ as soluble fusion proteins in pTZ18R led to the investigation of another expression vector - pET 11a. This vector is designed to produce fusion proteins under the control of the bacteriophage T7 promoter. The T7 RNA polymerase is highly selective for specific T7 promoters and termination signals which are rarely found in non-T7 DNA. Consequently, it is able to make complete transcripts of most DNA sequences under the control of the T7 promoter. This RNA polymerase also has a much higher activity than *E.coli* RNA polymerase (responsible for transcription from the lac z promoter in pTZ18R), which means that production of the fusion protein in this system out-strips the production of many of the bacterial proteins. In the pET expression system, T7 RNA polymerase is generated by a lysogenic strain of *E.coli* - BL21(DE3). This strain is deficient in the lon protease and omp T, both of which can cause degradation of proteins during their production. The bacteriophage DE3 is constructed such that the T7 RNA polymerase gene that it carries is controlled by the IPTG inducible lac UV5 promoter. Hence induction with IPTG leads to expression of T7 RNA polymerase, which can then direct transcription of the sub-cloned gene from the T7 promoter. Such a tightly inducible system seemed to be ideal for the expression of the 150-kDa protein PtdIns-PLCβ₁.

4.1.1 Subcloning PtdIns-PLCβ₁ into pET 11a

A 3.8 kb fragment of the bovine cDNA clone for PtdIns-PLCβ₁ was subcloned into pET 11a using a series of restriction digests. The PtdIns-PLCβ₁ in PMT-2 was digested with Apal I and blunt-ended using Klenow enzyme. The vector (pET 11a) was digested with Nhe I and blunt-ended using mung-bean nuclease digestion to generate a blunt end (see 2.2.1.2). Both linearised plasmids were then digested with BamH I, to generate complimentary 'sticky ends', and gel purified (see 2.2.1.4). This allowed the PtdIns-PLCβ₁ fragment to be inserted into pET 11a using an asymmetric blunt (N-
terminus) sticky (C-terminus) ligation. This generated a clone (β1-pET 11a) in which PtdIns-PLCβ1 (minus the first 8 amino acids) was 'in frame' with the T7 promoter of pET 11a. Restriction digest analysis was used to check that the PtdIns-PLCβ1 sequence had inserted correctly into the pET 11a vector.

4.1.2 Expression of PtdIns-PLCβ1

The clone β1-pET 11a was transformed into E.coli strain BL21(DE3) and grown in M9ZB medium (see 2.3) at 37°C to an A600 of 0.86, before induction with 1mM IPTG for 0, 1, 2 or 3 hours. Each culture was harvested into Laemmli buffer and separated on a 7% polyacrylamide gel, which was then Western blotted and probed with a polyclonal antiserum raised against a unique carboxy-terminal epitope of PtdIns-PLCβ1 (fig 4.1). An immunoreactive band of 150-kDa was seen in all three IPTG induced cultures of β1-pET 11a transformed cells, but not in the cultures which had been transformed with vector alone (pET 11a). A similar time-course of induction was repeated for induction times of 0 to 60 minutes. This showed that expression of the PtdIns-PLCβ1 protein peaked after 45 to 60 minutes induction with IPTG.

To test for PtdIns-PLC activity in the expressed protein, supernatants were prepared, as for the PtdIns-PLCδ1 fusion protein (see 2.2.3) from a 10 ml test culture which had been grown as above and induced with 1mM IPTG for 1 hour at 37°C. Samples of this crude lysate were assayed in the standard PtdIns-PLC assay, confirming that the expressed protein was active and therefore suitable for purification.

4.1.3 Purification of PtdIns-PLCβ1

The expression and extraction was scaled up for preparation of pure PtdIns-PLCβ1. Two 9 cm plates of pET 11a-PtdIns-PLCβ1 transformed BL21(DE3) cells were used to seed four 500ml cultures. The cultures were grown at 37°C for approximately 2 hours to an A600 of between 0.6 and 1.0 and then induced for 1 hour with 1mM IPTG.
FIGURE 4.1 Expression of Triton-soluble PtdIns-PLCβ₁ in E. coli.

2ml cultures of BL21 (DE3) cells transformed with pET 11a-PtdIns-PLCβ₁ or vector alone (pET 11a) were grown at 37°C and induced with 1mM IPTG as described in the text. The cells were harvested and extracted as described in 2.2.3 and supernatant samples were separated by SDS-PAGE on a 7% polyacrylamide gel (equally loaded on the basis of protein concentration). The proteins were transferred to nitrocellulose and probed with the PtdIns-PLCβ₁ polyclonal antisera. The odd numbered lanes (excluding 9) contain control supernatants from the vector transformed cells and the even numbered lanes contain supernatants from the pET 11a-PtdIns-PLCβ₁ transformed cells. Lanes 1 and 2 were not induced with IPTG; lanes 3 and 4 were induced for 1 hour; lanes 5 and 6 were induced for 2 hours; lanes 7 and 8 were induced for 3 hours. Lane 9 contains a sample of whole rat brain homogenate. The molecular weight markers on the left represent 200, 97 and 69 kDa and the arrow indicates the immunoreactive 150-kDa PtdIns-PLCβ₁.
The combined cultures were harvested and extracted as previously (see 2.2.3). The resulting cleared supernatant was then used to purify PtdIns-PLCβ1 through 3 chromatographic steps.

4.1.3.1 1ml Heparin-Sepharose

38 ml of the supernatant containing the expressed PtdIns-PLCβ1 protein were loaded, at a flow rate of 0.5 ml/min, onto a 1ml pre-packed heparin-Sepharose column which had been pre-equilibrated in buffer A.(see 2.3). The column was then washed with 7 ml of buffer A before eluting the bound protein (fig 4.1.1) with a 20 ml 0-1.0 M NaCl gradient. Fractions of 0.5 ml were collected and assayed for PtdIns-PLC activity. Much of the protein in the loaded sample did not bind to the column, but most of the protein that did bind co-eluted with the PtdIns-PLC activity at 500-600 mM NaCl. The active fractions were pooled and diluted into 10ml of Buffer A in preparation for loading onto the next column.

4.1.3.2 1ml Mono Q 5/5

The diluted pool was loaded in buffer A onto a pre-equilibrated 1ml Mono Q column at 0.5 ml/min. The column was washed in 8 ml buffer A and the bound protein eluted using a 12 ml 0-0.6 mM NaCl gradient (fig 4.1.2). Again fractions of 0.5 ml were collected and assayed for PtdIns-PLC activity. This time the activity eluted just before the bulk of the protein, between 300 and 400 mM NaCl. The two peak fractions were pooled and dialysed over-night into buffer D, thus reducing the pH of the samples to 5.5, for loading onto the cation exchange column.
FIGURE 4.1.1 1ml Heparin-Sepharose purification of PtdIns-PLCβ1.

38 ml of supernatant were prepared from an induced growing culture of BL21 (DE3) transformed with pET 11a-PtdIns-PLCβ1 (see text). This preparation was chromatographed on a 1ml heparin-Sepharose column. The protein (most of which did not bind to the column) is indicated by filled circles. A gradient of NaCl (-) from 0-900mM eluted the bound protein and the PtdIns-PLC activity (open boxes)
The fractions containing the peak of PtdIns-PLC activity from the heparin-Sepharose column were pooled and chromatographed on a 1ml Mono Q column. The bound protein (filled circles) eluted at 300mM on the 0-600mM NaCl gradient (-), together with the PtdIns-PLC activity (open boxes).
4.1.3.3 1ml Mono S 5/5

PtdIns-PLCβ1 protein was known to be susceptible to cleavage at the carboxy-terminus [Park et al., 1993b], yielding a 110-kDa protein which could not be activated by Gqα, although it was active in a standard cholate PtdIns-PLC activity assay. For the purpose of reconstituting G-protein coupled activation of this enzyme, it was therefore essential to ensure that the final preparation of purified enzyme contained only full length protein. Previously, a stepped NaCl gradient on a Mono S column had been used to separate 'clipped' PtdIns-PLCβ1 from the full length protein [Carozzi et al., 1993], when purifying the enzyme from bovine brain. Thus the same technique was employed in the purification of the bacterial enzyme.

The dialysed pool was loaded at 0.5 ml/min onto a 1ml Mono S column which had been pre-equilibrated in buffer D. The column was washed with 7 ml of buffer D and the remaining bound protein was eluted with a stepped NaCl gradient (fig 4.1.3). In the initial gradient (2.5ml 0-250mM NaCl followed by 5 ml at 250 mM NaCl) nearly all of the remaining protein was eluted, including a low level of PtdIns-PLC activity, believed to result from the 'clipped' PtdIns-PLCβ1. The bulk of the PtdIns-PLC activity eluted in the second step of the gradient at 600 mM NaCl - the ratio of these two peaks of activity varied in different preparations, although the second peak was always larger.

The first and second peaks of activity were analysed by SDS PAGE, followed by silver staining and Western blotting. The silver stain (fig 4.1.4) revealed a fairly pure protein of 150-kDa in peak II. By estimating the amount of PtdIns-PLCβ1 protein in the preparation, a figure for the specific activity was obtained - 165 μM/min/mg, very similar to the value quoted for purified bovine brain PtdIns-PLCβ1. Probing the Western blot with the PtdIns-PLCβ1- specific polyclonal antisera (fig 4.1.5) demonstrated that the 150-kDa band in peak II was immunoreactive.
FIGURE 4.1.3 1ml Mono S of Mono Q pool.

The two peak fractions of PtdIns-PLC activity from the Mono Q column were pooled, dialysed and chromatographed on a 1ml Mono S column. A stepped NaCl gradient (-) from 0-250mM and 250-1000mM was employed to elute the majority of the protein (filled circles) at 250mM NaCl. The PtdIns-PLC activity (open boxes), however eluted at approximately 600mM (fraction 24). The peak of contaminating protein was labelled I (fractions 9-15) and the peak of PtdIns-PLC activity was labelled II (fractions 23-25).
FIGURE 4.1.4 Silver Stain of Mono S activity peaks.

The peak pools I and II (1 and 2) from the Mono S column were separated by SDS-PAGE on a 7% polyacrylamide gel. The proteins were visualised by silver staining. The molecular weight markers on the left indicate 200, 97 and 69 kDa and the arrow indicates the 150-kDa purified PtdIns-PLCβ1 protein.
FIGURE 4.1.5 Immunoreactivity of the purified protein

The samples from peaks I and II of the Mono S column were separated by SDS-PAGE on a 7% polyacrylamide gel, transferred to nitrocellulose and probed with the PtdIns-PLC$_{\beta_1}$ polyclonal antisera. Lane 1 contains a sample of whole rat brain homogenate, lane 2 a sample of peak I and lane 3 a sample of peak II. Molecular weight markers on the left indicate the immunoreactive 150-kDa PtdIns-PLC$_{\beta_1}$ protein.
This purification procedure achieved a 29,000 fold purification, yielding an almost homogenous preparation of PtdIns-PLCβ1, which could be stored for several months at 4°C or for longer at -20°C (1:1 with ethane-diol). The data for the purification are shown in the table below:

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Protein (mg)</th>
<th>Activity (Units)</th>
<th>Specific Activity (U/mg)</th>
<th>Purification (fold)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>38</td>
<td>243</td>
<td>1386</td>
<td>5.7</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>5.5</td>
<td>1,000</td>
<td>182</td>
<td>32</td>
<td>72</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>0.94</td>
<td>895</td>
<td>949</td>
<td>166</td>
<td>65</td>
</tr>
<tr>
<td>4</td>
<td>0.5</td>
<td>0.006</td>
<td>1,064</td>
<td>165,000</td>
<td>29,000</td>
<td>77</td>
</tr>
</tbody>
</table>

Units are nmoles/min. The protein concentration of the final purified sample was estimated from the silver stain shown in fig. 4.1.4.

The increases recovery between steps 2 and 4 was probably due to the removal of a contaminating protein which was inhibitory towards PtdIns-PLCβ1.

4.1.4 Characterisation of PtdIns-PLCβ1 expressed in bacteria

Having extensively characterised the PtdIns-PLCβ1 fusion protein the costly studies of the substrate specificity for the PtdIns-PLCβ1 purified from bacteria were deemed unnecessary. However, a calcium titration was performed with PtdIns(4,5)P2 as the substrate, for comparison with the published eukaryotic enzymes (fig 4.1.6). The profile is very similar to that obtained with bovine PtdIns-PLCβ1 [Katan and Parker., 1987], with half-maximal activation at about 0.2 μM Ca2+.
FIGURE 4.1.6 Ca$^{2+}$ dependence of PtdIns-PLC$\beta_1$.

The PtdIns-PLC activity of pool II was measured under standard conditions in the cholate assay, but at different Ca$^{2+}$ concentrations, employing the buffer system described by Katan and Parker [1987].
The successful expression and purification of a second PtdIns-PLC isozyme in bacteria confirmed that such a system was a valid means to generate these enzymes for in vitro studies. Interestingly, the specific activity of the PtdIns-PLC\(\beta_1\) isozyme expressed in bacteria was very similar to that of the bovine enzyme, suggesting that post-translational modifications are probably not necessary for the intrinsic catalytic activity of PtdIns-PLC\(\beta_1\), although they may be for PtdIns-PLC\(\delta_1\) (see 3.4.5). This does not exclude the possibility that a post-translational modification such as phosphorylation could be used to regulate the interaction of the enzyme with another protein, such as G\(\alpha\), or indeed a lipid.

4.2 Generation of G\(\alpha q\) for in vitro reconstitution with PtdIns-PLC\(\beta_1\)

The method used to produce G\(\alpha q\) was an adaptation of the method of Wu et al., [1992a]. COS-1 cells were transfected with 40 \(\mu\)g plasmid containing the cDNA for G\(\alpha q\) under the control of the CMV promoter and grown for up to 72 hours in normal growth medium. The cells were harvested, after washing in ice-cold PBS, in buffer E and homogenised on ice, using dounce B for 30 strokes. The homogenate was centrifuged at 500 rpm for 10 minutes, to remove nuclei and cell debris. The supernatant was then centrifuged for 10 minutes in an ultra-centrifuge at 70,000 rpm. The pellet was re-suspended in buffer F and tumbled on a wheel for 2 hours at 4°C, before centrifuging again at 70,000 rpm for 10 minutes. This step serves to remove any extrinsic protein from the membranes. The pellet was washed in buffer H twice and then resuspended in buffer H (400 \(\mu\)l for every two 190mm plates harvested). The protein content of the membranes was assessed by the method of Bradford [1976] and the remaining preparation frozen in aliquots at -20°C. The presence of G\(\alpha q\) in such a membrane preparation had previously been verified by Western blotting with antisera directed against a unique epitope in the carboxy-termini of G\(\alpha q\) and G\(\alpha 11\) [Lee et al., 1992].
4.3 Reconstituting Gqα activation of PtdIns-PLCβ1 in vitro

The reconstitution assay was based on that described by Wu et al., [1992a] and is described in 2.2.2.2. Essentially, the PtdIns-PLCβ1 purified from E.coli or bovine brain was mixed with the membrane preparation (in the absence or presence of activatory AlF4⁻) and sonicated lipid vesicles containing 200μM Ptd-Ethanolamine, 200μM Ptd-Serine and 20μM PtdIns(4,5)P₂. The presence of Ptd-Ethanolamine and Ptd-Serine and the low Ca²⁺ concentration (0.05μM) in the assay serve to depress the PtdIns-PLC activity in the absence of any activator, permitting Gqα-dependent activation to be observed.

4.3.1 Titration of the Gqα membrane preparation

The PtdIns-PLCβ₁ purified from E.coli was incubated in the above assay for 30 minutes at 37°C, with varying amounts of Gqα. The PtdIns-PLC activity was assessed in the absence or presence of AlF₄⁻, using the standard assay procedure to measure the release of [³H]IP₃ (fig 4.3). As the Gqα concentration increased, the PtdIns-PLC activity in the presence of AlF₄⁻ also increased, although the background activity in the absence of AlF₄⁻ remained stable. The AlF₄⁻ is clearly required to activate the activator - Gqα, as in its absence an activity similar to that seen in the absence of Gqα, can be measured. The AlF₄⁻ is believed to work by mimicking the final phosphate moiety of GTP, when GDP is bound to the α subunit, thus switching the G-protein into its active conformation.

Having established that the bacterial enzyme could be activated in this assay, membrane preparations from αq or vector alone transfected COS-1 cells were compared in their ability to activate PtdIns-PLCβ₁ in the absence or presence of AlF₄⁻ (10 μM) (fig 4.3.1). Both membrane preparations demonstrated an AlF₄⁻ dependent activation of PtdIns-PLCβ₁, although the Gqα membranes activated to a much greater degree. This indicated the presence of low levels of endogenous PtdIns-PLCβ₁-activatory G-proteins in the membrane preparations, (which may include all members of the Gqα family), however the majority of the activatory effect was evidently contributed by the over-expressed Gqα protein.
FIGURE 4.3 Gαq membrane dependence of PtdIns-PLCβ1.

Gαq membranes prepared as described in 4.2 were titrated into the reconstitution assay described in 2.2.2.2, in the absence (filled circles) or presence (open circles) of 10μm AIIF4-. The PtdIns-PLCβ1 used in the assays was diluted, based upon its activity in the standard cholate assay (2.2.2.1), to give 0.47 units of cholate activity per assay tube. The values plotted are means of duplicate samples and the variance is indicated by the error bars.
FIGURE 4.3.1 PtdIns-PLCβ_1 activation by control COS-1 membranes.

Samples of the purified bacterially expressed PtdIns-PLCβ_1 (0.5 units as measured in the cholate assay) were treated with 2μg of COS-1 membranes prepared from control (vector) transfected or Gqα transfected cells in the reconstitution assay described in 2.2.2.2. The PtdIns-PLC activity was measured in the absence (filled bars) or presence (striped bars) of 10μM AlF_4-. The values plotted are means of triplicate samples and the variance is indicated by the error bars.
4.3.2 Titration of PtdIns-PLC\(\beta_1\) enzymes in the reconstitution assay.

To establish a suitable concentration of PtdIns-PLC (measured as units of activity in the standard cholate assay) for use in the reconstitution assay, PtdIns-PLC\(\beta_1\) was titrated into the assay (fig 4.3.2) and the AI\(F^4\) dependent PtdIns-PLC activity measured. It was observed that as the amount of PtdIns-PLC\(\beta_1\) was increased, the 'background' activity seen in the absence of AI\(F^4\) also increased, above that seen in the absence of activatory membrane. This background must be composed of the true background - created by lipids being naturally degraded and carried over during the assay - plus an additional background of intrinsic, non-activatable catalytic activity, the result of high levels of PtdIns-PLC\(\beta_1\) in the assay, which cannot be fully depressed under the assay conditions. If such an assay is to be used to demonstrate inhibition of coupling, it is important to choose a PtdIns-PLC\(\beta_1\) concentration which is high enough to give a visible activation in the assay, but that does not push the 'background' up beyond that seen in the absence of activatory membranes.

4.3.3 Comparison of bacterial and bovine PtdIns-PLC\(\beta_1\)

Having established optimal conditions for the reconstitution assay, it was important to compare G\(\alpha\)-coupled activation of PtdIns-PLC\(\beta_1\) expressed in and purified from \textit{E.coli} with the G\(\alpha\)-coupled activation of PtdIns-PLC\(\beta_1\) purified from bovine brain. The bovine brain enzyme had been purified by A.Carozzi using the method established by Katan et al., [1987]. The comparison was done by titrating in G\(\alpha\) membrane with each of the two enzymes, which had been diluted to the same activity, (as determined in the standard cholate PtdIns-PLC assay), to normalise the two preparations. Each PtdIns-PLC preparation was diluted in buffer G so that 0.5 U activity (measured in the standard cholate assay) could be used in each 60\(\mu\)l reaction. Each reaction was incubated (with varying membrane concentrations) for 30 minutes at 37\(^\circ\)C. These titrations (fig 4.3.3) suggested that there might be a difference in the G\(\alpha\) activation of the two PtdIns-PLC\(\beta_1\) preparations.
FIGURE 4.3.2 Titration of bacterially expressed PtdIns-PLCβ1.

The bacterially expressed PtdIns-PLCβ1 preparation was titrated into the reconstitution assay described in 2.2.2.2, with 2μg of a Gαq membrane preparation, in the absence (open circles) and presence (filled circles) of 10μM AlF4-. The concentration of PtdIns-PLCβ1 is expressed as the standard cholate units in each 60μl reaction. The values plotted are the means of duplicate reactions and the variance is indicated by the error bars.
FIGURE 4.3.3 Comparison of Bacterial and Bovine PtdIns-PLCβ1.

The bacterially expressed PtdIns-PLCβ1 preparation was compared with a bovine brain PtdIns-PLCβ1 preparation in the reconstitution assay described in 2.2.2.2. Both preparations were diluted to give 0.5 cholate units per reaction and a Gαq membrane preparation was titrated into the reactions. The PtdIns-PLC activity values plotted are means of duplicates and the variance is indicated by the error bars. The total protein content of the membrane preparation was measured using the method described by Bradford [1976].
The titration curve for the bacterial enzyme was slightly to the left of that for the bovine enzyme. One possible explanation for this difference was that modification of residues in the PtdIns-PLCβ1 from bovine brain might desensitise the coupling between the enzyme and the G-protein.

4.3.4 The effect of dephosphorylation on bovine brain PtdIns-PLCβ1

To determine whether the different Gαq responses for the bovine and bacterial enzymes were due to differences in their phosphorylation status, the bovine enzyme was incubated with an excess of phosphatase for 15 minutes at 30°C in the presence or absence of the phosphatase inhibitor microcystin (50 μM). The phosphatase was in fact a mixture of 2 forms of PP2A (PCSH, PCSL) and PP1C, diluted in buffer K to give 40 U of each in the final incubation with PtdIns-PLCβ1. The reaction was stopped with buffer C containing microcystin to give a final concentration of 80 μM in the treated preparation.

The AlF4⁻-dependent Gαq activation of the phosphatase-treated PtdIns-PLCβ1 samples was measured in the reconstitution assay and compared with untreated bovine brain and bacterial PtdIns-PLCβ1 (fig 4.3.4) at two concentrations of Gαq membranes. Bearing in mind the range of experimental error when analysing the results of this experiment, there was no significant difference between the untreated bacterial and bovine enzymes in their response to activated Gαq membranes at either concentration (lanes 1 and 2). Secondly, there is no difference at either membrane concentration between lane 3 (containing the bovine enzyme treated with active phosphatase) and lane 4 (containing the bovine enzyme treated with microcystin inactivated phosphatase). This implies that treatment of the eukaryotic PtdIns-PLCβ1 with this mixture of phosphatases did not alter its sensitivity to activation by Gαq membranes.
FIGURE 4.3.4 Phosphatase treatment of PtdIns-PLCβ₁.

PtdIns-PLCβ₁ samples were treated with a phosphatase mixture containing PP2A (PCSH and PCSM) and PP1C as described in the text. The ability of two different concentrations of a Goαq membrane preparation (0.5μg (filled bars) and 0.25μg (striped bars)) to activate phosphatase treated and untreated PtdIns-PLCβ₁ preparations was assessed using the reconstitution assay described in 2.2.2.2. Lanes 1 represents untreated bacterially expressed PtdIns-PLCβ₁; lane 2 represents untreated bovine brain PtdIns-PLCβ₁; lane 3 represents bovine brain PtdIns-PLCβ₁ which had been treated with the phosphatase mixture; lane 4 represents bovine brain PtdIns-PLCβ₁ which had been treated with microcystin-inactivated phosphatase. The activation values represent the difference between the activities of each sample in the presence and absence of 10μM AlF₄⁻. Each value is a mean of the results from duplicate reactions, with variance indicated by the error bars.
These two observations together suggest that if there is any difference in the level of phosphorylation between the bacterially expressed and bovine enzymes, this is unlikely to result in a difference between the activation of these enzymes by Goq. However, one final observation from this experiment is the slight difference between the untreated bovine enzyme (lane 2) and the enzyme treated with microcystin inactivated phosphatase, particularly noticeable at the lower Goq membrane concentration.

This result could be explained by the difference in microcystin concentration in the two sets of samples. The samples in lane 4 received a double dose of microcystin, as the phosphatase was treated with microcystin initially and the 15 minute reaction was terminated as with the samples in lane 3 by addition of a quench buffer containing microcystin. It is possible that contaminating phosphatases in the Goq membrane preparation, (distinct from the ones tested in this experiment), normally slowly de-phosphorylate the bovine enzyme during the 30 minute reconstitution assay, raising its activity nearer to that of the unphosphorylated bacterial enzyme. The higher concentrations of microcystin in the lane 4 samples might inhibit this reaction, thus maintaining the difference in activity between the two PtdIns-PLCβ1 preparations. This suggestion would also explain the variation in results between the experiment described in 4.3.3 and the experiment described here, as the phosphatase content might vary in different membrane preparations.

The effect of microcystin on PtdIns-PLCβ1 activity in the reconstitution assay was investigated (fig 4.3.5). When microcystin was added to the reconstitution assay a small effect was observed. At both membrane concentrations tested, microcystin treatment of the two PtdIns-PLCβ1 preparations appeared to exaggerate the difference in activation between the two. However, the observed effect was not large enough to exclude the possibility that it was due to experimental variations, although such an effect was seen to a greater or lesser degree in every experiment performed. Such inconclusive results meant that the effects of phosphorylation on G-protein-PtdIns-PLCβ1 coupling would have to be assessed using a different approach.
FIGURE 4.3.5 The effect of microcystin on PtdIns-PLCβ1 activation.

The effect of microcystin (50μM) on the activation of both PtdIns-PLCβ1 preparations by two concentrations of a Gαq membrane preparation was assessed. The filled bars represent samples of the bacterially expressed PtdIns-PLCβ1 and the striped bars represent samples of the bovine brain PtdIns-PLCβ1. The black and diagonally striped bars represent untreated samples; the grey and horizontally striped bars represent samples to which microcystin was added prior to assaying in the reconstitution assay described in 2.2.2.2. Values plotted are the means of duplicate results and the variance is indicated by the error bars.
4.4 Gαq activation of other PtdIns-PLC isozymes

Having confirmed that PtdIns-PLCβ1 could be activated by Gαq in the reconstitution assay, it was of interest to test other PtdIns-PLC isozymes for their ability to be activated in a similar manner. Work done by Amanda Carozzi in the laboratory, using the same assay, demonstrated that PtdIns-PLCβ1 purified from HeLa cells was activated by Gqα proteins in an AlF4⁻-dependent manner. Comparison of the bovine brain PtdIns-PLCβ1 with the HeLa PtdIns-PLCβ3 preparation showed that both isozymes can be strongly activated by Gαq to similar extents (the ratio of β1 to β3 in terms of cholate units added to the assay was 5 : 3). However, when 0.5 units of purified PtdIns-PLCδ1 fusion protein were tested in the assay, no activation above that seen in the absence of added PtdIns-PLC enzyme (fig 4.4), was measured. It is interesting to note that the Gαq membrane preparation evidently contains low levels of endogenous COS-1 PtdIns-PLC, as even in the absence of added enzyme there is an AlF4⁻ dependent increase in PtdIns(4,5)P₂ hydrolysis. Western blotting of COS-1 extracts by Catherine Webster in the laboratory had previously revealed that the principal PtdIns-PLC isozyme present in these cells is PtdIns-PLCβ3.
FIGURE 4.4. Effect of Gαq on PtdIns-PLCδ1 activity.

Bacterially expressed PtdIns-PLCδ1 (0.5 units of cholate activity) or buffer (no enzyme) were mixed with 2μg of a Gαq membrane preparation in the reconstitution assay described in 2.2.2.2. The PtdIns-PLC activity was measured in the absence (filled bars) and presence (striped bars) of 10μM AlF4-. The means of triplicate results are plotted and the variance indicated by error bars.
General Discussion

Following the successful expression and purification of the PtdIns-PLCδ1 fusion protein discussed in chapter 3, the expression of PtdIns-PLCβ1 in \textit{E.coli} confirmed the value of such an approach. The failure to express PtdIns-PLCβ1 using pTZ18R and subsequent success with the pET system raises an important point. Clearly not all proteins are easily expressed in \textit{E.coli} - a method that works for one enzyme cannot be guaranteed to work for another, not even a protein of the same family. It is widely held that the greater the size of the protein to be expressed, the harder it is to express soluble protein. However, the successful expression of the 150-kDa PtdIns-PLCβ1 protein using the pET 11a vector has demonstrated that it is by no means impossible to express large proteins in a soluble and extractable form.

The brief characterisation of the purified PtdIns-PLCβ1 protein demonstrated its similarity to the bovine brain enzyme, even in its specific activity. This contrasted with the distinct difference observed between the specific activities of eukaryotic and bacterially expressed PtdIns-PLCδ1. This contrast may reflect an underlying difference between the regulation mechanisms of the β and δ class PtdIns-PLC enzymes. PtdIns-PLCδ1 may yet prove to be subject to a more basic level of regulation, possibly through post-translational modifications. The β class of PtdIns-PLC enzymes, on the other hand, appear to be regulated through interactions with other proteins (as are the γ PtdIns-PLC enzymes) and although post-translational modifications may be important in the regulation of this process, they may act by altering the interactions between the PtdIns-PLC and its regulatory proteins, rather than directly altering the intrinsic catalytic activity of the enzyme.

Production of Goq for use in a reconstitution assay using the method established by Wu et al., [1992a] was straightforward. Using a membrane preparation for reconstitution is advantageous from the point of view of providing a ‘semi \textit{in vivo}’ environment in which to activate the PtdIns-PLC. However, some of the advantages of using a pure PtdIns-PLC in such an assay are lost when it is mixed with a crude membrane preparation, containing many more proteins than the overexpressed Goqα. The small
activation of the PtdIns-PLCβ₁ by membranes prepared from control COS-1 cells demonstrated the presence of PtdIns-PLC-specific G-proteins in the preparations. The AIF⁴⁻ dependent increase in PtdIns(4,5)P₂ hydrolysis seen in Gqα membranes in the absence of added PtdIns-PLC confirmed the presence of low levels of endogenous PtdIns-PLCβ₁ and the possible effect of microcystin on the reconstitution assay indicated that there might be phosphatases present in such preparations.

Another drawback with the membrane preparations is the inability to properly quantify their Gαq content. Lee et al., [1992] used antisera against the Gαq protein to quantitate the amount of enzyme in a fixed concentration, claiming that 1µg of membrane contain 15ng of αq. However, transformation efficiency and presumably the level of expression of the transfected cDNA, varies in different preparations. For this reason, ideally only experiments performed with the same membrane preparation can be compared, although different membrane preparations can be roughly normalised on the basis of protein concentration.

In spite of these drawbacks, the reconstitution assay was used to demonstrate Gqα directed activation of PtdIns-PLCβ₁ and PtdIns-PLCβ₁. The assay also allowed confirmation of previous reports that PtdIns-PLCδ₁ cannot be activated by Gαq. The apparently lowered activity of bovine brain PtdIns-PLCβ₁ in the reconstitution assay hinted that phosphorylation may have a role in the regulation of Gqα coupling to PtdIns-PLCβ₁ isoforms. However, because the observed differences were small and variable, probably due to the limitations mentioned above, an alternative approach to investigate the role of phosphorylation in regulating this activation process was taken as described in the following chapter.
CHAPTER 5 - REGULATION OF BETA ISOZYMES THROUGH Gqα

Introduction

The successful expression of PtdIns-PLCβ1 in *E.coli* and reconstitution of its activation by Gαq *in vitro* provided a tool with which to investigate the mechanisms involved in this process. The discovery of the Pertussis toxin insensitive Gq family of heterotrimeric G-proteins and demonstration that the GTP-bound α subunits of this family were able to activate the different PtdIns-PLCβ isozymes to varying extents *in vitro* (see 1.3.3.), led to a search for the region of these enzymes which enabled interaction with the Gq α subunits. Alignments of all the known PtdIns-PLC isozymes had revealed that the PtdIns-PLCβ isozymes all possessed an elongated C-terminus which was not present in the γ or δ PtdIns-PLC family isozymes. As neither the γ or δ PtdIns-PLC isozymes could be activated by the Gq α subunits [Taylor et al., 1991], the elongated C-terminus seen in the β isozymes was a prime candidate for a region involved in the G-protein coupled activation of these enzymes. To establish whether the C-terminus of PtdIns-PLCβ1 was able to interact with activated Gαq, it was proposed to test the ability of this domain to block the *in vitro* reconstituted coupling between Gαq and full length PtdIns-PLCβ1. The pGEX-2T expression vector was chosen to express the PtdIns-PLCβ1 carboxyterminus as a GST fusion protein in *E.coli.*. The GST portion of this fusion protein would allow it to be easily purified from bacterial extracts using glutathione-Sepharose beads and could subsequently be used to recover the protein from incubations such as phosphorylation reactions.

Results and Discussion

5.1 Subcloning the PtdIns-PLCβ1 carboxyterminus into pGEX-2T

The region of PtdIns-PLCβ1 to be expressed was chosen on the basis of an alignment of the β and δ PtdIns-PLC sequences. The positions of the catalytic X and Y domains (see 1.3.4) in the PtdIns-PLC protein sequence had previously only been loosely defined by measuring the catalytic activity of several deletion mutants [Bristol et al., 1988].
Using the Dot Plot Sleuth program the protein sequences of bovine PtdIns-PLCs \( \beta_1 \) and \( \delta_1 \) were compared, enabling the X and Y domains to be picked out as regions of high homology between the two different isozymes (see below).

The plot above indicates regions of homology between the two sequences (PtdIns-PLC\( \beta_1 \) and PtdIns-PLC\( \delta_1 \)) with dots. Two long stretches of homology (labelled X and Y) can be seen and are known to represent the two catalytic domains of these enzymes. The end of the Y domain is not obvious from this alignment and therefore the start of the carboxyterminus was defined as the point at which there was no homology of sequence between the different isozymes - the point where the \( \delta_1 \) sequence ended.

A 1.5 kb fragment encoding the C-terminus of PtdIns-PLC\( \beta_1 \) (which included the epitope for the polyclonal antisera directed against that isozyme) was cut out of \( \beta_1 \)-pET 11a for insertion into the pGEX-2T vector. The 3' end was generated by cutting with BamH I and blunt-ended using mung-bean nuclease (see 2.2.1.2). The 5' end was generated by cutting at the \( Nhe \) I site which produced a C-terminal fragment 64 amino acids longer than the above 'defined C-terminus'. The vector was prepared by digesting with \( Nco \) I, blunt-ending with mung-bean nuclease followed by digestion with \( Xba \) I to generate a complimentary 'sticky end' for ligation with the \( Nhe \) I 'sticky end' of the PtdIns-PLC\( \beta_1 \) fragment. This enabled an asymmetric sticky/blunt ligation to be performed (see 2.2.1.5),
generating pGEX-β₁COOH, a clone encoding a protein composed of GST fused 'in frame' to 477 amino acids of the PtdIns-PLCβ₁ carboxyterminus.

5.2 Expression of the GST-β₁COOH fusion protein

The pGEX-β₁COOH construct was checked by restriction enzyme analysis and transformed into the *E.coli* strain BB4. Test cultures of 1ml were seeded from overnight cultures (1/10) and grown in L-B medium (ampicillin at 100μg/ml) at 37°C for 2 hours to an A₆₀₀ of 1.0 (see 2.2.6). The cells were harvested by centrifugation, resuspended in 100μl Laemmli buffer and 50μl of each sample separated on an 8% polyacrylamide gel. Staining the gel with coomassie blue (fig 5.2) did not reveal any significant difference between the control extract, from cells transfected with vector alone, (lane 1) and the extract from the a clone thought to contain pGEX-β₁COOH (lane 2).

Having failed to see any expression of the fusion protein by coomassie staining, an experiment to investigate the best growth and induction conditions was undertaken. Two 1ml cultures of pGEX-β₁COOH transformed *E.coli* cells, one containing IPTG at a concentration of 1mM, were grown at 37°C overnight and harvested using the lysozyme protocol (see 2.2.3). The final supernatants were mixed 1:1 with Laemmli sample buffer and analysed by Western blotting (lanes 2 and 3 of fig 5.2.1). In addition, six 1ml cultures were inoculated at an A₆₀₀ of 0.01 from a 9cm L-B plate containing pGEX-β₁COOH transformed *E.coli* cells. The cultures were grown to an A₆₀₀ of 0.7 (lanes 4-6) or 1.0 (lanes 7-9) and either harvested at that point (lanes 4 and 7) or grown for a further hour in the absence or presence of 1mM IPTG before harvesting. All the samples, including the control sample from the previous experiment (lane 1), were separated by SDS PAGE on an 8% polyacrylamide gel which was Western blotted and probed with the polyclonal antisera raised against the C-terminal epitope of PtdIns-PLCβ₁ (fig 5.2.1).
FIGURE 5.2 Expression of the carboxyterminus in *E.coli*.

BB4 bacteria transformed with the pGEX-β1COOH construct (lane 1) or pGEX-2T (lane 2) were grown in 1ml test cultures at 37°C for 2 hours. The cells were harvested by centrifugation, resuspended in Laemmli sample buffer, separated by SDS-PAGE on an 8% polyacrylamide gel and the proteins stained with coomassie blue. The molecular weight markers on the right represent 97, 66 and 45 kDa.
FIGURE 5.2.1 IPTG Induction of fusion protein expression.

Samples of Triton X 100 extracted bacterial cultures were generated as described in the text, separated by SDS-PAGE on an 8% polyacrylamide gel, transferred to nitrocellulose and probed with the polyclonal antisera directed against PtdIns-PLCβ1. Lanes 1 and 2 contained samples from 16 hour cultures of pGEX transformed (1) and pGEX-β1COOH transformed (2 and 3) bacteria grown in the absence (1 and 2) or presence of 1mM IPTG. All the other lanes contain samples from pGEX-β1COOH transformed bacteria grown to an A600 of 0.7 (4, 5 and 6) or 1.0 (7, 8 and 9). The cultures represented in lanes 4 and 7 were then harvested, whilst the other cultures were grown for a further hour in the absence (5 and 8) or presence (6 and 9) of 1mM IPTG, before harvesting. The molecular weight markers on the left represent 97, 66 and 45 kDa and the arrow indicates the expressed fusion protein.
A band just below the 97-KDa marker was observed in the samples from pGEX-β1COOH transformed cells (3-9) which was not visible in the control sample (1). The 92-kDa fusion protein could not be seen in the overnight culture grown in the absence of IPTG (lane 2), suggesting that IPTG was necessary for expression of the fusion protein. However, the other samples indicated that IPTG was not required for expression of the fusion protein, as the protein was expressed to the same levels in the presence or absence of the inducer. The quantity of soluble protein produced only seemed to be a function of the culture growth time, lanes 8 and 9 containing more of the fusion protein than lanes 5 and 6. Several different strains of E.coli were tested for expression of the fusion protein (BL21, TOP2, lon- and HMS174), but there did not seem to be any significant difference in the levels of fusion protein produced in any of these bacterial strains, hence the BB4 strain was used for all future purifications.

As two different antisera were available to probe Western blots for the fusion protein, their ability to recognise it was compared. Equal amounts of control or GST-β1COOH supernatant were electrophoresed on an 8% polyacrylamide gel which was Western blotted and cut into 3 strips containing both samples. The blots were probed with either the antisera raised against a C-terminal epitope of PtdIns-PLCβ1 (in the presence or absence of competing peptide) or the antisera raised against GST (fig 5.2.2). Both antisera recognised a 92-kDa protein in the GST-β1COOH supernatant but not in the control supernatant (from BB4 E.coli transformed with vector alone). The β1COOH peptide was able to compete out the band, demonstrating that the immunoreactivity was specific. The bands common to both supernatants were not competed out, suggesting that they were simply non-specific bacterial proteins. As the immunoreactivity seen with the anti-GST antisera was better than that seen with the β1COOH antisera, the anti-GST antisera was chosen for use during the purification of the protein. This antisera also had the advantage that it would also recognise fusion protein which had been 'clipped' in the β1COOH portion.
FIGURE 5.2.2 Testing the immunoreactivity of GST-\(\beta_1\)COOH.

Supernatant samples were prepared from pGEX (c) or pGEX-\(\beta_1\)COOH (f) transformed bacteria as described in 2.2.3 and separated by SDS-PAGE on an 8% polyacrylamide gel. The proteins were transferred to nitrocellulose and the nitrocellulose divided into 3 pieces, each containing both samples. The nitrocellulose strips were probed with the polyclonal antisera against PtdIns-PLC\(\beta_1\) in the absence (1) or presence (2) of competing peptide, or with the anti-GST polyclonal antisera (3). The molecular weight markers on the left represent 200, 97 and 69 kDa and the arrow indicates the immunoreactive GST-\(\beta_1\)COOH fusion protein.
5.3 Purification of GST-β₁COOH

Cultures of transformed bacteria were set up from overnight cultures and grown to an A₆₀₀ of between 1.0 and 2.0 at 37°C and supernatant was prepared using the lysozyme procedure (see 2.2.3). Typically 40ml of supernatant would be extracted from 2l of culture for purification. However, initially several test purifications were performed on a smaller scale.

5.3.1 Binding the fusion protein to glutathione-Sepharose

Generally GST fusion proteins can be purified using a single step - binding the fusion protein to glutathione-Sepharose beads through the GST, washing the beads and eluting the bound protein with reduced glutathione (see 2.2.6). Therefore 20ml of GST-β₁COOH supernatant were mixed with 1ml glutathione-Sepharose beads (washed in buffer G) and tumbled in a 50ml falcon tube at 4°C on a wheel for 16 hours. The beads were washed 3 times in buffer G and the bound protein eluted into 2ml of buffer H (by tumbling for 1 hour at 4°C). Figures 5.3.1 and 5.3.1.1 show samples of the original supernatant (lane 1), the supernatant after tumbling with the beads (lane 2) and the 2ml elution (lane 3) which were analysed by SDS PAGE followed by Western blotting (5.3.1) with polyclonal antisera raised against the GST protein or staining with coomassie blue (5.3.1.1).

The Western blot demonstrated that the fusion protein was removed from the supernatant (lane 2) after tumbling with the beads and could be eluted into buffer H (lane 3). The purified 92-kDa fusion protein (arrow) was seen by coomassie staining, together with several smaller bands which were recognised by the anti-GST antisera, suggesting that they had resulted from degradation in the β₁COOH part of the protein. Despite the success of this expression system for other proteins and domains, the fusion protein was only produced at low levels compared with the other bacterial proteins. In order to see the fusion protein by coomassie staining, 60μl of the elution sample had to be loaded onto the gel (the equivalent of 600μl of neat supernatant). Estimates of the amount of full length fusion protein on the gel suggested that it was at a concentration of about 2μg/ml.
FIGURE 5.3.1 Binding GST-β₁COOH to Glutathione-Sepharose.

20ml of Triton-soluble supernatant prepared from pGEX-β₁COOH transformed BB4 bacteria was incubated with 1ml of glutathione-Sepharose beads at 4°C for 16 hours as described in the text. The supernatant was removed from the beads which were washed as described and the bound protein was eluted into 2ml. Samples of the original supernatant (1), the supernatant after incubation with the beads (2) and of the final elution (3) were separated by SDS-PAGE on an 8% polyacrylamide gel. The proteins were transferred to nitrocellulose and probed with the anti-GST antisera. The molecular weight markers on the right represent 200, 97 and 69 kDa and the arrow indicates the full length GST-β₁COOH fusion protein.
FIGURE 5.3.1.1 Binding GST-\(\beta_1\)COOH to Glutathione-Sepharose.

Samples from the same extracts that were probed in figure 5.3.1 were separated by SDS-PAGE as before and stained with coomassie blue. Lane 1 contained the original supernatant, lane 2 the supernatant after incubation with beads and lane 3 the eluted protein. The molecular weight markers represent 200, 116, 97, 69 and 45 kDa and the arrow indicates the purified full length GST-\(\beta_1\)COOH fusion protein.
The yield of purified fusion protein from 1 litre of culture was approximately 4μg. The reconstitution assay was designed to accommodate 5μl of 'inhibitor', which, based on the estimated sample concentration, would allow no more than 0.1 pmoles of the fusion protein to be added to the assay. This meant that the sample would have to be concentrated if it was to be used as an inhibitor in the reconstitution assay.

To process the increased volume of supernatant required to generate enough fusion protein and allow elution of the bound fusion protein into a smaller volume, a 1ml column of glutathione-Sepharose beads was prepared and washed with 5ml buffer G. The supernatant (40ml) containing the expressed fusion protein was recirculated through the column for 16 hours using a pump. The column was washed in buffer G and the remaining bound protein eluted using 2ml buffer H washed through with buffer G. Fractions of 0.5ml were collected and analysed by SDS PAGE. The silver stain (fig 5.3.2) of the elution profile revealed that the fusion protein eluted from fraction 3 onwards. Although the fusion protein (long arrow) was more concentrated in the column fractions than it had been in the previous batch elution, the preparation appeared to be less pure (the small arrow marks the position of a large contaminating band). For this reason several other chromatography columns were tested for their ability to concentrate and purify the fusion protein.
FIGURE 5.3.2 1ml Glutathione-Sepharose column.

40ml of supernatant prepared from GST-β₁COOH expressing bacterial cultures were re-circulated through a 1ml glutathione-Sepharose column for 16 hours. The column was washed and the bound protein eluted in buffer G at a flow rate of 0.5ml/min into 5 1ml fractions (1-5). Samples of the fractions were separated by SDS-PAGE on an 8% polyacrylamide gel, which was silver stained. The molecular weight markers on the left represent 116, 97, 69 and 45 kDa. The arrows indicate full length GST-β₁COOH (upper) and alarge contaminating protein band (lower)
### 5.3.2 1ml Mono Q 5/5

Supernatant containing the fusion protein was prepared as previously and diluted 1:1 in buffer A, to reduce the viscosity of the sample prior to loading onto the column. 20ml of this sample were loaded at 0.5 ml/min onto a 1ml Mono Q column which had been pre-equilibrated in buffer A. The column was washed with buffer A until the base-line of the A$_{280}$ trace had been restored. The bound protein was eluted using a 20 ml 0 - 1.0 M NaCl gradient. The elution had to be terminated at 890 mM NaCl due to the column overpressuring (a common problem when purifying bacterial extracts on such a column). Samples of the 0.5 ml fractions were analysed by Western blotting using the anti-GST antisera (fig 5.3.3). Evidently much of the fusion protein in the load did not bind to the column and was detected in the run-through (lane 4), although the protein that had bound eluted in a peak between 300 and 500 mM NaCl (lanes 6, 8, 10 and 12). Comparison of the A$_{280}$ traces (fig 5.3.4) for purification of control extract (open circles) and extract containing the fusion protein (closed circles) revealed a sharp peak of protein eluting in fraction 16 (400mM NaCl) of the fusion protein purification but not in the control purification. This peak in the protein profile corresponded with the peak of fusion protein identified by Western blotting.

The Mono Q gave a good purification of the fusion protein, but the capacity of the column was too low for 10 ml of supernatant to be purified on it. Thus even an 8ml Mono Q (used to purify 10 ml of PtdIns-PLC δ1 supernatant) would not have had the capacity to allow purification of large volumes of GST-β1COOH supernatant.
FIGURE 5.3.3 1ml Mono Q Western blot

20ml of supernatant prepared from GST or GST-β₁COOH expressing bacterial cultures were chromatographed on a 1ml mono Q column. Samples of the 0.5ml fractions were separated by SDS-PAGE on 8% polyacrylamide gels, transferred to nitrocellulose and probed with anti-GST antisera. The odd numbered lanes contained fractions from the GST extract and the even numbered lanes contained fractions from the GST-β₁COOH extract. Lanes 1 and 2 contained samples of the supernatants prior to separation on the column and lanes 3 and 4 contained samples of the pooled flow-through fractions. The other lanes contained fractions from the column (lanes 5 and 6 fraction 13, lanes 7 and 8 fraction 15, lanes 9 and 10 fraction 17, lanes 11 and 12 fraction 19 and lanes 13 and 14 fraction 21). The molecular weight markers on the left of each blot represent 200, 97, 69 and 45 kDa and the arrow indicates the GST-β₁COOH fusion protein.
FIGURE 5.3.4 1ml Mono Q column profile.

The $A_{280}$ traces of the Mono Q purifications of GST (open circles) and GST-$\beta_1$COOH (filled circles) described in figure 5.3.3 are shown. The protein elution from the GST-$\beta_1$COOH purification peaks at fraction 16, agreeing with the data from the Western blot.
5.3.3 High capacity columns

The partially successful purification achieved on the 1ml Mono Q column led to the trial of a high capacity Q-Sepharose column. 100ml of supernatant was easily loaded onto this column, but very little of the fusion protein actually bound to it. However, the fusion protein did bind to a high capacity S-Sepharose column (equilibrated in buffer B) from which it could be eluted into two 5ml fractions on a 1M NaCl gradient. Unfortunately, the pH 5.5 buffer (B) used in this purification caused the protein to irreversibly coagulate when dialysed or diluted into a neutral buffer. Fig 5.3.5 shows that most of the protein eluted from the S-sepharose column (L) was precipitated and appears in the particulate fraction (P), leaving very little in solution (S). When the purification was performed at pH 7.0 to avoid this problem, very little of the fusion protein bound to the column.

5.3.4 1ml Heparin-Sepharose

Finally, a 1ml Heparin-Sepharose column pre-equilibrated at pH 7.5 was tested. Supernatant (3ml) containing GST-β1COOH was loaded at 0.5 ml/min onto a 1ml heparin-Sepharose column in buffer A. The column was washed with 5 ml of buffer A and the bound protein eluted with a 10 ml 0-1.0 M NaCl gradient. Samples of the 0.5 ml fractions were analysed by SDS PAGE (fig 5.3.6). The Western blot, probed with anti-GST antisera, revealed that all of the fusion protein in the load had bound to the column and been eluted into 3 fractions at 700 mM NaCl (fractions 16, 17 and 18). The silver stain (fig 5.3.6.1) of fraction 16 showed that although a significant amount of contaminating protein had been removed during the purification, the fusion protein was still not very pure. For this reason a two-step purification was devised, using a 1ml glutathione-Sepharose column (see 5.3.1) followed by a 1ml heparin-Sepharose column. Figure 5.3.7 shows the Western blot of the glutathione-Sepharose column with the lower arrow again marking the position of the large contaminating band which was observed by ponceau staining (see fig 5.3.2 for the silver stain).
FIGURE 5.3.5 Western blot of S-Sepharose pools.

100ml of GST-β₁COOH supernatant (L) were chromatographed on a high capacity 50ml S-Sepharose column at pH 5.5. Fractions of 5ml were collected and the peak of eluted GST-β₁COOH identified by western blotting samples of the fractions. The peak fractions were pooled and dialysed into a pH 7.5 buffer. The resulting soluble (S) and particulate (P) fractions were analysed by SDS-PAGE on an 8% polyacrylamide gel, followed by Western blotting and probing with anti-GST antisera. The molecular weight markers on the left represent 200, 97, 69 and 45 kDa and the arrow indicates the GST-β₁COOH fusion protein.
FIGURE 5.3.6 Western blot of 1ml Heparin-Sepharose.

3ml of GST-β₁COOH supernatant (lane 1) were chromatographed on a 1ml heparin-Sepharose column as described in the text and the 0.5ml fractions collected were sampled and separated by SDS-PAGE on an 8% polyacrylamide gel. The proteins were Western blotted and probed with anti-GST antisera. Lane 2 contained a sample of the pooled flow-through fractions and lanes 3-11 contained fractions 4, 6, 8, 10, 12, 14, 16 and 18 respectively. The molecular weight markers represent 200, 97 and 69 kDa and the arrow indicates the GST-β₁COOH fusion protein.
FIGURE 5.3.6.1 Silver stain of 1ml Heparin-Sepharose peak fraction.

A sample of the peak fraction from the 1ml heparin-Sepharose separation, 16, was separated by SDS-PAGE on an 8% polyacrylamide gel and the proteins were silver stained. The molecular weight markers represent 200, 116, 97, 69 and 45 kDa and the arrow indicates the GST-β₁COOH fusion protein identified by Western blotting.
FIGURE 5.3.7 Western blot of 1ml Glutathione-Sepharose column.

40ml of GST-β1COOH supernatant were recirculated through a 1ml glutathione-Sepharose column for 16 hours and eluted as described in 2.2.6 into 1ml fractions. Samples of these fractions were separated by SDS-PAGE on an 8% polyacrylamide gel, transferred to nitrocellulose and probed with anti-GST antisera. Fractions 2-4 are shown. The molecular weight markers represent 200, 116, 97 and 69 kDa and the upper arrow indicates the immunoreactive GST-β1COOH. The lower arrow indicates the position of the contaminating protein band seen in figure 5.3.2, which was visible by ponceau staining of the blot prior to probing with the antisera.
Figure 5.3.8 shows the elution profile of a 1ml heparin-Sepharose at the end of such a two-step purification of 40 ml of GST-β1COOH supernatant, the large contaminating band having been removed. The peak fractions from this column were pooled and subjected to buffer exchange and concentration into buffer J, using the Amicon centrifcon concentrators. A sample from this final concentrated pool was Western blotted and silver stained to test its integrity (fig 5.3.9). Total purification of the full length fusion protein away from degraded fusion protein was not possible, as GST and its fusion proteins form strong homo or hetero-dimers, preventing the use of purifications based on size selection.

5.4 Inhibiting Gαq activation of PtdIns-PLCβ1

Prior to the purification of the GST fusion, the ability of crude bacterial lysates from control cells (transformed with vector alone) and cells transformed with pGEX-β1COOH to inhibit Gαq directed activation of PtdIns-PLCβ1 was assessed. The coupling assay was set up as described in 2.2.2.2 with the supernatants diluted to a total protein concentration of 0.5 mg/ml in 50mM Hepes pH 7.0. The effect of the two supernatants on PIP2 hydrolysis was compared with that of the Hepes dilution buffer (fig 5.4). The control supernatant (2) had no inhibitory effect, whereas the supernatant containing GST-β1COOH (3) gave a 25% inhibition of the AIF induced activation. This preliminary experiment demonstrated that GST-β1COOH was able to inhibit the Gαq activation of PtdIns-PLCβ1.

Having purified the GST fusion of the PtdIns-PLCβ1 carboxyterminus, the effect of this domain on Gαq directed activation of full length PtdIns-PLCβ1 was further investigated. The GST-β1COOH preparation was titrated into the reconstitution assay described in 4.3.1. and the PtdIns-PLC activity was measured (fig 5.4.1). The GST-β1COOH preparation was diluted in buffer J, which was also used for the zero GST-β1COOH point. As a control, equivalent amounts of purified GST protein were tested in the assay, but found to have no effect.
FIGURE 5.3.8 Western blot of 1ml Heparin-Sepharose.

The fractions containing GST-β₁COOH from the 1ml glutathione-sepharose column were pooled and diluted into buffer A. The pool was then chromatographed on a 1ml heparin-Sepharose column using a 10ml 0-1M NaCl gradient. Samples of the 0.5ml fractions collected were separated by SDS-PAGE on an 8% polyacrylamide gel, transferred to nitrocellulose and probed with the anti-GST antisera. Lanes 1-5 contained samples of fractions 15-19 where the elution of GST-β₁COOH peaked. The molecular weight markers represent 200, 116, 97 and 69 kDa.
FIGURE 5.3.9 Silver stain and Western blot of purified GST-β1COOH.

The peak fractions from the 1ml heparin-Sepharose column shown in figure 5.3.8 were pooled and samples were separated by SDS-PAGE on an 8% polyacrylamide gel and either Western blotted and probed with anti-PtdIns-PLCβ1 antisera (lane 1) or silver stained (lane 2). The molecular weight markers represent 116, 97, 69, 45 and 31 kDa and the arrow indicates the full length purified GST-β1COOH fusion protein.
FIGURE 5.4 Inhibition of G\(\alpha_q\) activation of PtdIns-PLC\(\beta_1\).

Triton soluble lysates were prepared from GST and GST-\(\beta_1\)COOH expressing cultures and tested for their ability to inhibit G\(\alpha_q\) activation of bacterially expressed PtdIns-PLC\(\beta_1\) in the reconstitution assay described in 2.2.2.2. 5\(\mu\)l of dilution buffer (1), GST lysate (2) or GST-\(\beta_1\)COOH lysate (3) were included in the standard 60\(\mu\)l assay and the PtdIns-PLC activity was measured in the absence (filled bars) or presence (striped bars) of 10\(\mu\)M AIF\(^{4+}\). The two lysates were diluted to the same total protein concentration (1mg/ml) before being tested in the assay. The values plotted are the means of duplicate reactions and the variance between duplicates is indicated by the error bars.
FIGURE 5.4.1 Titration of purified GST-β1COOH.

The purified GST-β1COOH fusion protein was titrated into the Gαq reconstitution assay and the PtdIns-PLC activity was measured in the absence (filled circles) or presence (empty circles) of 10μM AlF4⁻. The values plotted are the means of duplicate reactions and the variance between duplicates is indicated by the error bars.
The GST-\(\beta_1\)COOH was able to completely inhibit the PtdIns-PLC\(\beta_1\) activity seen in the presence of the activatory AIF\(^4^+\), with an \(I_50\) value of 2nM but did not affect the 'background activity' seen in the absence of AIF\(^4^+\). This implied that the inhibitory effect was exerted on the G-protein stimulated activity, confirming that the carboxyterminus of PtdIns-PLC\(\beta_1\) can interact with G\(\alpha\)q and presumably through this interaction is able to activate the enzyme.

**5.5 Identification of an inhibitory peptide**

In an effort to overcome some of the disadvantages of using crudely purified membranes as a source of G\(\alpha\)q in the reconstitution assay (see section 4.5), an alternative approach based upon work done by Rarick et al., [1992], whilst studying the activation of phosphodiesterase (PDE) by transducin, was tested. PDE is normally activated by the GTP bound \(\alpha\) subunit of the heterotrimeric G-protein transducin (G\(\alpha\)t). Using a series of deletion mutants of G\(\alpha\)t they identified the region of this protein which was required to activate its effector (PDE) and were able to use a 22 amino acid peptide from this region to activate PDE by 40% in vitro.

To look for such a region in G\(\alpha\)q an alignment of several G-protein \(\alpha\) subunit sequences was made using Clustal [Genetics Computer Group, 1991] (fig 5.5). This revealed that in the region of G\(\alpha\)t that contained the activatory 22 amino acid sequence, the amino acid sequences of G\(\alpha\)q and G\(\alpha\)11 were absolutely conserved. This was interesting because both proteins are members of the Gq family of G-proteins and both have the same specificity with regard to the PtdIns-PLC\(\beta\) isoymes (activating PtdIns-PLC\(\beta_1\) the most strongly). A 25 amino acid peptide was made from the sequence in this region and tested for its ability to substitute for the G\(\alpha\)q membranes in the reconstitution assay. The effect of the peptide on the standard reconstitution assay using G\(\alpha\)q membranes as the activator was also investigated (fig 5.5.1). Although the peptide had no effect on PtdIns-PLC activity in the absence of activatory G\(\alpha\)q membranes (1), it had an inhibitory effect on the G\(\alpha\)q mediated activation of PtdIns-PLC\(\beta_1\) (3).
FIGURE 5.5 Alignment of Heterotrimeric G-protein α subunit sequences

The amino acid sequences of 24 heterotrimeric G-protein α subunits were aligned using the GCG programme CLUSTAL. The amino termini of those aligned sequences are shown, with asterisks indicating absolutely conserved residues and stops indicating residues which are similar in all sequences. All of the known members of the Gq family are included: αq (GQALPH), α11 (G11ALPH), α14 (G14), α15 (G15) and α16 (G16). The underlined sequences in GQALPH and G11ALPH represent the sequence of the peptide used in the experiments described in 5.5.
FIGUR 5.5.1 Testing the effects of the Gαq peptide.

The Gαq peptide was added to the reconstitution assay at 167μM, in the absence (1) or presence (2 and 3) of Gαq membranes. The empty bar of 1 and both bars of 2 represent reactions performed in the absence of the Gαq peptide. The PtdIns-PLC activity was measured in the absence (striped bars) or presence (filled bars) of 10μM AlF4—. The values plotted are the means of duplicate reactions and the variance between duplicates is indicated by the error bars.
A titration of the peptide (fig 5.5.2) showed that 40% inhibition was achieved at approximately 300µM. Increasing the peptide concentration further, did not increase the inhibition beyond 50%. This result suggested that the Gαq peptide was able to compete with Gαq for a site on PtdIns-PLCβ1 which is involved in the activation of that enzyme. The failure of the peptide alone to activate PtdIns-PLCβ1 indicated that a larger region than the 22 amino acid peptide of Gαq is involved in the activation process. The fact that the inhibition by the Gαq peptide was never greater than 50% may be due to the existence of another AIF4-dependent PtdIns-PLC activation pathway in the reconstitution assay. Alternatively, it may suggest that there is more than one site of activation on the PtdIns-PLCβ1 protein, to which Gαq binds during the activation process, which is consistent with the lack of an activatory effect of the peptide.

5.6 The effect of basic peptides on PtdIns-PLCβ1 activation

To control for the effect of the Gαq peptide described in 5.5, a peptide denoted PSL was tested in the reconstitution assay. This peptide had been made as a pseudosubstrate peptide from PKCα and was chosen as a control because it had been HPLC purified in the same manner as the Gαq peptide. When added to the assay at 167µM it caused a 3-fold enhancement of the AIF4-dependent PtdIns-PLCβ1 activation (fig 5.6).

The sequence of PSL: NH2-AAYADFIAAGRAARRNSVRRG-COH2 contains many basic residues (5 Arginines) with a predicted α helical region at the aminoterminus. A variant of this peptide - PSS: NH2-GRAARRNSVRRG-COH2, lacked the α helical region at the aminoterminus and was therefore compared with PSL in the reconstitution assay, to establish whether the effect was mediated through the α helical or the basic region of the sequence (fig 5.6.1). The PSS peptide demonstrated the same if not better activatory effect as the PSL peptide, indicating that the high concentration of basic residues in the two peptides was probably responsible for the effect on the reconstitution assay. Controls containing no membrane or no PtdIns-PLCβ1 were not affected by the peptides, demonstrating that the activatory effect of the peptide was dependent upon the presence of both components of the assay.
FIGURE 5.5.2 Titration of the Gαq peptide.

The Gαq peptide was titrated into the reconstitution assay and the PtdIns-PLC activity in the absence and presence of 10μM AlF4− was measured. The activation was calculated as the difference between the activities in the absence and presence of AlF4−. Full activation was defined as that in the absence of peptide and the % of total activation was plotted for each concentration of peptide.
FIGURE 5.6 Measuring the activatory effect of PSL.

The PtdIns-PLC activity of the bacterially expressed PtdIns-PLCβ1 was measured in the Gαq reconstitution assay in the absence (empty bars) or presence (filled bars) of 10μM AlF₄⁻. The assays were performed with (2) or without (1) 166μM PSL (see text). The values plotted are the means of triplicate reactions and the variance is indicated by the error bars.
FIGURE 5.6.1 Comparing the effects of PSL and PSS.

The PtdIns-PLC activity of the bacterially expressed PtdIns-PLCβ₁ was measured in the Gₛq reconstitution assay in the absence (empty bars) or presence (filled bars) of 10μM AlF₄⁻. The assays were performed in the absence (1) or presence of 166μM PSL (2) or PSS (3). The values plotted are the means of triplicate reactions and the variance is indicated by the error bars.
To exclude the possibility that PSS was acting on a component of the membranes other than Goq, a titration of control and Goq membranes was performed in the presence of PSS (fig 5.6.2). The A1F4-dependent activation of PtdIns-PLCβ1 by either membrane preparation was increased in the presence of PSS, however the previously observed difference between the level of activation with each membrane preparation was conserved. This suggested that PSS exerted its effect through both the endogenous Goq in control cells and the overexpressed Goq, but not through any other component of the membranes.

The effect of these basically charged peptides was compared with the effects of titrating polyarginine and spermine into the reconstitution assay (fig 5.6.3 and 5.6.4 respectively). The polyarginine had an activatory effect which was independent of A1F4-activation and peaked at about 30nM. The spermine effect was dependent upon A1F4-activation, peaking at 83µM. When included in the reconstitution assay at this concentration, spermine did not interfere with the % inhibition observed upon addition of the β1COOH fusion protein, demonstrating that it is able to increase the signal to background ratio in the assay, without appearing to alter the interaction between the activatory G-protein α subunit and the PtdIns-PLC.

The effect of the PSS peptide (167 µM) was tested on PtdIns-PLCβ1 and δ1 activation in the standard cholate assay (fig 5.6.5). An activatory effect was observed when PSS was added to either of the isozymes in the assay, but the effect was much less than that previously observed in the reconstitution assay.
FIGUR 5.6.2 Membrane titrations in the presence of PSS.

Membrane preparations from COS-1 cells transfected with CMV vector (squares) or Gαq (circles) were titrated into the reconstitution assay containing the bacterially expressed PtdIns-PLCβ1 and PSS at 166μM. The PtdIns-PLC activity was measured in the absence or presence of 10μM AlF4⁻.
FIGURE 5.6.3 Poly Arginine titration.

Poly arginine was titrated into the Goq reconstitution assay and the PtdIns-PLC activity of PtdIns-PLCβ1 was measured in the absence (open circles) or presence (closed circles) of 10μM AlF4−. The values plotted are the means of duplicate results.
FIGURE 5.6.4 Spermine Titration.

Spermine was titrated into the Gαq reconstitution assay and the PtdIns-PLC activity of PtdIns-PLCβ1 was measured in the absence (open circles) or presence (closed circles) of 10μM AlF4-. The values plotted are the means of duplicate results.
FIGURE 5.6.5 Effect of PSS on PtdIns-PLC activity in the cholate assay.

PtdIns-PLCβ₁ (1) and PtdIns-PLCβ₁ (2) were assayed in the standard cholate assay described in 2.2.2.1 in the absence (empty bars) or presence (filled bars) of 166μM PSS. The values plotted are the means of duplicate assays and the variance is indicated by the error bars.
The results obtained with polyarginine suggested that a high concentration of basic charge can interfere with the assay itself, raising the background of PtdIns(4,5)P₂ hydrolysis in the absence of AlF₄⁻ activated Gαq. This effect is probably due to the alteration of substrate presentation, possibly by relieving the suppression of PtdIns-PLC activity by the lipids in the assay. The effects seen with spermine and the two pseudosubstrate peptides seem to be more specific, acting only on the AlF₄⁻ stimulated Gαq activation of PtdIns-PLCβ₁ and may genuinely reveal something about the mechanisms involved in the activation process. The small effect on the cholate assay suggested that the effect of these basic peptides is probably exerted on the intrinsic catalytic activity of the PtdIns-PLC enzymes, but is not able to overcome the suppression in the reconstitution assay in the absence of AlF₄⁻ stimulated Gαq.

General Discussion

The low yield of GST-β₁COOH expressed in E.coli meant that large quantities of bacteria had to be harvested and extracted to produce sufficient quantities of fusion protein for the experiments to be performed. Although some proteins can be expressed at high levels in the pGEX-2T system, it is not uncommon to experience the problem of low expression levels. The size of a GST fusion protein has been shown not only to influence the expression levels of soluble fusion protein, but also its ability to bind to glutathione-Sepharose beads, during purification [Frangioni and Neel, 1993]. Thus the larger the construct, the harder it is to obtain large amounts of purified GST fusion protein. Another factor which contributed to the low yields of GST-β₁COOH was the 'clipping' in the PtdIns-PLCβ₁ C-terminus which is known to occur in the full length protein [Suh et al., 1988c] and does not seem to be prevented by the cocktail of protease inhibitors included in the extraction buffers, suggesting that it occurs in vivo, before extraction.

The two step purification enabled large volumes of starting extract to be concentrated and purified, although the strong propensity of GST fusion proteins to form dimers prevented the purification of the full length fusion protein away from its degradation products. The presence of partly degraded fusion protein in these preparations meant that
the concentration of full length fusion protein could only be estimated from silver stained
gels. For this reason the results of the inhibition experiments have to be viewed as more
qualitative than quantitative.

Despite these problems, the fusion protein was successfully used to inhibit PtdIns-
PLCβ1 activation by Gαq in the established reconstitution assay. This inhibition was
competitive, indicating that the full length PtdIns-PLCβ1 and the C-terminal fusion protein
were both competing for the same site on the Gαq subunit. This confirmed the idea that the
C-terminus of PtdIns-PLCβ1 was the region which interacted with Gαq, leading to the
activation of the enzyme and PtdIns(4,5)P2 hydrolysis.

During the course of these studies, Wu et al., [1993a] used a different approach,
co-expressing different deletions mutants of PtdIns-PLCβ1 with Gαq in COS-7 cells and
measuring PtdIns(4,5)P2 turnover. Using this method they also demonstrated that the C-
terminus of PtdIns-PLCβ1 was required for its activation and went on to demonstrate that
the C-terminus could be subdivided into two domains - P and G. The P region (residues
903 to 1030) was shown to be required for membrane association, whereas the G region
(residues 1030 to 1142) was required for activation by Gαq. The drawback of this loss of
function approach was that there was no way of assessing whether the introduced deletions
simply caused mis-folding of the protein and prevented it from coupling to Gαq
artificially.

Park et al., [1993b] avoided this problem by using two C-terminally clipped species
of PtdIns-PLCβ1, which had previously been observed during the purification of the full
length enzyme. For this experiment the 100 and 140-kDa proteins were generated by
cleaving the full length protein with calpain. They demonstrated that although both the full
length and truncated proteins retained full catalytic activity in the standard cholate assay,
only the 150-kDa protein could be fully activated by Gαq in vitro. The 140-kDa protein
could be partially activated in the presence of Gαq, whereas the 100-kDa protein could not
be activated at all in this assay. They concluded from these results that the region of PtdIns-
PLCβ1 required for its activation by Gαq is located in the C-terminus beyond residue 881
(where calpain cleaves the enzyme) and that the last 10-kDa of the C-terminus is not
absolutely required for activation, although full activation is only achieved with the intact 150-kDa enzyme.

In addition to this growing body of evidence that the C-terminus of the PtdIns-PLCβ isozymes was the region of interface with the G-protein α subunits, the discovery that the Gαq peptide was able to inhibit the activation process suggested that the C-terminus of Gαq contained a region which was able to interact with its effector, PtdIns-PLC. This had previously been demonstrated for Gαq in its activation of PDE and it now seems likely that many G-protein α subunits contain the regions required for effector activation within their C-termini [Berlot and Bourne, 1992]. The inability of the Gαq peptide to inhibit PtdIns-PLCβ1 activation by more than 50% suggested that, in the case of Gαq, there may be more than one point of contact with the effector. This could explain why, in the experiments conducted by Park et al., loss of the most C-terminal 10-kDa of PtdIns-PLCβ1 causes a reduction but not total ablation of its activation by Gαq.

Unlike in the Gαt/PDE system, the short Gαq peptide tested was not sufficient to activate PtdIns-PLCβ1, which may be a reflection of the different levels of complexity of the activating mechanisms employed in the two systems. Gαt is able to activate PDE (composed of αβ and two γ subunits) by binding to and subsequently removing the inhibitory γ subunits away from the αβ subunits. There is no evidence that PtdIns-PLCβ is composed of subunits and is therefore unlikely to be activated in the same manner, although it is possible that the enzyme is held in an inactive conformation by an inhibitory sub-domain, similar to the mechanism suggested for PtdIns-PLCγ regulation, by Rhee [1991]. The GTP charged Gαq could then bind to the C-terminus of the PtdIns-PLC and activate it by altering the interaction with the putative inhibitory sub-domain.

The results obtained with the basic peptides are difficult to interpret but suggest that charge plays an important role in the activation mechanism. It remains to be determined whether basic proteins such as spermine are involved in the activation process in vivo. The effects of basic proteins on PtdIns-PLCδ1 activity have been well documented [Haber et al., 1991], with spermine having an activatory effect at similar concentrations to those tested in the reconstitution assay. Clearly the activatory effect of spermine and the basic
peptides in the reconstitution assay was not achieved by directly mimicking the $G_{\alpha q}$ activation process, as spermine alone was unable to activate the PtdIns-PLC. The magnified activation seen in the presence of spermine could be inhibited to the same extent, with the C-terminal fusion protein, as the standard activation seen in the absence of spermine, indicating that the effect is probably exerted on the catalytic activity of the PtdIns-PLC once it has been activated. The small activatory effect of PSS on the cholate assay may be achieved through the same mechanism. As spermine is present in the cell at the concentrations used in these experiments, it will be interesting to see whether the effects observed in vitro are relevant to PtdIns-PLC activity in vivo.
CHAPTER 6 - THE POTENTIAL ROLE OF PHOSPHORYLATION IN PtdIns-PLCβ1 ACTIVATION

Introduction

The successful expression of both full length and C-terminal PtdIns-PLCβ1 in bacteria provided the tools with which to investigate the effects of the reported PKC phosphorylation of this isozyme \textit{in vitro}. Proteins expressed in bacteria were not believed to be post-translationally modified, yet it had been demonstrated that eukaryotic PtdIns-PLCβ1 was a phospho-protein \textit{in vivo} [Ryu et al., 1990], which became further phosphorylated when cells were stimulated with TPA. Ryu et al., identified a site in the C-terminus of PtdIns-PLCβ1 which they believed was phosphorylated by PKC \textit{in vitro}, the C-terminal fusion protein included this region of the protein and was therefore expected to be a substrate for PKC phosphorylation. Therefore attempts were made to phosphorylate both the full length bacterially expressed PtdIns-PLCβ1 and the fusion protein \textit{in vitro} to allow the effect of this modification on PtdIns-PLC activity to be investigated.

Results and Discussion

6.1 PKC phosphorylation bacterial PtdIns-PLCβ1

A phosphorylation reaction was set up (described in 2.2.8) to contain 2.5 units of purified PKC α, β or γ which was incubated with 30ng (0.2pmoles) of purified full length bacterial PtdIns-PLCβ1 or buffer and $^{32}$P labelled ATP at a specific activity of 3500 cpm/pmole for 5 minutes. The reactions were quenched in Laemmli sample buffer and electrophoresed on a 7% polyacrylamide gel. The gel was dried and exposed to film for 20 hours (fig 6.1). A faint band (arrow) of 150-kDa (presumed to represent phosphorylated PtdIns-PLCβ1) was seen in all the samples containing PtdIns-PLCβ1 (lanes 2, 4 and 6), the strongest band being visible in the sample that had been treated with PKCβ1 (lane 4).

The stoichiometry of the phosphorylation was calculated by measuring the counts incorporated into the 150-kDa band, giving a ratio of 0.05 moles of phosphate/mole of PtdIns-PLCβ1, which is a very low value.
FIGURE 6.1 PKC Phosphorylation of bacterially expressed PtdIns-PLCβ1.

30ng of PtdIns-PLCβ1 were treated with three different purified PKC preparations as described in the text, for 5 minutes. The reactions were mixed with Laemmli sample buffer and separated by SDS-PAGE on a 7% polyacrylamide gel. The gel was dried and autoradiographed for 20 hours. Reactions 1 and 2 were incubated with PKCα, reactions 3 and 4 with PKC β1 and reactions 5 and 6 with PKC γ. The incubations were performed in the absence (1, 3 and 5) or presence (2, 4 and 6) of bacterially expressed PtdIns-PLCβ1. The molecular weight markers represent 200, 97 and 6 kDa and the arrow indicates a faint band of phosphorylated PtdIns-PLCβ1 in lanes 2, 4 and 6.
In a separate experiment, 1pmole of purified GST-\(\beta_1\)COOH or GST was incubated with PKC \(\beta_1\) as before, at 30\(^\circ\)C for 15 minutes with \(^{32}\)P labelled ATP at a specific activity of 3100 cpm/pmole. The reactions were quenched in 6 reaction volumes of buffer K and mixed with 1 reaction volume of glutathione-Sepharose beads (equilibrated 1:1 in buffer H). After tumbling the samples on a wheel at 4\(^\circ\)C for 1 hour they were centrifuged, the supernatant removed and the beads were then washed 3 times in buffer N. The beads were resuspended in Laemmli sample buffer and boiled for 10 minutes, after which they were centrifuged again allowing the supernatant to be loaded onto an 8\% polyacrylamide gel. After separating the samples by electrophoresis, the gel was dried and exposed to film for 112 hours. A faint band (arrow) of the size of the GST-\(\beta_1\)COOH fusion protein could be seen (fig 6.1.1) only in the sample containing the fusion protein (lane 2). The level of phosphorylation in this band was so low that the stoichiometry could not be calculated.

As the levels of phosphorylation seen with PKC \(\beta_1\) were so low, an alternative PKC preparation was tested for its ability to phosphorylate the PtdIns-PLC\(\beta\) proteins. PKC \(\alpha\beta\gamma\) mix was incubated with \(^{32}\)P ATP at a specific activity of 2500 cpm/pmole and 1.3pmoles of full length PtdIns-PLC\(\beta_1\) or 2pmoles of either GST-\(\beta_1\)COOH or GST. The 5 minute reaction was terminated by addition of Laemmli sample buffer, followed by separation on an 8\% polyacrylamide gel. Autoradiography was then performed on the dried gel (fig 6.1.2). After 15 hours a faint band was visible just above the band of autophosphorylated PKC at about 96-kDa (arrow) in the GST-\(\beta_1\)COOH sample which had been treated with PKC. This band was not visible in the other lanes and the stoichiometry of this phosphorylation was estimated to be no better than the previously calculated ratio of 0.05 moles phosphate / mole of protein.

Another strongly phosphorylated band could be seen between the 200 and 97-kDa markers in the lanes containing full length PtdIns-PLC\(\beta_1\) - exposure of the autoradiograph for just 1 hour (fig 6.1.3) was enough to see the band in the PKC treated sample (lane 1). However, this strongly phosphorylated band appeared to be too small to represent the full length PtdIns-PLC\(\beta_1\) protein and was therefore thought to represent partially 'clipped' PtdIns-PLC\(\beta_1\) protein.
FIGURE 6.1.1 Phosphorylation of pGEX-β1COOH by PKC.

PKC β1 was incubated with purified GST (1), purified GST-β1COOH (2) or dilution buffer (3) as described in the text, for 15 minutes. The reactions were quenched and incubated with glutathione-Sepharose beads for 1 hour at 4°C. After washing the beads 3 times, the bound protein was eluted in Laemmli sample buffer. The samples were separated by SDS-PAGE on an 8% polyacrylamide gel which was dried and autoradiographed for 112 hours. The molecular weight markers represent 200, 97 and 69 kDa and the arrow indicates a very faint band in lane 2, though to be the GST-β1COOH fusion protein.
FIGURE 6.1.2 Phosphorylation with PKC αβγ mix.

PKC αβγ mix was incubated with dilution buffer (1), GST-β1COOH (2) or GST (3) as described in the text and the 5 minute reactions were terminated by the addition of Laemmli sample buffer. The reactions were separated by SDS-PAGE on an 8% polyacrylamide gel which was dried and autoradiographed for 15 hours. The molecular weight markers represent 200, 97 and 69 kDa and the arrow indicates a faint band of phosphorylated GST-β1COOH fusion protein.
FIGURE 6.1.3 Phosphorylation of PtdIns-PLCβ1 by PKC αβγ mix.

PKC αγβ mix was incubated with PtdIns-PLCβ1 (1) or buffer (2) for 5 minutes as described in the text. The reactions were terminated by addition of Laemmli sample buffer and the samples were separated by SDS-PAGE on an 8% polyacrylamide gel. The gel was dried and autoradiographed for 1 hour. The molecular weight markers represent 200, 97, 69 and 45 kDa and the arrow indicates a 140-kDa band which was highly phosphorylated after the PKC treatment.
A time course was performed, incubating the full length PtdIns-PLCβ₁ for 10, 15, 30 and 45 minutes with PKC αβγ mix and processing the samples as before (fig 6.1.4). The Mwt of the phosphorylated protein (arrow) was estimated from the autoradiograph to be approximately 140-kDa - the same size as one of the known partial degradation products of PtdIns-PLCβ₁. The level of phosphorylation in this band was quantified by cutting out the strips of gel underneath the autoradiograph bands and counting them (fig 6.1.5). This confirmed that the phosphorylation was almost complete after 45 minutes incubation (lane 5). Probing Western blots of the PtdIns-PLCβ₁ preparation with the C-terminal antibody prior to incubation with PKC had demonstrated that the full length enzyme was present, yet this 150-kDa protein never appeared as a phosphorylated band on the autoradiographs. One explanation for this was that the phosphorylation caused the enzyme to be 'clipped' to the smaller form. Alternatively, there may have been some of the 'clipped' form already present in the PtdIns-PLCβ₁ preparation (despite the use of the Mono S purification to remove it), which acted as a better substrate for the kinase than the full length protein. It was therefore necessary to determine whether the phosphorylated band was indeed 'clipped' PtdIns-PLCβ₁.

6.2 Establishing the identity of the phosphorylated band

The C-terminal antibody used to probe Western blots for PtdIns-PLCβ₁ was only able to recognise enzyme with an intact C-terminus. Therefore an alternative antiserum which had been raised against an epitope in the catalytic domain, common to all the PtdIns-PLC isoforms, was used to probe for PtdIns-PLCβ₁ which had lost part of the C-terminus. The PtdIns-PLCβ₁ preparation used in the phosphorylation reactions was Western blotted and probed with both antisera in the absence and presence of competing peptide (fig 6.2). Clearly the only specific immunoreactive band seen with either of the antisera was the 150-kDa full length PtdIns-PLCβ₁. This confirmed that only full length PtdIns-PLCβ₁ was initially present in the phosphorylation reactions.
FIGURE 6.1.4 Time course of PKC phosphorylation of 140-kDa band.

1.3 pmoles of PtdIns-PLCβ1 were incubated with PKC αβγ mix in the reaction described in 2.2.8 for 10 (lane 2), 15 (lane 3), 30 (lane 4) and 45 (lane 5) minutes. The reaction seen in lane 1 did not contain PKC and was incubated for 45 minutes in the same assay. The reactions were terminated by addition of Laemmli sample buffer and the samples were separated by SDS-PAGE on a 7% polyacrylamide gel. The gel was dried and autoradiographed for 1 hour. The molecular weight markers represent 200, 97 and 69 kDa and the arrow indicates the phosphorylated 140-kDa band.
FIGURE 6.1.5 Time-course of 140-kDa band phosphorylation.

The phosphorylated 140-kDa band from each of the time-points shown in figure 6.1.4 was cut out of the gel and the $[^{32}\text{P}]$ phosphate contained in each gel slice was measured by scintillation counting. The specific activity of the $[^{32}\text{P}]$ ATP used in the phosphorylation reaction was calculated at 17300 cpm/pmol, enabling the pmoles of ATP incorporated into the 140-kDa protein to be calculated for each time-point and plotted.
FIGURE 6.2 Western blots of PtdIns-PLCβ₁.

Samples of the purified bacterially expressed PtdIns-PLCβ₁ used in the phosphorylation experiments were separated by SDS-PAGE on a 7% polyacrylamide gel, transferred to nitrocellulose and probed with polyclonal antisera against the unique C-terminal epitope of PtdIns-PLCβ₁ (a) or a pantopic epitope common to all PtdIns-PLC isozymes (b) in the absence (-) or presence (+) of competing peptide. The molecular weight markers represent 200, 97 and 69 kDa and the arrow indicates an immunoreactive band of 150-kDa which is recognised by both antisera and competed out by the relevant peptides.
The same experiment was performed on PtdIns-PLCβ1 which had been incubated with PKC for 30 minutes in a 'cold' phosphorylation (using unlabelled ATP). There was no change in the amount of full length PtdIns-PLCβ1 seen with either antisera (fig 6.2.1) after the phosphorylation and the pantopic antiserum (lane 2) did not recognise the 140-kDa band which was normally phosphorylated. This result suggested that the PKC phosphorylatable band of 140-kDa was not related to the PtdIns-PLCβ1 protein and must therefore be an E.coli protein which co-purifies with PtdIns-PLCβ1 and is a good substrate for PKC phosphorylation.

As the stoichiometry for phosphorylation of the 150-kDa PtdIns-PLCβ1 by PKC was so poor, PKC treatment was not expected to affect the activation of the enzyme by Gqα and a brief experiment proved this to be the case.

6.3 Phosphorylation PtdIns-PLCβ1 by MAPKinase

The reported TPA stimulation of PtdIns-PLCβ1 phosphorylation in vivo implied that such a phosphorylation was performed by a kinase which is activated in response to TPA. PKC was the obvious candidate, being directly activated by TPA, however MAP kinase had also been shown to be activated in response to TPA [Adams and Parker, 1991] and was therefore tested for its ability to phosphorylate the bacterial PtdIns-PLCβ1. MAPKinase (0.625 U/ml final concentration) was incubated with 32P ATP at a specific activity of 2500 cpm/pmole and 1.3pmoles of full length PtdIns-PLCβ1 or 2pmoles of either GST-β1COOH or GST. The 5 minute reaction was terminated by addition of Laemmlli sample buffer, followed by separation on an 8% polyacrylamide gel. Autoradiography was then performed on the dried gel (fig 6.3) for 15 hours. A band of 140-kDa was seen in the reaction containing MAPKinase and PtdIns-PLCβ1 and a faint band (arrow) of the size of PtdIns-PLCβ1 was also visible. However, when this experiment was repeated a band of similar intensity was also seen in the control lane containing kinase alone. Thus it was concluded that MAP Kinase was probably not responsible for the TPA stimulated phosphorylation of PtdIns-PLCβ1.
FIGURE 6.2.1 Western blots of PKC treated PtdIns-PLCβ1.

Samples of the bacterially expressed PtdIns-PLCβ1 were incubated with PKC αβγ mix for 30 minutes as described in the text and the reactions were terminated by the addition of Laemmli sample buffer. The samples were separated by SDS-PAGE on a 7% polyacrylamide gel, transferred to nitrocellulose and probed with either the anti-PtdIns-PLCβ1 antisera (2) or the pantopic PtdIns-PLC antisera (2). The molecular weight markers represent 200, 97 and 69 kDa and the arrow indicates full length PtdIns-PLCβ1.
FIGURE 6.3 Phosphorylation of PtdIns-PLCβ₁ by MAPKinase.

MAPKinase was incubated with dilution buffer (1), GST-β₁COOH (2), GST (3) or PtdIns-PLCβ₁ (4) as described in 2.2.8 for 5 minutes. The reactions were terminated by addition of Laemmli sample buffer and separated by SDS-PAGE on an 8% polyacrylamide gel. The gel was dried and autoradiographed for 15 hours. The molecular weight markers represent 200, 97 and 60 kDa and the arrow indicates the faint 150-kDa band.
6.4 Phosphorylation of eukaryotic PtdIns-PLCβ1 by PKC

The failure of the bacterial PtdIns-PLCβ1 proteins to be significantly phosphorylated might have been due to their lack of post-translational modification. It had been shown [Ryu et al., 1990] that all the PtdIns-PLC isozymes were basally phosphorylated in vivo, therefore it seemed possible that such modifications were necessary to 'prime' the enzyme for further modifications during mitogenesis. Ryu et al. had used purified rat brain PtdIns-PLCβ1 in their in vitro studies, therefore a source of eukaryotic PtdIns-PLCβ1 was sought.

6.4.1 Expression and purification of PtdIns-PLCβ1 in COS-1 cells

COS-1 cells were transfected with PMT-2 containing the cDNA for bovine PtdIns-PLCβ1 and grown for 72 hours. Three 20 cm plates of transfected cells were washed in ice cold PBSA and harvested by scraping into 3 ml of buffer D. After homogenisation, the extract was centrifuged at 70,000 rpm for 10 minutes. The supernatant was purified using a 1ml heparin-Sepharose (fig 6.4) followed by a 1ml Mono S as in the purification of the bacterial PtdIns-PLCβ1 (fig 6.4.1). The fractions from the activity peaks of both columns were Western blotted (fig 6.4.2) and revealed that the PtdIns-PLC activity had coeluted with the overexpressed PtdIns-PLCβ1. The peak fractions 30, 31 and 32 (lanes 7, 8 and 9) were pooled for the phosphorylation experiments.

6.4.2 Phosphorylation of PtdIns-PLCβ1 from COS-1 cells

Equal amounts (2 cholate units) of PtdIns-PLCβ1 from E.coli or COS-1 cells were incubated with PKC αβγ in the standard phosphorylation reaction (containing 32P labelled ATP) for 20 minutes and analysed by autoradiography (fig 6.4.3).
FIGURE 6.4 1ml Heparin-Sepharose of COS-1 cell extract.

COS-1 cells expressing PtdIns-PLCδ1 were harvested and extracted as described in the text. The supernatant from the extract was chromatographed on a 1ml heparin-Sepharose column using a 20ml 0-1.0M NaCl gradient (-). A flow rate of 0.5ml/min was used and 0.5ml fractions were collected. Most of the protein (filled circles) was separated from the PtdIns-PLC activity (open squares) which eluted at approximately 550mM NaCl. The fractions containing the activity peak (fractions 23-27) were pooled and diluted into buffer E.
FIGURE 6.4.1 1ml Mono S of 1ml Heparin-Sepharose pool.

The fractions containing the peak of PtdIns-PLC activity from the 1ml heparin-Sepharose column were chromatographed on a 1ml Mono S column in buffer E. A stepped NaCl gradient (-) similar to that used in 4.1.3.3 was used to elute the bound protein (closed circles) away from the PtdIns-PLC activity (open squares). The initial 2.5 ml gradient from 0-0.25M NaCl followed by 5ml of 0.25M NaCl served to remove any 'clipped' PtdIns-PLCβ1. The second 15ml gradient from 0.25-1M NaCl enabled elution of the PtdIns-PLC activity at approximately 480mM NaCl. Fractions 30-33 contained the peak of PtdIns-PLC activity.
FIGURE 6.4.2 Western blot of COS-1 cell PtdIns-PLCδ1 purification.

Samples of fractions 22-27 from the 1ml heparin-Sepharose column (lanes 1-6) and fractions 30-33 from the 1ml Mono S column (lanes 7-10) were separated by SDS-PAGE on a 7% polyacrylamide gel and transferred to nitrocellulose. The blots were probed with the anti PtdIns-PLCβ1 antisera. The molecular weight markers represent 200, 116, 97 and 69 kDa and the arrow indicates the purified PtdIns-PLCβ1 protein. Fractions 30-32 were pooled for the phosphorylation experiment.
FIGURE 6.4.3 Phosphorylation of COS-1 expressed PtdIns-PLCβ₁.

PKC αβγ mix was incubated with dilution buffer (1), the bacterially expressed PtdIns-PLCβ₁ (2) or the COS-1 expressed PtdIns-PLCβ₁ pool (3) as described in the text. The reactions were terminated by addition of Laemmli sample buffer and separated by SDS-PAGE on a 7% polyacrylamide gel. The gel was dried and autoradiographed for 16 hours. The molecular weight markers represent 200, 97, 69 and 45 kDa and the arrow indicates the 140-kDa band in the PKC treated bacterial extract (2).
No phosphorylation of full length PtdIns-PLCβ1 was seen in either of the samples and the phosphorylated 140-kDa phosphorylated band (arrow) was only visible in the bacterial sample (lane 2). This provided more evidence that the 140-kDa phosphorylated band was a bacterial protein and not the shortened form of PtdIns-PLCβ1. The failure of PKC αβγ to phosphorylate either the COS-1 or bacterial PtdIns-PLCβ1 in these experiments implies that this PtdIns-PLC isozyme is not a direct target for PKC phosphorylation, in contrast with the results obtained by Ryu et al.

General Discussion

The failure to phosphorylate PtdIns-PLCβ1 from two sources suggested that it is not a good substrate for PKC phosphorylation in vitro. Several possible explanations for the difference between these conclusions and those of Ryu et al., [1990] are discussed below.

One obvious explanation is that the bacterially expressed enzyme cannot be phosphorylated because it is incorrectly folded, making the target residue inaccessible to PKC. This seems unlikely, as the thorough characterisation of bacterially expressed PtdIns-PLCβ1 showed it to be catalytically intact, suggesting that this PtdIns-PLC isozyme can be folded correctly when made in E.coli. Similarly, the bacterially expressed PtdIns-PLCβ1 exhibited catalytic properties almost identical to that seen with the bovine brain enzyme. More importantly, the bacterial PtdIns-PLCβ1 could be activated by Goq in the reconstitution assay, its performance in this reconstitution of coupling being indistinguishable from that of the purified bovine brain PtdIns-PLCβ1 preparation. This implies that the enzyme is correctly folded when expressed in E.coli and therefore incorrect folding is probably not the reason why it cannot be phosphorylated by PKC in vitro.

It is accepted that bacterial proteins are not post-translationally modified and therefore it is possible that the bacterial PtdIns-PLCβ1 lacks a vital modification which would normally prime it for phosphorylation by PKC. This possibility was essentially ruled out by the failure of PKC to phosphorylate PtdIns-PLCβ1 which had been
overexpressed in COS-1 cells and would have undergone any necessary post-translational modifications.

A more likely explanation is that the target of the PKC phosphorylation could have been another PtdIns-PLCβ isoform. A single *in vitro* phosphorylation experiment performed by A.Carozzi with PtdIns-PLCβ3 purified from HeLa cells [Carozzi et al., 1992] suggested that this isozyme might be a substrate for PKCβ1 phosphorylation. In this experiment the PtdIns-PLC was efficiently phosphorylated with a stoichiometry of >1 mole of phosphate / mole of PtdIns-PLCβ3. It is not clear whether the phosphorylation of PtdIns-PLCβ seen *in vivo* in response to TPA treatment was specific to the β1 isozyme, as the monoclonal antisera used to immunoprecipitate the phosphorylated 150-kDa protein were not tested for their ability to cross-react with the other PtdIns-PLCβ isoforms, which at that time were undiscovered.

The best explanation is that the phosphorylation observed by Ryu et al., may have been performed by another kinase which was a contaminant in the PKC preparation. This would explain why the site identified in PtdIns-PLCβ1 as the target for the observed phosphorylation [fig] does not seem to be a good consensus sequence for PKC phosphorylation. Work by Marais et al., [1990], assessing the ability of different peptides to be phosphorylated by PKC, indicated that the presence of strongly basic residues, in particular arginine, in close proximity to the target residue is required for efficient phosphorylation by PKC. If the number of basic residues is reduced to two or less, the Km for phosphorylation of such a peptide is dramatically increased and the reaction becomes very inefficient. The target sequence in PtdIns-PLCβ1 contains only one basic residue, lysine, in close proximity to the target serine.

If the kinase responsible for phosphorylating PtdIns-PLCβ1 is not PKC, the un-modified bacterial PtdIns-PLCβ1 could be usefully employed to screen fractionated cell extracts for a kinase which can phosphorylate it, enabling the real modulator of PtdIns-PLCβ phosphorylation to be identified.
CHAPTER 7 - REGULATION OF Ptdlns-PLC ISOZYMES BY G-PROTEIN βγ SUBUNITS

Introduction

The discovery that the α subunits of the Gq heterotrimeric G-protein family could activate PtdIns-PLCβ isoymes accounted for in vivo observations that PIP2 hydrolysis was stimulated by GTP and its analogues in place of the normal agonists (see 1.3.6). Experiments with PTX revealed that 2 different G-protein coupled pathways of PtdIns-PLC activation operated in cells, one PTX insensitive and one PTX sensitive. None of the α subunits belonging to the Gq family are substrates for ADP ribosylation by Pertussis toxin and the already known Pertussis toxin sensitive G-protein α subunits (αi, αo and αt) are unable to activate PtdIns-PLC isozymes in vitro [Wu et al., 1992a; Blank et al., 1992]. However, Camps et al., [1992a], demonstrated that a PtdIns-PLC found in HL60 cells, subsequently identified as PtdIns-PLC β2 [Camps et al., 1992b; Katz et al., 1992], could be activated in vitro by purified βγ subunits from retina and brain. They proposed that the existence of such a mechanism in vivo could account for the Pertussis toxin sensitive pathways identified. If, for example, the source of activatory βγ subunits was activated Gi, such a mechanism would be disrupted by Pertussis toxin, as the ADP ribosylation of αi would cause it to bind irreversibly to its βγ subunits. Thus the complex formed in response to Pertussis toxin would prevent both the α subunit and the βγ subunits from interacting with their appropriate effector enzymes.

The discovery of this new mechanism of PtdIns-PLC activation prompted a search for the regions of interaction between the βγ subunits and the PtdIns-PLC enzymes which they activate. The work of several laboratories had indicated that the C-terminus of PtdIns-PLCβ isoymes was not required for activation of the enzymes by βγ subunits in vitro [Schnabel et al., 1993; Wu et al., 1993b]. The possible candidates for the regions of PtdIns-PLCβ involved in βγ subunit interactions are therefore an acidic 'hinge-like' region between the catalytic X and Y domains and the aminoterminus. Both of these regions are possessed by all classes of PtdIns-PLC, but even within the classes there is little homology.
of these sequences between isozymes (see 1.3.4), compared with the highly conserved catalytic X and Y domains.

A region of sequence which can bind βγ subunits was identified in the G-protein coupled receptor kinase, βARK (see 1.1.6), which, by virtue of its βγ binding, is translocated to the membrane, the site of its substrate receptors. The region of βARK required for interaction with βγ subunits was found to be located in the carboxy-terminus [Koch et al., 1993]. A series of mutant βARK proteins were generated, leading to the identification of a 125 amino acid sequence which was required for binding to βγ subunits. This region of the βARK C-terminus has recently been identified as part of a plextrin homology (PH) domain [Musacchio et al., 1993]. These loosely defined domains have been identified in many proteins including rac kinase, sos, Ras-GAP and several PtdIns-PLC isozymes. A split PH domain was identified in the hinge region PtdIns-PLCγ1, straddled either side of the SH2 and SH3 domains and an aminoterminal PH domain was identified in the sequence of PtdIns-PLCδ1. However, there was no evidence for βγ activation of either of these PtdIns-PLC isozymes in vitro, although Liang and Garrison [1991] and Yang et al., [1991] had observed that EGF stimulated inositol phosphate production by PtdIns-PLCγ, in rat hepatocytes, was sensitive to Pertussis toxin. On the basis of this evidence it was proposed to test the bacterially expressed PtdIns-PLCδ1 preparation for its ability to be activated by βγ subunits in vitro. As βγ activation of the PtdIns-PLCβ class isozymes had already been demonstrated in vitro, it was proposed to search the amino acid sequences of the PtdIns-PLCβ isozymes for potential βγ-binding sequences, by looking for regions with homology to the known PH domains. A GST fusion of such a region of the protein could be made and tested for its ability to bind to βγ subunits, together with another PH domain GST fusion protein, containing the rac kinase PH domain.
Results and Discussion

7.1 Reconstitution of $\beta\gamma$ activation of PtdIns-PLC isozymes \textit{in vitro}

The ability of $\beta\gamma$ subunits to activate different PtdIns-PLC isozymes \textit{in vitro} was assessed using the system established by Camps et al., [1992a] (see 2.2.2.3). Bacterially expressed PtdIns-PLC $\beta_1$ and $\delta_1$ were compared with PtdIns-PLC $\beta_3$ purified from Hela cells [Carozzi et al., 1992] in their ability to be activated by $\beta_3\gamma_\text{t}$ (fig 7.1). To normalise the samples, the amount of each isozyme tested in the $\beta\gamma$ assay was 0.8 units as determined in the standard cholate assay. The results showed that both the bacterial $\beta_1$ and Hela $\beta_3$ could be activated by $1.6\mu\text{M} \beta_3\gamma_\text{t}$, although the $\beta_1$ was only activated 1.5-fold compared with over 4-fold for the $\beta_3$ isozyme. The effect of $\beta\gamma$ on the activity of the $\delta_1$ preparation could not be assessed because its basal activity (in the absence of activator) was not sufficiently suppressed in the assay.

The result obtained with the $\beta_1$ and $\beta_3$ isozymes was consistent with previously reported data [Camps et al., 1992b; Carozzi et al., 1993], confirming that PtdIns-PLC$\beta_1$ is very poorly activated by $\beta\gamma$ subunits. The failure to suppress the basal PtdIns-PLC$\delta_1$ activity in this assay may reflect the increased calcium sensitivity of this class of PtdIns-PLC enzymes. In order to examine the effect of $\beta\gamma$ subunits on the $\delta$ class an alternative assay which could suppress the basal activity had to be used. PtdIns-PLC$\delta_1$ basal activity had previously been successfully suppressed in the G$\alpha$ reconstitution assay, hence this assay was adapted to reconstitute $\beta\gamma$ activation. The membrane preparation normally used in that assay was replaced with water and the $\beta\gamma$ subunits ($1.6\mu\text{M}$) were substituted for the inhibitors previously tested (5.4 and 2.2.2.2). Although the basal activities of both $\beta_3$ and $\delta_1$ were suppressed, neither could be activated with $\beta\gamma$ subunits in the adapted assay, implying that the suppression was too severe to be overcome by the activator.
FIGURE 7.1 Activation of PtdIns-PLC isozymes by βγ subunits.

Each PtdIns-PLC preparation was assayed in the βγ assay (see 2.2.2.3) at a dilution which enabled 0.8 units of cholate assay activity to be used in each reaction in the absence (empty bars) or presence (filled bars) of 1.6μM βγ. The results shown are the means of duplicate reactions and the variance between results is indicated by the error bars.
The identification of a PH domain in the aminoterminus of the rat brain PLC III enzyme (PtdIns-PLCδ₁), together with the studies of the βARK βγ subunit interaction domain suggested that PtdIns-PLCδ₁ might bind βγ subunits. This led to investigations into the ability of βγ subunits to activate PtdIns-PLCδ₁. Park et al., [1993a] demonstrated a slight activation of rat brain PtdIns-PLCδ₁ by βγ subunits in vitro, using a different assay to the ones described above, but was unable to demonstrate any activation of PtdIns-PLCγ₁ in the same assay. In an effort to repeat this result, the bacterial PtdIns-PLCδ₁ was tested in the same assay (see 2.2.2.3) alongside the purified Hela PtdIns-PLCβ₃ (fig 7.1.1). The PtdIns-PLCβ₃ was activated in this assay, but not to the extent previously seen in the βγ assay or reported by Park et al., [1993a] using this assay. The bacterial PtdIns-PLCδ₁ was not activated to any extent which may have been due to slight differences in the assay conditions used. If such differences caused the perceived activation of PtdIns-PLCβ₃ to be lower than previously observed, it is quite likely that the small activation of PtdIns-PLCδ₁ seen by Park et al., would not be measured.

Such a discrepancy of results emphasises the dangers of relying solely upon in vitro data when defining which isozymes can be activated by a particular mechanism. Aside from the obvious problem of mimicking conditions within the cell (in particular lipid concentrations), assay sensitivity is a consideration when interpreting such data. In the same way that a positive result in vitro does not guarantee that such a mechanism exists in vivo, a negative result in vitro does not entirely rule out the possibility of such a mechanism occurring in vivo. It is therefore possible that γ class PtdIns-PLC isozymes can also be activated by βγ subunits, but that the assay conditions hitherto used in vitro have not been appropriate.
FIGURE 7.1.1 $\beta\gamma$ subunit activation of PtdIns-PLC$\delta$$_1$ and PtdIns-PLC$\beta$$_3$. 

Purified bacterially expressed PtdIns-PLC$\delta$$_1$ (1) and Hela cell PtdIns-PLC$\beta$$_3$ were assayed in the assay described by Park et al., [1993a] in the absence (empty bars) or presence of 0.05$\mu$M (striped bars) and 2.2$\mu$M (filled bars) $\beta\gamma$. Essentially the assay was the same as that described in 2.2.2.3, except that the lipid mixture was prepared from PtdEth, PtdSer and PtdIns(4,5)P$_2$ in a molar ratio of 1:1:1. The results shown are the means of triplicate reactions and the variance between results is indicated by the error bars.
7.2 Identifying the regions of PtdIns-PLCβ2 which interact with βγ subunits

On the basis of the loosely defined consensus for PH domains set out by Mayer et al., [1993] and Haslam et al., [1993], an alignment of the amino termini of representatives from each of the PtdIns-PLC families was performed using Clustal [Genetics Computer Group, 1991] and compared with the PH domain consensus (fig 7.2) [Parker et al., 1994]. As with previous alignments of PH domains, the only absolutely conserved residue is the tryptophan (bold). If however the charge and lipophilicity of the amino acids is considered, there appears to be a 'PH like' domain in the aminoterminus of each of the sequences examined. The two members of the PtdIns-PLCβ class which could be strongly activated in vitro by βγ subunits were β2 and β3 but no full length cDNA clone of PtdIns-PLCβ3 had been isolated, therefore the amino-terminal 'PH like' domain of PtdIns-PLCβ2 was selected for expression as a GST fusion protein.

7.2.1 Subcloning PtdIns-PLCβ2-PH into PGEX-2T using PCR

A 640bp aminoterminal fragment of the cDNA clone of PtdIns-PLCβ2 was generated by PCR to contain an Nco I restriction site at the 5' end start site (modifying the second amino acid from serine to alanine) and a Hind III restriction site at the 3' end. This fragment encoded the first 212 amino acids of PtdIns-PLCβ2, believed to include a PH domain. The PCR fragment and the pGEX-2T vector were prepared for ligation by digestion with Nco I and Hind III. After gel purification, the two pieces of DNA were ligated to generate a plasmid encoding the GST-PLCβ2-PH fusion protein. The ligation was transformed into the HB101 strain of E.Coli and transformants analysed by restriction enzyme digestion to identify 3 clones containing the pGEX-β2-PH plasmid.
FIGURE 7.2 Alignment of PtdIns-PLC aminoterminal PH domains.

This alignment was performed by Peter Parker using the GCG Clustal programme and is taken from Trends Biochem. Sci. 19, 54, (1994). It shows the amino termini of all the known mammalian PtdIns-PLC sequences aligned with the PH domain consensus (above) defined by Shaw [1993] and with subdomains 1-6 indicated. The only totally conserved residue (tryptophan) is indicated by an asterisk.

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7.2.2 Expression of the GST-PLCβ2-PH fusion protein

The clones encoding GST-rac-PH and GST-PLCβ2-PH were transformed into HB101 *E.coli*, together with control pGEX-2T, grown in a 500ml culture to an A600 of 0.6 and then grown for a further 2 hours in the absence of IPTG at 37°C. The cells were harvested and extracted as described in 2.2.3 and the resulting supernatants were purified on glutathione-Sepharose as described in 2.2.6. The final samples were separated by SDS-PAGE and transferred to nitrocellulose by Western blotting. The transferred proteins were visualised using ponceau stain and then probed with anti-GST antisera (fig 7.2.1). The 28-kDa GST band was observed in the control sample (lane 1), with the two PH domain GST fusion proteins visible as 43-kDa (GST-rac-PH) and 52-kDa (GST-PLCβ2-PH) bands (lanes 2 and 3 respectively). The fainter lower molecular weight bands in each of the lanes probably represent degradation products. The concentrations of the GST fusion proteins in these preparations were approximated from total protein concentrations measured by the the method of Bradford [1976] and estimations from ponceau stains and Western blots of the proportion of full length fusion protein in each preparation. The estimated concentrations were : GST (2.1µg/µl), GST-rac-PH (0.04µg/µl) and GST-PLCβ2-PH (0.05µg/µl).

7.3 Screening GST fusion proteins for βγ subunit interactions.

The inhibitory approach used to define the region of PtdIns-PLCβ1 involved in Gqα interactions was deemed inappropriate to screen for the region of PtdIns-PLCβ2 involved in βγ subunit interactions, as high concentrations of βγ1 are required to measure activation of PtdIns-PLCβ2 in vitro (1.6µM). Thus at least 50 pmoles of fusion protein (>2µg of GST-PLCβ2-PH) would have to be added to the reconstitution assay to be able to perceive any inhibitory effect on the βγ subunit directed activation. Aside from the difficulties of producing highly concentrated preparations of these fusion proteins, it would be impossible to distinguish a real inhibitory effect from the non-specific effect observed when high concentrations of GST are added to such an assay. Therefore a more direct approach to screen for βγ binding was taken.
FIGURE 7.2.1 Expression of PH domain fusion proteins.

The finally concentrated and purified preparations of GST (lane 1), rac-PH-GST (lane 2) or PLC\(\beta\)2-PH-GST (lane 3) were separated by SDS-PAGE on a 12.5% polyacrylamide gel and transferred to nitrocellulose by Western blotting. The transferred proteins were visualised by ponceau staining and then probed with anti-GST antisera. The molecular weight markers on the left are 66, 45, 31 and 21 kDa.
Glutathione-sepharose beads were loaded with rac-PH-GST fusion protein or control GST and 30μl of these beads were then incubated with 2.5μg of purified βγt for 20 minutes at 4°C. The beads were washed twice in PBS containing 0.02% Triton and any remaining bound proteins were eluted into buffer I. Samples of each stage were separated on 12.5% polyacrylamide gels which were transferred to nitrocellulose and probed with antisera directed against the β subunit (fig 7.3) or GST (fig 7.3.1). When probed with anti-β subunit antisera the β subunit was only detected in the supernatant after incubation with the GST beads and in the supernatant and first wash after incubation with the Rac-PH-GST beads. This experiment was repeated and gave exactly the same result, suggesting that there may be a very slight difference between the affinity of the βγ subunits for the control and the Rac-PH-GST beads. However the interaction is evidently not strong enough to carry the βγ subunits through all the washing procedures, suggesting that it is very weak under the assay conditions. The anti-GST blot revealed that some of the GST and fusion protein did not remain bound to the beads, even after the initial incubation with βγ subunits, although the majority of the GST and fusion protein did remain bound. It is possible that the βγ subunits did bind the fusion protein, but mainly that which was in solution rather than the bead immobilised protein. Similar results were obtained with GST-β2-PH loaded glutathione-Sepharose beads.

On the basis that the interaction between the βγ subunits and the PH domain were very weak, several crosslinkers were tested to try to complex the PH domain to any protein with which it was bound. The cross linking agents tested were: DSS, EGS, DSP and SANPAH. The GST or GST-rac-PH loaded glutathione-Sepharose beads were mixed with βγ subunits as before and incubated with the different crosslinkers at 3mM for 15 minutes at room temperature. Controls containing βγ subunits alone or fusion proteins alone were also incubated in the same manner. Each sample was then mixed with Laemmli sample buffer and separated by SDS-PAGE on a 12.5% polyacrylamide gel, which was Western blotted onto nitrocellulose and probed with an antisera raised against G-protein β subunits.
FIGURE 7.3 Screening for $\beta\gamma_t$ binding domains.

Glutathione-Sepharose beads loaded with GST (c) or rac-PH-GST (f) were incubated with $\beta\gamma_t$ as described in the text, washed 3 times and treated with Laemmli sample buffer to elute any bound protein. The samples from each stage were separated by SDS-PAGE on a 12.5% polyacrylamide gel, transferred to nitrocellulose and probed with antisera raised against the $\beta$ subunit of $\beta\gamma_t$. Samples of supernatant taken after incubation of the beads with $\beta\gamma_t$ are labelled 1; the samples from the three washes are labelled 2, 3 and 4 and the final elutions are labelled 5. The molecular weight markers on the left indicate 66, 45, 31 and 21 kDa and the arrow indicates the $\beta$ subunit.
FIGURE 7.3.1 Screening for $\beta\gamma_\text{I}$ binding domains.

The blot shown in figure 7.3 was subsequently probed with anti-GST antisera. Lanes labelled c contained samples from GST-loaded beads and lanes labelled f contained samples from rac-PH-GST loaded beads. Samples of supernatant taken after incubation of the beads with $\beta\gamma_\text{I}$ are labelled 1; the samples from the three washes are labelled 2, 3 and 4 and the final elutions are labelled 5. The molecular weight markers on the left indicate 66, 45, 31 and 21 kDa and the arrows indicate GST (lower arrow) or rac-PH-GST (upper arrow).
No change in molecular weight of the β subunits was observed after treatment with any of the cross-linkers, indicating that the βγ subunits had not been complexed with either GST alone or the PH domain fusion protein. The experiment was repeated with free GST and fusion protein, but again no complexes with the βγ subunits were observed. This negative result still did not rule out the possibility that rac-PH can interact with βγ subunits, as the cross-linkers tested may not have been appropriate to react with the βγ-PH domain complex.

7.3.1 Testing for a βγ-PH domain interaction with an affinity column

To further investigate the affinity of βγ subunits for the Rac-PH domain an affinity column approach was used. The objective was to compare the rates of movement of βγ subunits through a glutathione-Sepharose column which had been pre-loaded with either control GST or rac-PH-GST fusion protein, expecting that if the βγ subunits had any affinity for the Rac-PH domain, their passage through a column loaded with such a domain would be retarded with respect to their passage through a control column.

Two 1ml columns of glutathione-Sepharose were prepared in PBS (+0.02% Triton X 100) and loaded with either GST or the fusion protein. The loading was performed by re-circulating 10ml of supernatant extracted from bacteria expressing the desired protein, overnight (see 5.3.1). As the GST protein was overexpressed to higher levels than the fusion protein, the GST supernatant was diluted to give a concentration of GST which was similar to that of the fusion protein in the supernatant. This dilution was performed on the basis of a concentration estimated from a coomassie-blue stained phast gel analysis of the two supernatants.

The columns were washed with 5 column volumes of PBS containing 0.02% Triton X 100, before loading with 10μg of βγ subunits. The βγ subunits were then passed through the columns at a flow rate of 0.2 ml/min for 20 minutes. Fractions of 200μl were collected and analysed by Western blotting. To complete the double experiment, each column was stripped of its load by elution with 2ml of bufferX and then washed with 3M NaCl in PBS followed by 0.02% Triton X 100 in PBS. The columns were then re-loaded
with fresh supernatants, this time the other way round, so that both columns had been loaded with both of the test proteins. The whole procedure was repeated and the two profiles from each column were compared. Fig 7.3.2 shows the protein profiles measured from column A, which are very similar to those from column B. The protein elution profiles with either control (GST) or fusion protein bound to the column are the same, with a protein detected in fractions 8 to 10 from column A and in fractions 11 to 14 from column B. The Western blots of these fractions revealed the presence of the β subunit in these same fractions (see fig 7.3.3). The results indicated that under these experimental conditions βγτ subunits do not specifically interact with an immobilised GST fusion protein of the Rac-PH domain. This lack of measurable interaction could be due to the wrong environment for example, the absence of lipids which would normally be present at the cell membrane. The presence of detergent Triton X 100 at low levels (needed to prevent the βγ subunits from sticking to surfaces) may also disrupt the interaction.
FIGURE 7.3.2 Protein profiles of $\beta\gamma_t$ binding to affinity columns.

A 1ml glutathione-Sepharose column loaded with either GST (closed circles) or rac-PH-GST (open circles) was subsequently loaded with 10$\mu$g of $\beta\gamma_t$. Buffer was pumped through the column for 20 minutes at a flow rate of 0.2ml/min and 0.5ml fractions were collected. The $A_{280}$ traces for the two column runs are shown. A protein can be seen to elute in fractions 11 to 18, peaking in both cases at fraction 14.
FIGURE 7.3.3 Elution of $\beta\gamma_t$ from affinity columns.

Equal volumes of the fractions from the peak of the protein elution profiles in fig 7.3.2 were separated by SDS-PAGE on a 12.5% polyacrylamide gel and transferred to nitrocellulose. The blots were probed with antisera raised against G-protein $\beta$ subunits. Lanes 1-4 contain fractions 11-14 from the GST column and lanes 5-8 contain fractions 11-14 from the rac-PH-GST column. The molecular weight markers represent 97, 66, 45 and 31 kDa and the arrow indicates the $\beta$ subunit. The $\beta$ subunit can only be faintly seen in lanes 3 and 4, which may be due to a slightly lower loading of $\beta\gamma$ onto the GST column.
General Discussion

The alignment of the amino termini of the different families of PtdIns-PLC isoforms with the PH domain consensus suggested that such a domain might be present in all of the isoforms. No function has yet been assigned to the PH domain, although it seems to be common to many proteins involved in signalling and may perform a vital role in such mechanisms. The co-localisation of the βγ subunit binding domain of βARK and its PH domain suggests that binding to these heterotrimeric G-protein subunits may be part of the function of PH domains, although it is uncertain whether all of the diverse proteins found to contain PH domains are involved in interactions with βγ subunits. The initial discovery of these domains in members of the γ and δ classes of PtdIns-PLC isoforms and not in the β class of PtdIns-PLC isoforms which are known to be activated by βγ subunits was surprising, leading to the search for PH domains in those isoforms and for evidence of βγ activation of the γ and δ class PtdIns-PLC isoforms.

It is difficult to assess the significance of the data from Park et al., which shows a slight activation of PtdIns-PLCδ1 by βγ subunits in vitro. Combined with the finding of a PH domain in the aminoterminus of this enzyme it is tempting to believe that the link between βγ binding and the presence of a PH domain demonstrated for βARK can also be applied to the PtdIns-PLC isoforms. However, the activation of PtdIns-PLCδ1 by βγ subunits has not yet been observed in any other reconstitution assays and even in the assay used by Park et al., it is less than the activation seen for PtdIns-PLCβ1, the β isozyme least activated by βγ and thought to be mainly activated by the α subunits of Gq. In a series of in vivo experiments Katz et al., [1992] coexpressed PtdIns-PLCβ1 or PtdIns-PLCβ2 with β1γ1 subunits in COS-7 cells, only measuring a stimulation of inositol phosphate production when PtdIns-PLCβ2 was expressed. They concluded that free βγ subunits can not activate PtdIns-PLCβ1 in the cell. This conclusion suggests that the in vitro activation of PtdIns-PLCδ1 by βγ subunits reported by Park et al., is probably not relevant in vivo.

The failure to demonstrate binding of βγ subunits to the rac kinase and PtdIns-PLCβ2 PH domain fusion proteins need not necessarily exclude the possibility that this
domain mediates interactions with G-protein βγ subunits. The high concentrations of βγ₁ subunits (1.6μM) required to activate PtdIns-PLCβ₂ in vitro imply that the interactions between these proteins may be very weak, preventing the formation of detectable complexes on the glutathione-Sepharose beads in the above experiments. Another possible explanation for the lack of interaction in the experiments described is that the conditions in vitro do not provide an optimal environment for interaction. Possibly the presence of the detergent Triton X 100 (necessary to maintain the βγ subunits in solution) or the absence of other proteins or lipids normally present in the cell prevented the βγ subunits from binding to the PH domains. It is also possible that the lack of post-translational modifications in the bacterially expressed domains prevent them from binding βγ subunits, although low level activation of the full length bacterially expressed PtdIns-PLCβ₁ protein was demonstrated in vitro (see 7.1), making this unlikely. In support of this, the binding of bovine brain βγ and βγ₁ subunits to βARK carboxyterminus [Pitcher et al., 1992] was demonstrated using a GST fusion protein that had been expressed in E.coli. In this binding assay, βγ₁ had to be used at a concentration 4 times greater than that of the bovine brain βγ subunits, implying that binding of PH domains to βγ₁ is weaker than to other βγ subunits. This may explain why no binding was observed in the above experiments. Touhara et al., [1994] have recently reported the binding of bovine brain βγ subunits to GST fusions of PH domains from 9 different proteins, including the second half of the PtdIns-PLCγ₁ split PH domain. However, in these experiments the bovine brain βγ subunits were used at a 5 times greater concentration than in the original experiments with βARK, yet the concentration of the PH domains remained similar. Even at these concentrations, the new PH domain fusion proteins did not bind βγ as strongly as the βARK fusion. Thus in vitro it seems that the interactions between βγ subunits and PH domains are very weak therefore it will be important to assess such interactions in an in vivo environment.
CHAPTER 8 - STUDIES OF PtdIns-PLC REGULATION IN VIVO

Introduction

The studies described in chapters 3 to 7 provided useful data about possible mechanisms of regulation for the PtdIns-PLC enzymes, using in vitro techniques to dissect out some of the reactions which combine to form complex signalling pathways in vivo. However it became apparent, from this work and that of other laboratories, that in order to understand the processes that occur in living cells, such data must be combined with and tested in an in vivo system. This chapter describes the use of a 'semi' in vivo system - transient transfection in COS-1 cells to assess some of the findings of the in vitro work already described.

Several laboratories have shown that the muscarinic ACh receptors which belong to the seven-transmembrane class of receptors couple through selected G-proteins to a variety of signalling pathways [reviewed in Hulme et al., 1991]. Activation of the M2 and M4 receptors leads to the inhibition of adenylylcyclase, stimulation of K+ currents and low level release of IP3. These pathways can all be blocked by Pertussis toxin, indicating that they are probably mediated through Gi and or Go. Conversely, activation of the M1, M3 and M5 receptors leads to substantial IP3 release in a Pertussis toxin insensitive pathway. The M1 pathway has been shown to operate through the α subunit of Gq [Mullaney et al., 1993].

Katz et al., [1992] demonstrated that both pathways could be reconstructed in COS-7 cells by overexpression of the M2 receptor with PtdIns-PLCβ2 or the M1 receptor with PtdIns-PLCβ1. The carbachol induced inositol phosphate release could be blocked by PTX treatment only in the M2 expressing cells, demonstrating the difference in activation mechanisms between the two pathways. Total reconstruction of the pathways by transfecting in cDNAs for the α, β and γ subunits confirmed that the effect of Pertussis toxin on inositol phosphate production was exerted through the βγ subunits. This co-transfection system seemed an ideal one in which to test the effects of different regions of
the PtdIns-PLC proteins on these coupling mechanisms, using a more physiological *in vivo* system than the *in vitro* systems already described.

It was proposed to transfect the cDNA for M1 or M2 receptors into COS-1 cells and measure the carbachol stimulation, through the transfected receptors, of inositol phosphate production by the endogenous PtdIns-PLC. Western blotting of cell lysates had shown that the major PtdIns-PLC in COS-1 cells was the β3 isozyme, which was ideal to study both pathways, as *in vitro* studies had shown that it could be strongly activated by either Gqα or Gβγ subunits. The roles of the amino and carboxy termini of the PtdIns-PLCβ isozymes in the two activation mechanisms could then be assessed by coexpression of those domains with the receptor cDNAs, in an effort to block one or other of the pathways.

**Results and Discussion**

8.1 Establishing a PtdIns-PLC activation pathway *in vivo*

Various concentrations of plasmids, containing the cDNA sequences for the M1 and M2 receptors, under the control of the CMV promoter, were transfected into COS-1 cells using electroporation. The electroporated cells were diluted in 20ml of growth medium and for each electroporation 6 wells of a 24 well plate were seeded with 300µl of this suspension. The cells were grown in standard growth medium for 24 hours after which time the medium was removed and replaced with labelled medium. After 16 hours growth in this medium, the cells were treated with a mixture of 20mM LiCl and 10µM carbachol, or 20mM LiCl alone. After 25 minutes incubation at 37°C the treated cells were harvested as described in 2.2.11 and the levels of inositol phosphates released in each of the cell samples were measured by scintillation counting for 10 minutes.

Fig 8.1 shows the results of the titrations, which revealed that when as little as 5µg of plasmid containing the M1 cDNA was transfected into the COS-1 cells, a good carbachol induced PtdIns-PLC activation was measured. In comparison, transfection of the COS-1 cells with up to 20µg of M2 plasmid did not yield a measurable carbachol induced PtdIns-PLC activation.
FIGURE 8.1 Titration of M1 and M2 constructs in COS-1 cells.

Increasing amounts of plasmid encoding the M1 (circles) and M2 (squares) receptors were transfected into COS-1 cells by electroporation as described in the text. The transfected cells were grown for 24 hours, after which time they were labelled with $[^3H]$ inositol and stimulated with 10μM carbachol. The inositol phosphate production in the cells was measured as described in 2.2.11 and the text. The empty shapes represent cells that were not treated with carbachol and the filled ones represent those that were stimulated with carbachol. The values plotted represent the means of triplicate results.
These results confirmed reports [Katz et al., 1992] that although the M1 directed activation of PtdIns-PLC through Gqa is very strong, the M2 directed activation coupled through βγ subunits is poor in comparison. Koch et al., [1994] reported that increasing the density of M2 receptors in cells can increase the level of βγ directed activation of PtdIns-PLC. Based upon this report, greater amounts of M2 plasmid were transfected into the COS-1 cells and the carbachol induced activation of PtdIns-PLC measured as before. To improve the likelihood of measuring M2 directed PtdIns-PLC in this system, the effect of increasing the numbers of transfected cells grown per well was also assessed (fig 8.1.1).

It was clear from the results presented in fig 8.1.1 that transfection of 60μg of M2 - encoding plasmid gave a slight carbachol inducible activation of PtdIns-PLC activity in the COS-1 cells. Doubling the number of transfected cells grown per well also helped to make the carbachol response more measurable. It was therefore decided that for future experiments 60μg of M2 plasmid would be electroporated into the cells and that each electroporation (containing 6x10^6 cells) would be plated at 4x10^5 cells per well, based upon the pre-electroporation cell density. Little more than 10% of the cells survive electroporation, therefore providing that the medium containing the dead cells is removed and replaced as soon as the surviving cells have adhered to the plate (in practice about 4 hours), the wells do not become overcrowded. The experiment presented in fig 8.1.1 also revealed that cotransfection of a plasmid encoding full length PtdIns-PLCβ2 did not improve inositol phosphate production in response to carbachol through the M2 receptor, simply raising the background. As PtdIns-PLCβ2 is known to be activated by βγ subunits, it might be expected that expression of this protein in the M2 transfected cells would improve the carbachol stimulated response, as reported by Katz et al., [1992]. However, the large amounts of plasmid transfected into these cells by electroporation may have prevented full expression of the PtdIns-PLCβ2 protein with the M2 receptor, limiting its contribution to the carbachol induced inositol phosphate response (see 8.2.6.3 and General Discussion at the end of this chapter).
FIGURE 8.1.1 Carbachol stimulated PtdIns-PLC activation through M2.

COS- cells were transfected with the plasmid encoding the M2 receptor at 60μg per electroporation (columns 2 and 6) or 40μg per electroporation (all other columns). Some cells were also co-transfected with 20μg (3 and 7) or 40μg (4 and 8) of plasmid encoding PtdIns-PLCβ2. The electroporated cells were mixed with 3ml of growth medium and plated into 24 well dishes at two densities (50μl or 100μl per well). They were then grown for 24 hours, labelled with [3H] inositol and treated with (filled bars) or without (empty bars) 10μM carbachol before harvesting. The inositol phosphate production in each well was measured as described in 2.2.11. The values plotted represent the means of triplicate results and the variance between those results is indicated by the error bars.
As cell survival after electroporation is poor and variable, a method was established to 'normalise' the counts extracted from each well with respect to the numbers of cells growing in each well prior to harvesting. It was discovered that, as well extracting the soluble inositol phosphates for measurement, the treatment of the cells with 3% perchloric acid during harvesting (see 2.2.11) caused the cells themselves to be fixed to the plastic plates. Subsequent treatment of these 'fixed' cells with 0.2 M NaOH solubilised the remaining material containing the majority of the incorporated [³H]. Scintillation counting of this final extract revealed that the counts retained in the well were proportional to the number of cells originally plated in the well. Thus in all further experiments using this system, the results are expressed as a proportion of the total counts in the well.

8.2 *In vivo* studies of the domains involved in the G-protein coupled activation of PtdIns-PLCβ isozymes

Having successfully established an *in vivo* system in which PtdIns-PLCβ3 could be activated through either a βγ or αq coupled pathway, it was proposed to generate constructs of the carboxy and aminoterminal domains of PtdIns-PLCβ1 and PtdIns-PLCβ2 respectively, with a view to co-expressing them with the M1 or M2 receptors. Based on the *in vitro* work with these domains, it was predicted that the expression of the carboxyterminal domain would inhibit the αq coupled PtdIns-PLC activation signalled by the M1 receptor. Likewise, if the aminoterminal domain of PtdIns-PLCβ2 contained a region of βγ subunit interaction, it would be expected that expression of this domain in the M2 transfected cells would interrupt the βγ coupled PtdIns-PLC activation in response to carbachol.
8.2.1 Subcloning the PtdIns-PLCβ1 carboxyterminal domain into pKS-I

The cDNA encoding the previously defined carboxyterminal domain (see 5.1) of PtdIns-PLCβ1 was cut out of β1-pET 11a, (the construct generated in 4.1.1), using the restriction enzymes Nhe I and Pst I. The eukaryotic expression vector pKS I was prepared by digestion with EcoR I and Pst I. The ligation of the carboxy fragment into this vector was achieved using the double-stranded oligonucleotides depicted below:

![Oligonucleotide diagram]

When assembled, using the annealing protocol described in 2.2.1.6 this linker possessed a 5' EcoR I sticky end and a 3' Nhe I sticky end.

This linker served to join the vector EcoR I sticky end to the insert Nhe I sticky end, allowing the complimentary Pst I sticky ends of vector and insert to complete the ligation. A series of different ratios of vector : oligo : insert had to be tested before successful ligation was achieved (see 2.2.1.5) to yield a clone β1-C-pKS I which encoded the last 477 amino acids of PtdIns-PLCβ1. The correct construction of the clone was verified by restriction digest, before transfecting it into COS-1 cells to look for expression.

8.2.2 Expression of the PtdIns-PLCβ1 carboxy domain in COS-1 cells

The newly created construct - β1-C-pKS I was electroporated into 70% confluent COS-1 cells (see 2.2.10.1) and the transfected cells grown in z medium. As a control an equivalent amount of empty vector - pKS I was transfected into COS-1 cells at the same time. After 48 hours growth both 9cm plates of cells were harvested into 150μl of Laemmli sample buffer and analysed by SDS PAGE on a 12.5% gel. The gel was transferred to nitrocellulose and probed with the antisera raised against the C-terminal epitope of PtdIns-
PLCβ1 (fig 8.2). A band of approximately 56-kDa (arrow) was observed in the sample from β1-C-pKS I transfected cells, but not in the control lane and therefore presumed to represent the expressed C-terminal domain.

8.2.3 Determining the sub-cellular localisation of the expressed domain

As the C-terminal domain only represented part of PtdIns-PLCβ1 it was of great concern that the expressed protein fragment might not be targeted to the cell membrane. Wu et al., [1993a] had demonstrated the existence of a region in the C-terminus of PtdIns-PLCβ1, denoted the P box, which was necessary for membrane association of the enzyme. However, it had not been determined whether this was the only region of PtdIns-PLCβ1 which was required for membrane localisation of the enzyme. As it was very important that the expressed domain was targeted to the membrane, its localisation in the COS-1 cells was assessed by subcellular fractionation (see 2.2.12).

Figure 8.2.1 shows a Western blot of the samples generated by subcellular fractionation, probed with the anti-PtdIns-PLCβ1 antiserum. The expressed domain (arrow) seen in the whole lysate can not be detected in the cytosol of the expressing cells (lane 4). Treatment of the particulate fraction with 1% Triton X 100 demonstrated that some of the expressed domain could be solubilised in this manner (lane 6), although the majority of the expressed protein was still present in the particulate fraction, which contains nuclei, membranes and cytoskeleton. These results indicated that the PtdIns-PLCβ1 carboxyterminal domain was at least in part membrane associated, confirming the existence of a membrane association region in the C-terminus of this enzyme.
FIGURE 8.2 Expression of PtdIns-PLCβ₁ carboxyterminus in COS-1 cells.

COS-1 cells were transfected with pKS I vector (v) or β1-C-pKS I (c) by electroporation and grown for 48 hours as described in 2.2.10. The cells were harvested into Laemmli sample buffer and separated by SDS-PAGE on a 10% polyacrylamide gel. The proteins were transferred to nitrocellulose and probed with the PtdIns-PLCβ₁ polyclonal antisera. Molecular weight markers on the left represent 97, 66, 45 and 31 kDa and the arrow indicates the expressed carboxyterminus in lane c.
FIGURE 8.2.1 Subcellular localisation of expressed carboxyterminus.

COS-1 cells transfected with pKS I vector or β1-C-pKS I were grown as before and harvested as described in the text and in 2.2.1.2. Samples of the different cellular fractions were separated by SDS-PAGE on a 10% polyacrylamide gel and transferred to nitrocellulose. The blot was probed with the PtdIns-PLCβ1 antisera. The odd numbered lanes represent vector transfected cells and the even numbered lanes contain samples from cells expressing the carboxyterminal domain. Lanes 1 and 2 contain whole cell lysate; lanes 3 and 4 contain the cytosolic fraction of the transfected cells; lanes 5 and 6 contain the Triton X 100 extracted fractions and lanes 7 and 8 contain the non extracted particulate fractions of the transfected cells. The molecular weight markers represent 97, 66, 45, 31 and 21 kDa and the arrow indicates the expressed carboxyterminal domain.
8.2.4 Subcloning the aminotermminus of PtdIns-PLCβ2 into pKS I

An aminoterminal cDNA fragment of PtdIns-PLCβ2 was generated by PCR (see 2.2.1.10) and ligated into pKS I for expression in COS-1 cells. To preserve the C-terminal epitope against which the PtdIns-PLCβ2 polyclonal antisera had been raised, a two step PCR strategy was used. The 640bp aminotermminus was generated using primers which inserted an aminoterminal EcoRI site and Kozak sequence 5' of the start site. The chosen 400bp C-terminal sequence was amplified using primers which inserted a 3' Pst I site. To join the PCR fragments together, the initial primers also contained a sequence to make the 3' end of the aminoterminal fragment complementary to the sequence included at the 5' end of the carboxyterminal fragment. The two primary PCR fragments were joined by a second round of PCR using the sense primer from the aminoterminal fragment and the anti-sense primer from the carboxyterminal fragment. Figure 8.2.2 shows the two initial PCR fragments of 400 and 640bp (a), and the final 1kb fragment (b) which was subcloned into pKS I. The subcloning was completed by restriction digestion of the PCR fragment and the vector with EcoRI and Pst I, followed by a sticky-ended ligation (see 2.2.1.5). Vent polymerase was used to catalyse the PCR reactions, to reduce the likelihood of mistakes being introduced. Nevertheless the new clone β2-A-pKS I was checked with restriction analysis (fig 8.2.2 c) and the entire insert sequenced using 4 primers. No mistakes were found in the sequence, therefore the construct was transfected into COS-1 cells to look for expression of the Amino terminal domain.

8.2.5 Expression of the Amino terminal domain in COS-1 cells

40μg of the β2-A-pKS I construct and the empty vector - pKS I were electroporated into COS-1 cells and grown on 15cm plates in z medium for 48 hours, after which time each plate was harvested into 500μl of Laemmli sample buffer. Samples were analysed by SDS PAGE on a 12.5% gel which was transferred to nitrocellulose and probed with the polyclonal antisera raised against a C-terminal epitope of PtdIns-PLCβ2 (fig 8.2.4a).
FIGURE 8.2.2 PCR subcloning of the PtdIns-PLCβ2 aminoterminal.

PCR was performed as described in the 2.2.1.10 and the products were separated on a 2% agarose gel (a). Reactions 3-8 contained the template (plasmid containing the cDNA for mammalian PtdIns-PLCβ2). Reactions 1 and 7 contained the sense and antisense primers for the aminoterminal fragment and reactions 2 and 8 contained the carboxyterminus sense and antisense primers. Lanes 3, 4, 5 and 6 contained each of the primers singly. Lane 7 contains a band of approximately 600bp (lower arrow) and lane 8 contains a band of approximately 400bp (upper arrow). Gel (b) shows the reactions from the second round of PCR. Lanes 1, 2, and 3 contained template (a 1:1 mixture of the 400bp and 640bp fragments from the first PCR reaction), with both sense and antisense primers in lanes 1 and 4. Lanes 2 and 3 contained individual primers. The kb markers on the left indicate that the large fragment in lane 1 (arrow) is approximately 1kb. This band was purified by electroelution and subcloned into pKS I.
FIGURE 8.2.4 Expression of PtdIns-PLCβ2 aminoterminus in COS-1 cells

a) COS-1 cells were transfected with β₁-C-pKS I (C) or β₂-A-pKS I (A) and grown for 48 hours, before harvesting into Laemmli sample. The samples were separated by SDS-PAGE on a 12.5% polyacrylamide gel and the proteins transferred to nitrocellulose. The blot was probed with the antisera raised against a unique C-terminal epitope of PtdIns-PLCβ₁ (1) or antisera raised against a unique C-terminal epitope of PtdIns-PLCβ₂ (2). The molecular weight markers on the left represent 66, 45, 31 and 21 kDa and the arrow indicates the expressed carboxyterminal fragment previously seen in fig 8.2.1. The expected molecular weight of the aminoterminnal fragment was 26-kDa although no such band can be seen in lane 2A. b) COS-1 cells were transfected with a plasmid encoding the full length mammalian PtdIns-PLCβ₂ protein, grown for 48 hours and harvested into Laemmli sample buffer. The sample was separated by SDS-PAGE on a 7% polyacrylamide gel, transferred to nitrocellulose and probed with the anti-PtdIns-PLCβ₂ antisera. The molecular weight markers on the left represent 200, 116, 97 and 69 kDa, indicating that the immunoreactive band is approximately 140-kDa.
There was no evidence of an immunoreactive band of the predicted size of the aminoterminal domain (25-30-kDa) which was unique to the β2-A-pKS I transformed cells. The efficacy of the antisera was tested by probing a Western blot of a sample from COS-1 cells which had been transfected with full length PtdIns-PLCβ2 (fig 8.2.4b). The antisera was able to recognise as little as 100ng of the full length enzyme. The sequence data confirmed that no mistakes had been made in the cDNA encoding the aminoterminus and that the carboxyterminal epitope had been correctly joined 'in frame' with the rest of the PtdIns-PLCβ2 reading frame, yet no expression of the domain in COS-1 cells could be detected, even when 60μg of plasmid were transfected into the cells. One possible explanation for this apparent lack of expression was that the domain was being expressed, but was subject to proteolytic cleavage resulting in the loss of the antibody epitope. Proteolysis in the aminoterminus has been observed during the purification of PtdIns-PLCβ2 [Camps et al., 1992b]. On the premise that this may be the case, the aminoterminal construct was tested along with the carboxyterminal construct for inhibitory activity in the M1 and M2 transfected cells.

8.2.6 Co-transfection of the domain constructs with the M1 and M2 receptors

To assess the effect of either domain on the activation of the endogenous COS-1 PtdIns-PLC through the M1 and M2 receptors, 5μg of the M1 receptor plasmid or 60μg of the M2 receptor plasmid were mixed with different combinations of vector, β2-A-pKS I (60μg) and β1-C-pKS I (40μg), ensuring that the total concentration of plasmid was maintained in each mixture. The plasmid mixtures were transfected into COS-1 cells by electroporation and plated out into 24 well plates as previously described. After 48 hours of growth, the medium was removed and 300μl of pre-warmed labelled medium (see 2.2.11) added to each well. The cells were grown in this medium for a further 16 to 20 hours, harvested and the levels of inositol phosphates measured as previously. The total counts remaining in the 'fixed' cells were also determined, allowing the calculation of a normalised figure representing PtdIns-PLC activity. For each sample the cpm of free inositol
phosphate was expressed as a percentage of the total cpm in the cells at harvesting. Each experimental condition was measured in triplicate and the figures presented represent the results of at least 3 experiments.

8.2.6.1 Inhibition of the M1 directed pathway

Fig 8.2.5 demonstrates the effect of the individual domains on the pathway activated by carbachol through the M1 receptor. Expression of the carboxyterminus in M1 expressing cells disrupted the carbachol stimulated PtdIns-PLC activation by approximately 50%. Due to the relative insensitivity of such an approach, it was not possible to perform a titration of the inhibition. However, since there is much evidence that the activation of PtdIns-PLC through M1 is coupled through Gqα, it seems likely that the over-expressed carboxy-terminal domain inhibited the pathway by competing with the endogenous PtdIns-PLCβ3 for activated Gqα subunits. This result was consistent with the in vitro data which had suggested that the C-terminus of PtdIns-PLCβ1 was able to interact with activated G-protein α subunits of the Gq family (see 5.).

Transfection of the amino-terminal construct into M1 expressing COS-1 cells clearly results in the expression of a protein which also has an inhibitory effect on the M1 directed pathway. This result is surprising as there has been no evidence to suggest that the aminoterminus of the PtdIns-PLC isozymes interacts with G-protein α subunits. It seems more likely that this inhibitory effect might be due to the domain binding to and hence sequestering the βγ subunits released when Gq is activated. This could result in the inhibition of PtdIns-PLCβ3 activation by 2 different mechanisms. The PtdIns-PLC isozymes have been shown to possess a GTPase activating activity [Berstein et al., 1992], which is thought to participate in a negative feedback mechanism, returning αq to its inactive GDP bound state. Thus unlike other G-protein activation cycles, where GTP hydrolysis is the rate-determining step, the GAP activity of the PtdIns-PLCβ isozymes may make the recombination with βγ subunits the rate-determining step in the Gq activation cycle in vivo.
FIGURE 8.2.5 Inhibition of the M1 pathway.

COS-1 cells were cotransfected with plasmid encoding the M1 receptor and pKS I or β1-C-pKS I (Carboxy) or β2-A-pKS I (Amino) as described in the text. The cells were grown for 24 hours, labelled with [3H] inositol and stimulated with (striped bars) or without (filled bars) 10μM carbachol as described in 2.2.11, before harvesting. The inositol phosphate release was measured and the cpm of inositol phosphate are expressed as a % of the total cpm counted per well. The values plotted are the means of triplicate results and the variance between results is indicated by the error bars.
Therefore, sequestration of the βγ subunits by the aminoterminal domain would have a dramatic effect on the re-cycling of αq and hence inhibit PtdIns-PLC activation. It is also possible that the Gqβγ subunits may be involved in the activation of the endogenous PtdIns-PLCβ3, as they do after stimulation of Gi through the M2 receptor. Data from in vitro studies has implied that the contribution of Gqβγ subunits to PtdIns-PLC activation is likely to be small, as compared with αq, much higher concentrations of βγ subunits are required to activate the PtdIns-PLCβ isozymes. It had therefore been assumed that the βγ subunits for PtdIns-PLC activation in vivo would originate from a different source to the αq subunits - probably a more abundant G-protein such as Gi. The M1 receptor is not known to couple to any G-protein other than Gq, therefore if the substantial inhibition by the aminoterminal domain in the COS-1 system is in part due to the interruption of a βγ activation pathway, the source and potency of the βγ subunits in vivo may be different from that suggested by in vitro studies, implying a synergistic action of the αq and βγ subunits in activating PtdIns-PLCβ isozymes.

8.2.6.2 Inhibition of the M2 directed pathway

Fig 8.2.9 shows the effect of both constructs on the carbachol stimulation of PtdIns-PLC activity through the M2 receptor. Neither the expression of the carboxyterminal nor the aminoterminal constructs had any effect on the carbachol stimulation of PtdIns-PLC activity through the M2 receptor. This result implies, that the C-terminus cannot interact with βγ subunits and makes sense in the light of evidence that C-terminally-truncated PtdIns-PLCβ2 mutants are still able to be activated by βγ subunits when expressed in COS-1 cells [Wu et al., 1993b]. The failure of the 'PH like' domain in the aminoterminal construct to inhibit the βγ mediated activation of the endogenous PtdIns-PLC upon stimulation of the M2 receptor, suggests that either the aminoterminal domain expressed does not contain a full βγ binding site, or it does contain a βγ binding site, but is not expressed at high enough levels to compete effectively with all of the βγ subunits released.
FIGURE 8.2.6 Inhibition of the M2 pathway.

COS-1 cells were cotransfected with plasmid encoding the M2 receptor and pKS I or β1-C-pKS I (Carboxy) or β2-A-pKS I (Amino) as described in the text. The cells were grown for 24 hours, labelled with [3H] inositol and stimulated with (striped bars) or without (filled bars) 10μM carbachol as described in 2.2.11, before harvesting. The inositol phosphate release was measured and the cpm of inositol phosphate are expressed as a % of the total cpm counted per well. The values plotted are the means of triplicate results and the variance between results is indicated by the error bars.
There is no direct evidence to support either explanation, however, based upon the alignment shown in fig 7.2 [Parker et al., 1994] the aminoterminal construct used in these experiments includes all of the potential βγ binding site defined by Koch et al., [1993]. Wu et al., demonstrated that a chimera of PtdIns-PLCβ1 and PtdIns-PLCβ2 which was composed of the first 250 amino acids of PtdIns-PLCβ2 sequence plus the rest of the PtdIns-PLCβ1 sequence, was not activated by βγ subunits, when overexpressed in COS-7 cells. The inability of the first 250 amino acids of PtdIns-PLCβ2 to confer βγ activatability may, however, have been due to the lack of sequences required for PtdIns-PLC activation, rather than the absence of a βγ binding domain. Given that Gi is more abundant in cells than Gq, it is quite probable that the M2 pathway generated many more βγ subunits than were produced by the M1 pathway, making it impossible for the expressed domain to have any measurable effect on the concentration of free βγ subunits in the M2 transfected cells.

### 8.2.6.3 Investigating the combined effect of the carboxy and aminoterminal constructs on the M1 directed pathway

To assess the nature of the inhibition of the M1 pathway by the aminoterminal construct, the combined effect of both constructs was measured. COS-1 cells (70% confluent) were transfected with 5μg of the M1 receptor construct in combination with either vector alone, either of the constructs alone or the two constructs combined. The total concentration of DNA was maintained in all the mixtures, using vector plasmid to make up the difference as shown in the table below:

<table>
<thead>
<tr>
<th>EXPERIMENT:</th>
<th>VECTOR</th>
<th>CARBOXY</th>
<th>AMINO</th>
<th>COMBINATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Construct (μg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pKS I</td>
<td>100</td>
<td>60</td>
<td>40</td>
<td>-</td>
</tr>
<tr>
<td>β1-C-pKS I</td>
<td>-</td>
<td>40</td>
<td>-</td>
<td>40</td>
</tr>
<tr>
<td>β2-A-pKS I</td>
<td>-</td>
<td>-</td>
<td>60</td>
<td>60</td>
</tr>
</tbody>
</table>
The cells were grown, labelled, stimulated with carbachol and harvested as previously. This experiment was repeated 5 times, each giving different results and unfortunately preventing the drawing of any meaningful conclusions. The inconsistency of these results was probably due to the large amounts of DNA being transfected into the cells (a total of 100μg), in order to get good levels of expression of the constructs. If these experiments were to be repeated it would be worth looking to see whether lower concentrations of the individual constructs will still give a visible effect, so that the total levels of DNA transfected into the cells could be reduced. Evidently a total of 60μg of plasmid (used in the single construct experiments) does not cause so many problems with variability. It is unfortunate that the amino construct cannot be recognised by Western blotting, as this would enable checks to be made on the levels of each protein in doubly transfected cells, as compared with cells transfected with only one construct.

General Discussion

The reconstruction of two PtdIns-PLC activation pathways in vivo confirmed several predictions made from in vitro results. As predicted from in vitro work, which showed that αq was over 40-fold more potent as an activator of PtdIns-PLC than βγ subunits, a 10-fold difference was observed between the carbachol stimulated PtdIns-PLC activation measured in M1 and M2 transfected COS-1 cells. Activation of the Gq proteins through the M1 receptor led to a substantial activation of the endogenous PtdIns-PLCβ3, whereas activation of the much more abundant Gi proteins through the M2 receptor caused only a modest increase in PtdIns-PLC activity. This indicates that in the absence of stimulation through a Gq coupled receptor, PtdIns-PLCβ3 activity is not greatly stimulated in vivo by the release of βγ subunits from G-proteins such as Gi. Thus the main mechanism for activation of PtdIns-PLCβ isozymes in these cells probably operates through activated Gqα subunits. However, activation of abundant G-proteins such as Gi or Go, through agonists normally associated with the activation of other signalling pathways, may contribute to a low level activation of PtdIns-PLCβ isozymes in vivo. This may not be
the case in all cell types, as PTX has been shown to completely inhibit inositol phosphate signalling through various hormones in other cell types, in particular those of haematopoietic lineage [Meldrum et al., 1991b].

The expression of the carboxyterminal domain of PtdIns-PLCβ1 and its association with the particulate fraction during cell fractionation confirmed the report of Wu et al., [1993a] that this region of the protein contained a sequence which enables membrane association. As PtdIns-PLCβ enzymes are mostly membrane bound in cells, compared with the mainly cytosolic PtdIns-PLCγ, this membrane association domain is clearly important for the enzyme to function properly within cells. Despite being unable to detect expression of the aminoterminal domain in transfected COS-1 cells, by Western blotting, transfection of the construct exerted a substantial inhibitory effect on the M1 receptor directed pathway, indicating that the domain, at least in part, had been successfully expressed.

The inhibitory effect of the carboxyterminal construct on the M1 pathway confirmed the \textit{in vitro} data which had indicated that this part of the PtdIns-PLCβ isozymes was responsible for interaction with Gqα subunits during receptor mediated activation of PtdInsP(4,5)P2 hydrolysis. The reason for the inhibition exerted by the amino terminal construct on the M1 pathway is not clear from these results and may represent a direct effect on the αq activation of the PtdIns-PLC through some sort of inhibitory interaction either with the carboxyterminus of the PtdIns-PLC or with the αq subunits. Alternatively the inhibitory effect may be achieved through the sequestration of Gqβγ subunits. This would almost certainly slow down the recycling of Gqα subunits and inhibit the M1 receptor mediated activation of the endogenous PtdIns-PLC. The failure of the aminoterminal domain to disrupt M2 directed PtdIns-PLC activation \textit{in vivo} implies that this domain cannot bind βγ subunits. However, activation of Gi through the M2 receptor probably causes the release of many more βγ subunits than are released by the activation of Gq through the M1 receptor. Thus the expression levels of the aminoterminal construct in the transfected COS-1 cells may be enough to sequester a significant proportion of the Gqβγ subunits, but not a significant proportion of the Giβγ subunits.
If it is assumed that the aminoterminal domain expressed in the COS-1 experiments contained a $\beta\gamma$ binding domain, the results in the M1 system might also suggest that the Gq$\alpha$ and G$\beta\gamma$ subunits activate the endogenous PtdIns-PLC in a synergistic fashion. From the M2 system it is evident that $\beta\gamma$ subunits alone do not activate the endogenous PtdIns-PLC to any great extent, however in the M1 system in the presence of activated $\alpha$q they may be able to activate the PtdIns-PLC to a greater extent. Thus when the $\beta\gamma$ subunits were sequestered by the aminoterminal construct, the contribution of the $\beta\gamma$ subunits to the activation of the endogenous PtdIns-PLC would be reduced. Such a synergism has been demonstrated in the activation of certain adenylate cyclase isozymes by G$\beta\gamma$ subunits [Federman et al., 1992]. Here it was shown that for $\beta\gamma$ subunits to activate adenylate cyclases II and IV $\alpha$s also had to be present.

The more likely explanation for the inhibitory effect of the aminoterminal construct on the M1 coupled pathway is that by binding to Gq$\beta\gamma$ subunits it disrupts the recycling and reactivation of $\alpha$q-GDP. This model is backed up by the effect of the same domain observed in vitro. The PtdIns-PLC$\beta_2$-PH-GST fusion protein (see 7.2.2) was titrated into the Gq$\alpha$ membrane reconstitution assay used to test the PtdIns-PLC$\beta_1$ carboxyterminal GST fusion protein (see 5.4.1), but found to have no effect on the AIF$^4^-$ stimulated activation of bacterially expressed PtdIns-PLC$\beta_1$. If the effect seen in vivo was due to disruption of a synergistic PtdIns-PLC activation by Gq $\alpha$ and $\beta\gamma$ subunits, a similar effect would be expected in the membrane reconstitution assay. Thus the lack of inhibitory activity of GST-PLC$\beta_2$-PH indicates that this is not a valid explanation. The only difference between the PtdIns-PLC activation in the in vitro and in vivo assays is the method of Gq activation. In the in vivo system, Gq is activated by the M1 receptor in response to carbachol and is subject to the normal G-protein activation cycle, however, in the in vitro assay, Gq is activated by AIF$^4^-$, leading to permanently dissociated $\alpha$q and $\beta\gamma$ subunits. Thus the in vitro assay is not dependent upon $\beta\gamma$ subunits for $\alpha$q mediated activation. This would explain why a $\beta\gamma$ subunit-binding domain is inhibitory to PtdIns-PLC activation in vivo, but not in the in vitro assay. Thus these experiments represent indirect evidence that the amino terminus of PtdIns-PLC$\beta_2$ contains a $\beta\gamma$-binding domain.
CHAPTER 9 DISCUSSION

Introduction

Since the initial measurements of PtdIns(4,5)P$_2$ hydrolysis by PtdIns-PLC, many isozyymes have been cloned and purified and much has been learnt about the mechanisms by which a diverse selection of cell surface receptors activate them, in response to a wide variety of agonists. It was calculated that if the PtdIns-PLC isozyymes exhibited the same levels of activity in vivo as had been measured in vitro, all of the inositol phospholipids in the cell would be rapidly used up. Thus it became obvious that all of the isozyymes must be subject to negative regulation in the cell and that receptor mediated activation of the different isozyymes would involve partial relief of this inhibition. Little is known about the mechanisms in the cell which keep the PtdIns-PLC isozyymes in check, although clearly the lipid composition of the cell membranes in which the substrate is located, are an important factor in this. Studies with mixed lipid monolayers have indicated that changes in surface pressure can dramatically alter PtdIns-PLC activity [Boguslavsky et al., 1994] and Sphingomyelin, a component of the cell membrane, has been shown to substantially inhibit PtdIns-PLC$_{\delta1}$ activity in vitro [Pawelczyk and Lowenstein, 1992]. Difficulties in assessing the precise composition of cell membranes and the pressures exerted within them have made it difficult to construct in vitro assays which truly mimic the in vivo environment. Therefore the study of receptor mediated PtdIns-PLC activation mechanisms has involved the use of a variety of different in vitro assays, some employing partially purified cell membranes or 'ghosts' to provide a more authentic environment for the enzyme and its regulators. The results of these experiments have been combined with those from in vivo work to build up a picture of PtdIns-PLC regulation.

Two types of coupling between receptor activation and PtdIns-PLC activation have been identified. One involves the translocation of cytoplasmic PtdIns-PLC$\gamma$ isozyymes to the membrane, where they associate with activated receptor tyrosine kinases through their SH2 domains and are phosphorylated on tyrosine residues, leading to the activation of the enzyme. The other mechanism involves the release of activated heterotrimeric G-protein
subunits which interact with and activate the PtdIns-PLCβ isozymes. The G-protein
mediated activation of PtdIns-PLC activity has been shown to occur through both α and βγ
subunits, accounting for the previously identified PTX insensitive and sensitive PtdIns-
PLC activation pathways. The PTX insensitive activation is mediated through a number of
different α subunits of the Gq family and much work has been done to establish the
specificity of the different α subunits for the different PtdIns-PLCβ isozymes. The source
and role of the βγ subunit directed PtdIns-PLC activation is less well established, although
there is much evidence to suggest that βγ subunits released by the activation of the PTX
sensitive Gi proteins are responsible for the PTX sensitive PtdIns-PLC activation
pathways.

Although the essential components of these activatory mechanisms have been
determined, the detailed mechanisms involved in PtdIns-PLC activation, are as yet
unknown and even less is known about the activation of the PtdIns-PLCδ isozymes.

9.1 Regulation of the PtdIns-PLCδ class isozymes by phosphorylation.

Expression of PtdIns-PLCδ1 in E.coli yielded a catalytically active enzyme, with
properties characteristic of the purified mammalian enzyme. The high specific activity of the
bacterially expressed enzyme, measured in the standard cholate assay, was also observed in
another laboratory [Ellis et al., 1993] and may indicate a requirement for a post-
translational modification to repress the activity of this enzyme in vivo. In the studies
described in chapter 3, neither purified PKC isozymes α, β, γ or ε nor Ca^2+/Calmodulin
kinase were able to phosphorylate the bacterially expressed enzyme. However, the
possibility that other kinases might phosphorylate PtdIns-PLCδ1 could not be excluded.
On the basis that native eukaryotic PtdIns-PLCδ1 is a phosphoprotein [Ryu et al., 1990]
further studies of this class of PtdIns-PLC isozymes might usefully employ the
'unmodified' bacterially expressed PtdIns-PLCδ1 to screen fractionated cell extracts for a
PtdIns-PLCδ1-kinase.

Although phosphatase treatment of PtdIns-PLCδ1 preparations did not alter their
activity, these experiments were not sufficient to rule out an effect of phosphorylation on
PtdIns-PLCδ₁ activity. The specific activity of the part purified COS-1 cell expressed enzyme was not much lower than that of the bacterially expressed enzyme, therefore to fully explore this question would require the purification of a PtdIns-PLCδ₁ preparation with a substantially lower specific activity than that of the bacterially expressed enzyme. This should be possible, using previously published purification procedures for PtdIns-PLCδ₁ from rat liver [Ryu et al., 1987] and rat brain [Homma et al., 1988] and including phosphatase inhibitors throughout the purification. The purification of overexpressed PtdIns-PLCδ₁ from COS-1 cells could also be repeated, but with transfected cells that were serum starved prior to extraction, to try to ensure that the PtdIns-PLCδ₁ is in its basal activation state. These low specific activity preparations could then be treated with phosphatases as before and the effects on their activity measured.

If PtdIns-PLCδ₁ is regulated by phosphorylation it is likely that changes in electrostatic charge within the enzyme might be responsible for mediating the effect. Stimulatory effects of polyamines and basic proteins on PtdIns-PLCδ₁ activation have been documented in vitro [Haber et al., 1991], however the relevance of such phenomena to PtdIns-PLCδ₁ activation in vivo has not been determined. As the substrates of the PtdIns-PLC isozymes are negatively charged, the observed activatory effects of positively charged molecules may be achieved through charge neutralisation, leading to a decrease in repulsive electrostatic interactions between the enzyme and its substrate. If this were the case, the introduction of negatively charged phosphate groups into regions of the enzyme responsible for substrate interactions might have an inhibitory effect. Phosphorylation of specific tyrosine residues of PtdIns-PLCγ by the PDGF receptor apparently has the opposite effect on the activity of this isozyme. Once phosphorylated, the enzyme is able to overcome the inhibitory effects of profilin on PtdIns(4,5)P₂ hydrolysis, possibly by interaction with a cluster of basic residues located on the surface of profilin. In both cases, the phosphorylation of the PtdIns-PLC molecule might change the conformation of the enzyme, through making or breaking intramolecular interactions, thus rendering it active or inactive. To determine whether this does occur, a strategy similar to that used to demonstrate conformational changes in proteins such as the nicotinic Ach receptor could be
used, employing a panel of monoclonal antibodies raised against different regions of the PtdIns-PLC sequence.

### 9.2 Regulation of the PtdIns-PLCδ class isozymes by G-proteins.

Like the PtdIns-PLCγ class isozymes, the PtdIns-PLCδ class isozymes do not possess the extended carboxyterminus through which the PtdIns-PLCβ isozymes are able to interact with the α subunits of the Gq family of G-proteins. In chapter 4 of this thesis, it was demonstrated that membranes containing Gαq were unable to activate bacterially expressed PtdIns-PLCδ1 in the reconstitution assay. Other laboratories have demonstrated the same null effect using the purified mammalian enzyme in several different assays [Taylor et al., 1991; Wu et al., 1992a; Hepler et al., 1993]. It therefore seems unlikely that the PtdIns-PLCδ isozymes are regulated by Gqα subunits.

As the PtdIns-PLCβ isozymes were shown to be activated by the Gq family of α subunits, which cannot be modified by PTX, it was suggested that the PtdIns-PLCδ isozymes might mediate the PTX sensitive signalling pathways in cells. This suggestion is backed-up by observations made during work with a Chinese hamster lung fibroblast cell line (CCL39). Wild-type CCL39 cells had been shown to express PtdIns-PLCδ1, however mutant CCL39 cells defective in thrombin stimulated PtdIns turnover and mitogenesis did not express this enzyme [Rath et al., 1990], suggesting that thrombin stimulated PtdIns-PLC activation in these cells was mediated through PtdIns-PLCδ1. This was subsequently demonstrated using a *Xenopus* oocytes microinjection system, in which PtdIns-PLC activation was assayed by measuring $^{45}$Ca$^{2+}$ efflux from oocytes injected with CCL39 mRNA. Co-injection of anti-PtdIns-PLCδ1 antibodies [Cho et al., 1993] selectively blocked thrombin stimulated $^{45}$Ca$^{2+}$ efflux from the oocytes, whereas injection of antibodies against PtdIns-PLCβ1 or PtdIns-PLCγ1 did not block this response. In a reciprocal experiment, only anti-PtdIns-PLCγ1 antibodies were able to block PDGF stimulated $^{45}$Ca$^{2+}$ efflux. This result in combination with the observation that thrombin-elicited PtdIns(4,5)P$_2$ hydrolysis in CCL39 cells is inhibited by PTX [Paris and
Pouyssegur, 1986], implied that PtdIns-PLCδ1 is activated by a PTX sensitive pathway in these cells.

Identification of the G-protein responsible for PTX sensitive PtdIns-PLC activation has proved difficult. The α subunit of the PTX sensitive Go, was considered to be a possible mediator of the PTX response. When puriﬁed GTPγS activated αo was injected into Xenopus oocytes, it caused stimulation of the IP3 mediated Cl− current and was able to enhance the Ach stimulated response through muscarinic receptors [Moriarty et al., 1990]. Injection of 3 different αi proteins did not produce either of these effects. Thus it was concluded that αo might be responsible for the PTX sensitive activation of PtdIns-PLC enzymes. However, subsequent in vitro assays using recombinant G-protein α subunits and in vivo overexpression experiments have shown that neither Goα nor any of the Giα subunits nor Gαs or Gαz are able to activate any of the PtdIns-PLC isoforms [Wu et al., 1992a; Blank et al., 1992; Hepler et al., 1993].

Recent evidence has suggested that G-protein βγ subunits derived from PTX sensitive G-proteins, such as Gi and Go, may be responsible for this mechanism of PtdIns-PLC activation. Most of the work on βγ subunit directed PtdIns-PLC activation has focused on the regulation of the PtdIns-PLCβ isoforms, however, Park et al., [1993a] have demonstrated a slight activation of PtdIns-PLCδ activity by puriﬁed bovine βγ subunits in their mixed micelle in vitro assay. The results presented in chapter 7 of this thesis show that this result could not be obtained with the bacterially expressed PtdIns-PLCδ1 and βγ either in the βγ assay used by Wu et al or in that developed by Camps et al [1992a]. The difference between these results may be due to a difference in the efficacy of βγ subunits compared with other βγ subunits. Such a difference has been observed in the binding of βγ subunits to βARK and probably reﬂects a degree of speciﬁcity between different βγ isoforms. This speciﬁcity is probably required to prevent PtdIns-PLC activation in response to the stimulation of every G-protein coupled receptor in a cell. Nevertheless, the activation of PtdIns-PLCδ1 by bovine brain βγ subunits observed by Wu et al was very small when compared with the activation of even the worst activated PtdIns-PLCβ isozymes. PtdIns-PLCβ1 and therefore the relevance of such an effect in vivo is
uncertain. In deed, when PtdIns-PLCβ₁ and PtdIns-PLCδ₁ expressing constructs were cotransfected into COS-1 cells together with constructs expressing G-protein β and γ subunits, no difference in inositol phosphate production was observed between control and transfected cells [personal communication from M.Simon]. The same experiment performed with PtdIns-PLCβ₂ however led to a dramatic increase in inositol phosphate production (see 9.5), indicating that in vivo PtdIns-PLCβ₂ is more likely to be βγ regulated than the other two isozyms.

Work on the regions of the PtdIns-PLCβ isozyms required for their activation by βγ subunits is at present incomplete, although the amino terminus seems to be the most likely location for such a region. All of the PtdIns-PLC isozyms possess a domain aminoterminal to the catalytic X domain, yet there is only 20% homology in this region between the different PtdIns-PLC isozyms. Within the β class, however, the % homology in this region is much higher at approximately 50%. If the sequences for interaction with βγ subunits do lie within this region of the enzyme, this might imply that in vivo, βγ activation is restricted to the β class of PtdIns-PLC isozyms. However, the identification of a PH domain in the amino terminus of PtdIns-PLCδ₁ and the co-localisation of the βγ subunit binding domain of BARK with a PH domain suggest that PtdIns-PLCδ₁ might be regulated by βγ subunits in vivo. The establishment of a role or roles for PH domains in proteins is therefore important to further the understanding of the regulation and action of PtdIns-PLCδ isozyms.

9.3 Structural analysis of PtdIns-PLCδ₁.

Aside from predictions about structure from sequence alignments, proteolysis and expression of deletion mutants of PtdIns-PLCδ₁ have provided useful data on the structure of these isozyms and the possible functions of different domains within the protein. Alignment of the PtdIns-PLCδ isozyme protein sequences with those of the other isozyms revealed the location of the conserved catalytic X and Y domains. Further information about the location of these domains has been obtained by expressing deleted PtdIns-PLCδ₁ mutant proteins in E.coli as GST fusions and measuring their activity in the standard in
Deletion of any part of the carboxyterminal sequence resulted in a catalytically inactive protein, indicating that the Y domain extended further than was previously suggested by sequence alignment with the other isozymes.

Limited proteolysis of a full length PtdIns-PLCδ1–GST fusion protein produced two fragments of 40-kDa and 30-kDa, which were later identified by sequencing to consist of residues 139-474 and residues 475-756 respectively. Thus the 40-kDa fragment included part of the aminoterminus, the whole of the X domain and most of the acidic region between the X and Y domains, with the 30-kDa fragment containing the remaining carboxyterminal part of the protein. The two fragments were able to associate under non-denaturing conditions and the complex possessed full catalytic activity. The accessibility of the acidic hinge to proteolysis and the tight association of the two fragments lends credence to the proposal that in the full length enzyme, the acidic region between the two catalytic domains loops out like a hinge, allowing association of the X and Y domains through non-covalent interactions, to form an active catalytic core.

A deletion mutant of PtdIns-PLCδ1 lacking the first 135 amino acids of the aminoterminus was catalytically active *in vitro* and the X fragment in the active complex also lacked the first 139 residues of the enzyme, suggesting that the extreme aminoterminus is not required for catalysis and may therefore be involved in regulation of the enzyme. Unlike the full length enzyme, the 70-kDa complex was unable to form the 160-kDa homodimers normally seen when full length PtdIns-PLCδ1 is analysed at physiological concentrations [Ryu et al., 1987]. A deletion mutant lacking the first 58 residues was able to dimerise, suggesting that between residues 58 and 139 of the aminoterminus there is a dimerisation domain. Inspection of the alignment of the aminotermini of the PtdIns-PLC isozymes with the PH domain consensus (figure 7.2) reveals that the first 60 amino acids of PtdIns-PLCδ1 contain the first 3 subdomains of the PH domain and that residues 58 to 139 contain the second 3 subdomains of the PH domain. This implies that part of the function of the PH domain in PtdIns-PLCδ1 is as a dimerisation domain, yet the same region of the PH domain in βARK facilitates βγ subunit binding. Thus it appears that one function of the PH domain is to enable protein-protein binding. It will be interesting to
determine the protein recognition sequence of these dimerisation domains and whether each PH domain directs dimerisation with specific protein targets.

Proteolysis studies in another laboratory [Cifuentes et al., 1993] yielded a 77-kDa complex composed of 45-kDa and 32-kDa fragments corresponding to the X and Y domains of PtdIns-PLCδ1. Sequencing of the aminoterminal fragment revealed that it began at residue 61. Again this complex was active, although it was reported to have a 100-fold lower affinity for its substrate PtdIns(4,5)P2 when presented in bilayer vesicles. In addition, the specific activity of the proteolysed complex in a micellar assay was only 10% of the specific activity of the full length enzyme. This indicated that the first 60 amino acids of PtdIns-PLCδ1 probably contained a region necessary for substrate recognition. This may correspond to the non-catalytic PtdIns(4,5)P2 binding site predicted by the two-site mechanism for PtdIns(4,5)P2 hydrolysis by the PtdIns-PLC isozymes. Such a site would be responsible for anchoring the enzyme in the lipid membrane, where it can hydrolyse PtdIns(4,5)P2 processively. It has recently been reported that the PH domain, which has structural similarities to lipid binding proteins, binds to PtdIns(4,5)P2 in PtdCh vesicles [Harlan et al., 1994]. The PH domains of plextrin, T-cell specific kinase, rasGAP and βARK were all tested and found to have PtdIns4P and PtdIns(4,5)P2 binding activity. Furthermore, by analysing the chemical shifts for 1H, 13C and 15N of the plextrin PH domain during PtdIns(4,5)P2 binding, they were able to predict the residues involved in the lipid interactions. All of these interactory residues are located in the aminoterminus of the PH domain at the lip of the β-barrel identified during studies of PH domain structure [Yoon et al., 1994]. It was suggested that 3 positively charged lysine residues in this region (subdomains 1 and 2) might interact directly with the negatively charged phosphates of PtdIns(4,5)P2. This proposal was supported by the inability of a PH domain containing acylated lysines to bind to PtdIns(4,5)P2 in the centrifugation assay. This work strongly suggests that the first part of the PH domain in the amino terminus of PtdIns-PLCδ1 might enable interaction of the enzyme with its substrate in the plasma membrane.

Thus the amino terminal PH domain of PtdIns-PLCδ1 apparently functions as a protein-protein dimerisation domain and as a lipid binding domain, with the remainder of
the molecule required for catalytic activity. The small size of the PH domain and its apparent
dual function seem incompatible, unless the domain could form a structure which allowed
one face to interact with the charged headgroups of certain membrane phospholipids, whilst
the other face mediated protein-protein interactions. Future work on the regulation of this
class of PtdIns-PLC isozymes will inevitably involve determining the relevance of
homodimerisation or heterodimerisation with proteins such as G-protein βγ subunits, to the
regulation of these enzymes. Such questions could be tackled by expressing chimeric
proteins in E.coli, in which the PH domains from different proteins are exchanged and
examining the ability of such mutants to dimerise with different proteins in vitro. Individual
domains could be expressed in the COS-1 cell system described in chapter 8 and their
ability to block the M1 and M2 receptor mediated activation of PtdIns-PLCβ3 tested. To
investigate whether the first part of the PH domain is responsible for membrane targeting of
the protein, through lipid interactions, various portions of the aminoterminus could be
expressed in COS-1 cells and their cellular localisation determined by cell fractionation. The
roles of individual charged residues in such a membrane interaction could then be assessed
through expression of a series of mutants in which each basic residue is converted to a
neutral one.

9.4 Regulation of PtdIns-PLCβ isozymes by Gαq proteins.

Once the Gq family of G-protein α subunits was cloned and αq / α11 directed
activation of the PtdIns-PLCβ isozymes had been demonstrated, much work was done to
establish the specificity of the interactions between the different Gqα subunits and PtdIns-
PLCβ isozymes. Using purified PtdIns-PLCβ isozymes in in vitro assays several
laboratories have concluded that PtdIns-PLCβ1 and PtdIns-PLCβ3 are the isozymes most
strongly activated by αq and α11, whilst PtdIns-PLCβ2 is only poorly activated by these
subunits (see figure 4.4 of this thesis) [Lee et al., 1992; Smrcka and Sternweis, 1993; Jhon
et al., 1993]. Both PtdIns-PLCβ1 and PtdIns-PLCβ3 appear to be widely expressed,
PtdIns-PLCβ1 being the most abundant in brain. PtdIns-PLCβ2 however, appears to be
restricted in its expression to cells of hematopoetic origin, suggesting that specificity of
PtdIns-PLCβ activation may in part be achieved through selective expression of certain isoforms in some cells.

Specificity of the Gqα response is probably also achieved through the different members of the Gq family. Using an in vitro assay similar to that described in 2.2.2.2, Lee et al., [1992] demonstrated that PtdIns-PLCβ1 is most strongly activated by αq and α11, but can also be activated to a lesser extent by α16 and α14. In the same assay, PtdIns-PLCβ2, can only be activated significantly by α16 although this activation is not as great as that seen with PtdIns-PLCβ1. Cotransfection experiments in COS-7 cells demonstrated that PtdIns-PLCβ2 could be activated by αq, α11, α14 and α16 to similar levels [Wu et al., 1993b]. The difference between these in vitro and in vivo results may simply reflect differences in expression levels and assay sensitivity, emphasising the importance of a dual in vitro / in vivo approach. Following the purification of PtdIns-PLCβ3 the same assay was used to demonstrate that αq, α11 and α16 can all activate this isozyme, although to a slightly lower level than PtdIns-PLCβ1 [Jhon et al., 1993]. The α16 directed activation of PtdIns-PLCβ3 was higher than that observed with PtdIns-PLCβ2. Similar results were obtained using α16 expressed in and purified from SF9 cells [Kozasa et al., 1993]. The activation pattern for PtdIns-PLCβ4, determined by overexpression of the Gqα subunits in COS-7 cells, is slightly different [Jiang et al., 1994]. This isozyme is strongly activated by αq, α11, α14 and α15, but poorly activated by α16. The α subunit responsible for activation of the turkey erythrocyte PtdIns-PLCβ-like enzyme was identified as Gα11 [Maurice et al., 1993], indicating that despite their similar specificity in vitro, αq and α11 are differently expressed in vivo. All of this data indicates that in vivo, there is specific activation of particular PtdIns-PLCβ isoforms by the different Gq family α subunits and that differential expression of both α subunits and the PtdIns-PLC isoforms may provide further response specificity. For example, PtdIns-PLCβ2 was first identified in and purified from uninduced HL60 cells [Kriz et al., 1990; Park et al., 1992] and α16 expression appears to be restricted to hematopoietic cells [Amatruda et al., 1991].

A role for α14, its mouse counterpart α15 and α16 has been suggested by studies in which interleukin 8 (II-8) receptors were coexpressed in COS-7 cells with various G-
protein subunits and the IL-8 stimulated release of inositol phosphates was measured [Wu et al., 1993c]. Both the α and β receptors were able to activate inositol phosphate release in response to IL-8, when co-expressed with α14, α15 or α16. However, coexpression with αq or α11 was not sufficient to reconstitute the IL-8 response. It was assumed that the main PtdIns-PLC isozyme in COS-7 cells was PtdIns-PLCβ1 from previous work [Katz et al., 1992], however, based upon expression in COS-1 cells (see chapter 8) it is likely that PtdIns-PLCβ3 is present in COS-7 cells. Thus the co-expressed α subunits might be interacting with either of these two PtdIns-PLC isozymes in response to IL-8. The same system was used to explore a PTX sensitive IL-8 stimulated PtdIns-PLC activation and is discussed below.

In addition to the IL-8 stimulated pathway, several other signalling pathways have been shown to involve coupling through Gαq subunits, including the Ach M1 receptor mediated pathway (through αq) [Mullaney et al., 1993], the thyrotropin-releasing hormone receptor (through αq and α11) [Aragay et al., 1992], the thromboxin A2 receptor [Shenker et al., 1991; Baldassare et al., 1993], the α1-adrenergic receptor [Wu et al., 1992b] and bradykinin stimulated activation of a potassium current in Neuroblastoma cells [Wilk-Blaszczak et al., 1994]. Thus the activation of PtdIns-PLCβ isozymes through the PTX insensitive Gq α subunits is widely used in many diverse receptor mediated signalling mechanisms.

9.4.1 The sites of interaction between PtdIns-PLCβ isozymes and Gαq subunits.

It is now well established that the extended carboxyterminus of PtdIns-PLCβ1 is the region of this protein responsible for interaction with and activation by Gαq subunits. The carboxyterminal domain was shown to disrupt PtdIns-PLCβ activation by Gαq in vitro (chapter 5) and in vivo (chapter 8), demonstrating that this domain can bind to Gαq subunits in competition with full length PtdIns-PLCβ isozymes. Other groups have demonstrated that truncated PtdIns-PLCβ1 lacking the carboxyterminus cannot be activated by Gαq in vitro [Park et al., 1993b] or in transfected COS-7 cells [Wu et al., 1993a].
Through expression of a series of deletion mutants of PtdIns-PLCβ1 the carboxyterminal domain has been further divided into two functionally different subdomains. The 'P' domain (residues 903-1030) is required for membrane localisation of the protein and mutants which lack this region are expressed as cytosolic proteins. The 'G' domain (residues 1030-1142) are required for activation by Gqα and mutants which lack this domain although localised in the particulate fraction, are unable to be activated by Gqα. Two peptides based upon sequences within the 'G' domain were able to fully inhibit Gqα activation of PtdIns-PLCβ1 in vitro, indicating that this region of the protein is able to interact with Gqα.

The identification of a peptide derived from the carboxyterminus of Gqα which possessed inhibitory activity towards the activation of PtdIns-PLCβ1 by Gqα in vitro, indicated that this region of Gqα is able to interact with PtdIns-PLCβ1, but that this interaction was not sufficient to activate the PtdIns-PLC (chapter 5). The failure of the peptide to exert more than 50% inhibition suggested that there is more than one site of interaction between Gqα and PtdIns-PLCβ1. It would be interesting to test the ability of the PtdIns-PLCβ1 'G' domain peptides to lift the inhibition exerted by the Gqα peptide or vice-versa, to establish whether they were able to bind to each other. This work could be followed up by a more detailed analysis of the Gqα carboxyterminus, either using a panel of peptides or GST fusion proteins based upon the sequences in this region and studying their effects upon PtdIns-PLCβ1 activation by Gqα in vitro. Alternatively deletion mutants of Gqα could be coexpressed with PtdIns-PLCβ1 in COS-7 cells and their ability to activate inositol phosphate production in response to AIF4 could be measured using the method established by Wu et al., [1992a], or the membranes from these cells could be purified and tested in the in vitro reconstitution assay described in 2.2.2.2. The same systems could also be used to test a more complete series of PtdIns-PLCβ1 deletion and point mutants for their ability to be activated by Gqα.

The effects of the basic peptides and polyamines on PtdIns-PLCβ1 activation presented in chapter 5 indicated that certain negatively charged molecules (peptides PSS and PSL and sørerminel) can increase the ability of PtdIns-PLCβ1 to hydrolyse its substrate.
but cannot activate the enzyme in a system that normally requires Goαq for activation in the absence of this activator. This effect is different from that of other negatively charged molecules such as polyarginine, which can activate PtdIns(4,5)P2 hydrolysis in the absence of activatory Goαq. The polyarginine effect is probably due to a non-specific disruption of the lipid micelles in the reconstitution assay and therefore has little physiological relevance. However the effect of spermine and the peptides appears to be more specific and may represent a real effect on the activated PtdIns-PLCβ1 enzyme, possibly affecting substrate binding or turnover at the catalytic site. The reported effects of polyamines on PtdIns-PLCδ1 (see 9.1) may act through the same mechanism. A similar effect was observed with a basic peptide derived from the carboxyterminus of the PtdIns-PLCβ2 X domain [Simoes et al., 1993]. This region of PtdIns-PLCβ2 has some similarity with the PtdIns(4,5)P2 binding sites of several actin binding proteins, including profilin and gelsolin, the proteins thought to be involved in the PtdIns-PLCγ activation mechanism (see 1.3.5). The peptide was able to stimulate PtdIns(4,5)P2 hydrolysis by PtdIns-PLCβ2, when added to an in vitro assay at 28μM. The preparation of PtdIns-PLCβ2 used in this experiment was a truncated mutant, lacking the Goαq-interactory carboxyterminus, demonstrating that the observed effect was not dependent upon Goαq activation or the presence of the carboxyterminus. It was suggested that the stimulation observed was a result of the basic peptide binding to PtdIns(4,5)P2 in such a way that it was easier for the PtdIns-PLC to bind to and hydrolyse it, possibly by aggregation. Alternatively it was suggested that this peptide might relieve an inhibitory constraint within the molecule similar to that observed in PtdIns-PLCγ1, exerted by the PCI (see 1.3.5). It will be interesting to determine whether the region of PtdIns-PLCβ2 represented by the peptide is involved in PtdIns(4,5)P2 binding, as the similar region in PtdIns-PLCδ1 did not bind to this lipid very strongly [Rebecchi et al., 1992], but as discussed below, the carboxy terminal portion of the PH domain clearly does. It remains to be determined whether part of the putative PH domain in the aminoterminus of PtdIns-PLCβ2 also contains a PtdIns(4,5)P2 binding domain. It may be easier to interpret these results once 3-D structural data is available for
the PtdIns-PLC isozymes and more is known about the mechanisms occurring in the active site of the enzyme.

9.5 Regulation of PtdIns-PLCβ isozymes by G-protein βγ subunits.

The discovery that a PtdIns-PLC isozyme from HL60 cells [Camps et al., 1992a] and one from bovine liver cytosol [Blank et al., 1992] could be activated by G-protein βγ subunits provided an explanation for the PTX sensitive PtdIns-PLC activation pathways that had been identified in various cell types [Meldrum et al., 1991b]. The HL60 isozyme was subsequently identified as PtdIns-PLCβ2 [Camps et al., 1992b] and other laboratories published similar findings for PtdIns-PLCβ2 [Katz et al., 1992], PtdIns-PLCβ3 [Carozzi et al., 1993] and the turkey erythrocyte PtdIns-PLCβ-like enzyme [Boyer et al., 1992]. It became clear that several of the PtdIns-PLCβ isozymes could be activated in this way and therefore, as with the Gqα activation, it was important to establish whether there was any specificity for this form of activation.

The results presented in chapter 7 of this thesis show that the bacterially expressed PtdIns-PLCβ1 isozyme can be activated to a small degree by βγ, although PtdIns-PLCβ3 from Hela cells is much better activated in this assay. Similar results were obtained with bovine brain PtdIns-PLCβ1 [Carozzi et al., 1993] and COS-1 cell expressed PtdIns-PLCβ2 [Camps et al., 1992b]. Full comparisons of the βγ activation of PtdIns-PLCβ isozymes [Park et al., 1993a; Smrcka and Sternweis, 1993] have indicated the following hierarchy: PtdIns-PLCβ3 > PtdIns-PLCβ2 > PtdIns-PLCβ1 and the newly discovered isozyme in this family, PtdIns-PLCβ4 does not appear to be activated to any extent by βγ subunits [Jiang et al., 1994].

Thus the combination of selective expression, differential sensitivity to the two types of G-protein activation mechanism and to the different Gqα subunits, provides much scope for diverse responses to stimulation of the many different receptors that are coupled to the PtdIns-PLCβ family isozymes. Specificity through selective expression is exemplified in the activation of PtdIns-PLC in response to IL-8 stimulation. During the maturation of neutrophils it has been observed that a greater proportion of PtdIns-PLC
activation becomes PTX sensitive [Baggiolini and Clark-Lewis, 1992] and the amount of 
G\(\alpha_{16}\) decreases substantially, whilst the amount of G\(\alpha_c\) protein increases [Amatruda et al., 1991]. This suggests that during maturation there is a shift from G\(\alpha_q\) directed activation of 
PtdIns-PLC to \(\beta\gamma\) activation of PtdIns-PLC.

Several laboratories are currently trying to establish whether there is any specificity of 
different combinations of \(\beta\) and \(\gamma\) subunits for the different PtdIns-PLC isozymes and 
other \(\beta\gamma\) regulated processes, such as activation of \(\beta\)ARK and adenylate cyclases II and IV. 
There is already some evidence that different \(\beta\) and \(\gamma\) subunit combinations have different 
affinities for different receptors [Kleuss et al., 1992]. Cotransfection experiments have 
been used to demonstrate some subunit specificity for PtdIns-PLC activation, revealing that 
G\(\beta_1\gamma_1\), G\(\beta_1\gamma_5\) and G\(\beta_2\gamma_5\) can activate PtdIns-PLC\(\beta_2\) when overexpressed \textit{in vivo}, but 
G\(\beta_2\gamma_1\) cannot [Wu et al., 1993b]. The specificity of \(\beta\gamma\)-effector and \(\beta\gamma\)-receptor 
interactions is important, as otherwise all \(\beta\gamma\) directed mechanism would be predicted to be 
activated in response to the stimulation of any G-protein coupled receptor. Evidently this 
does not occur. One reason for this may be the low sensitivity of the \(\beta\gamma\) response measured 
\textit{in vitro}, in comparison with the G\(\alpha_q\) stimulated response. If this low level of sensitivity to 
\(\beta\gamma\) subunits exists \textit{in vivo}, PtdIns-PLC isozymes may only be activated in this way when 
receptors are persistently stimulated. This seems unlikely as PTX treatment has been 
shown to have a substantial effect on some PtdIns-PLC mediated signalling pathways. An 
alternative explanation for the low sensitivity observed \textit{in vitro} may be that G\(\alpha\) subunits 
and \(\beta\gamma\) subunits act synergistically in the activation of PtdIns-PLC in a similar manner to 
adenylyl cyclase activation (see general discussion of chapter 8).

It has proven difficult to demonstrate any synergy using \textit{in vitro} assays, as the 
addition of \(\beta\gamma\) subunits may also have the effect of inactivating the G\(\alpha\) subunits in a 
similar mechanism to that proposed for Gi\(\beta\gamma\) subunits on G\(\alpha_s\) (see 1.2.1). Such an 
inhibitory effect of \(\beta\gamma\) subunits on the activation of the turkey erythrocyte PtdIns-PLC 
isozyme by G\(\alpha_{11}\) \textit{in vitro}, has been observed [Waldo et al., 1991]. A similar observation 
was made when activating PtdIns-PLC\(\beta_1\) and PtdIns-PLC\(\beta_3\) with a mixture of G\(\alpha_q/11\), at 
low (1mM) \(\text{Mg}^{2+}\) \textit{in vitro} [Smrcka and Sternweis, 1993]. However at higher \(\text{Mg}^{2+}\)
concentrations (2mM upwards) the effects of \( \beta \gamma \) subunits and G\( \alpha q/11 \) were additive for PtdIns-PLC\( \beta 3 \). This indicated that the two types of G-protein activation occur through different sites on the PtdIns-PLC. COS-7 Cotransfection experiments have provided some evidence for synergistic activation of PtdIns-PLC\( \beta 2 \) by G\( \alpha 16 \) and G\( \beta 1 \gamma 1 \) [Wu et al., 1993b].

The results presented in chapter 8 of this thesis could be interpreted as evidence of synergistic PtdIns-PLC activation by G\( \alpha q \) and \( \beta \gamma \) subunits. Expression of the G\( \alpha q \)-binding carboxyterminal domain of PtdIns-PLC\( \beta 1 \) resulted in inhibition of the carbachol stimulation of PtdIns-PLC activity through the M1 receptor, which exerts its effects through G\( \alpha q \). Similarly, the putative \( \beta \gamma \)-binding aminoterminal domain of PtdIns-PLC\( \beta 2 \) inhibited this pathway, implying that G\( \alpha q \) \( \beta \gamma \) subunits are required for the successful activation of PtdIns-PLC\( \beta 3 \) through the M1 receptor-G\( \alpha q \) pathway. Such a result can either be interpreted as evidence of a synergistic interaction or due to the disruption of the G\( \alpha q \) activation cycle (see general discussion of chapter 8), although the failure of the GST-PLC\( \beta 2 \)-PH fusion protein to inhibit PtdIns-PLC activation in the G\( \alpha q \) reconstitution assay favours the second explanation. In the light of work on the roles of PH domains in proteins discussed above, a third explanation for the inhibitory effect of the aminoterminal domain can be suggested. If the first part (subdomains 1-3) of PH domains does bind to PtdIns(4,5)P\( 2 \), the main substrate of the PtdIns-PLC enzymes, overexpression of such a domain might inhibit the action of any PtdIns-PLC in the cell, through competition for its substrate. However, a large amount of the domain would have to be produced in expressing cells in order to have a significant effect on PtdIns(4,5)P\( 2 \) availability. It therefore seems most likely that the aminoterminual domain exerts its inhibitory effect through binding to \( \beta \gamma \) subunits and preventing the re-cycling of \( \alpha q \) hydrolysis.

**9.5.1 Structural determinants for interaction of PtdIns-PLC\( \beta \) isozymes with G\( \beta \gamma \) subunits.**

The region of the PtdIns-PLC\( \beta \) isozymes responsible for interaction with G\( \beta \gamma \) subunits has yet to been determined. However, there is strong evidence that it lies
somewhere in the amino terminus. Deletion studies have shown that the carboxyterminus of PtdIns-PLCβ2 (from residue 818) is not required for βγ activation of the enzyme in vitro [Schnabel et al., 1993; Lee et al., 1993b]. Furthermore, mutation of the acidic glutamate residues between the X and Y catalytic domains to glutamine residues, had no measurable effect on the activation of PtdIns-PLCβ2 by Gαq or Gβγ subunits. This indicated that this 'hinge-like' region is not directly involved in the activation of the PtdIns-PLCβ isozymes. Expression of chimeric PtdIns-PLC proteins, composed of sequences from PtdIns-PLCβ1 and PtdIns-PLCβ2, in COS-7 cells demonstrated that residues 1-250 of the amino terminus of PtdIns-PLCβ2 are not sufficient to enable βγ activation of PtdIns-PLC activity [Wu et al., 1993b]. However, it is not clear whether this is due to the lack of a βγ interaction domain in this region or due to the lack of other 'activatory' regions. The discovery of a βγ-binding domain in the second half of the βARK PH domain prompted a search for a similar region in the PtdIns-PLCβ isozymes. It has not been possible to demonstrate directly βγ subunit binding to the putative aminoterminal PH domains of the PtdIns-PLC isozymes, however the indirect evidence from the COS-1 cell system implies that such an interaction does occur. Experiments using truncated PtdIns-PLCβ1 proteins lacking this region (see 9.3) have indicated that it may also be involved in the homodimerisation of PtdIns-PLC isozymes, although the physiological relevance of this phenomenon is unknown. Evidently further detailed deletion-and mutagenesis studies are required to establish the region of PtdIns-PLCβ2 responsible for its activation by βγ subunits.

9.6 The role of phosphorylation in PtdIns-PLCβ regulation.

There is much evidence for a negative feedback loop of PKC on PtdIns-PLC activation. TPA treatment of cells inhibits receptor-coupled activation of PtdIns(4,5)P2 hydrolysis and Ca2+ mobilisation [Sortino et al., 1987; Pfeilschifter and Bauer, 1987] and this inhibition could be prevented by incubation of the cells with PKC inhibitors [Hoek et al., 1988]. However, cells in which PKC had been down-regulated by prolonged phorbol ester treatment did not demonstrate the same inhibitory effects. The target of this feedback regulation is however uncertain. There is evidence for PKC phosphorylation of the α-
adrenergic receptor [Leeb-Lundberg et al., 1985], the EGF receptor [Downward et al., 1985], the Gαz subunit [Carlson et al., 1989], PtdIns-PLCβ1 [Ryu et al., 1990] and PtdIns-PLCγ2 [Taylor et al., 1991]. However, it has not been demonstrated that this phosphorylation affects PtdIns-PLC activation. The work described in chapter 6 of this thesis suggests that the site in PtdIns-PLCβ1 identified as a PKC phosphorylation site is in fact phosphorylated by an unknown kinase. It is likely that the kinase is activated in response to treatment of TPA and therefore any screen for the kinase should be performed on extracts from TPA treated cells. The unmodified bacterially expressed PtdIns-PLCβ1 could be used as a target substrate in such a screen.

The site (PAPGS*VKAP) does not closely represent any of the known kinase consensus sequences [Kemp and Pearson, 1990] and appears to be lacking the normal requirement of basic arginine residues. The location of the site between the end of the catalytic Y domain and the G-domain of the carboxterminus suggests that the introduction of a negatively charged phosphate group at this site could possibly disrupt Gαq activation, although without a 3-D structure for this enzyme it is difficult to predict what the role of this region of the molecule would be. Further studies might involve the generation of PtdIns-PLCβ1 mutants in which the serine is mutated to a non-phosphorylatable alanine, or a negatively charged glutamic acid. The effects of these mutations on PtdIns-PLCβ1 activation could be assessed using the in vitro Gαq and βγ coupling assays and the standard cholate assay, or in vivo using the coexpression system.

9.7 Summary

Much has been learnt about the regulation of the different PtdIns-PLC isozymes in cells, through the combination of in vitro and in vivo approaches. Expression of these enzymes in E.coli has proved a useful tool to produce single isozymes and domains for testing in vitro. It is now clear that the different isozyme families are activated through different mechanisms, although the precise details of such mechanisms remain unknown. It is becoming evident that for each of these mechanisms, gaining access to the substrate PtdIns(4,5)P2 is a very important part of PtdIns-PLC activation and may well involve
some form of insertion of the enzyme into the lipid membrane. Thus for the γ class
isozymes, activation requires association with a tyrosine kinase receptor, followed by
phosphorylation of specific tyrosine residues, which somehow enables the enzyme to gain
access to the lipid membrane and its substrate. The b-class enzymes are known to contain a
membrane attachment domain (the P-box) and association of these and possibly the δ
enzymes with G-protein α and βγ subunits might also enable access to the lipid substrate in
the membrane. If progress is to be made in the understanding of PtdIns-PLC activation, it
will be important to assess the effects of phosphorylation and G-protein subunit association
on substrate recognition in the cell membrane. The use of lipid monolayers may be one
method by which such questions could be tackled, using the well established bacterial
expression systems to produce pure components for testing in the monolayer.
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As a result of the work described in chapter 3, the following paper was published: