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By

Lee Mark Wheldon BSc. (Hons).

Department of Cell Physiology and Pharmacology,

University of Leicester.

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RECEPTOR-MEDIATED PHOSPHOINOSITIDE AND CALCIUM SIGNALLING IN THE HUMAN SH-SY5Y NEUROBLASTOMA CELL.

Lee Mark Wheldon

This study investigated G-protein coupled receptor (GPCR) and receptor tyrosine kinase (RTK)-mediated signal transduction via phospholipase C (PLC). Recent evidence suggested RTK-mediated Ca\(^{2+}\) signalling may be relevant in synaptic transmission and an understanding of acute PLC signalling by this receptor family is therefore essential. Neuronal preparations are heterogeneous populations often making interpretation of experiments difficult. This study used the human SH-SY5Y neuroblastoma cell line, providing a simpler model exhibiting many neuronal characteristics and expressing several GPCRs and RTKs. The muscarinic M\(_3\) receptor was used as the model GPCR whilst the platelet-derived growth factor (PDGF) receptor was identified and used as the model RTK.

It has been generally assumed that GPCRs activate PLC-\(\beta\) isoforms whilst RTKs activate PLC-\(\gamma\). However, GPCR-mediated activation of PLC-\(\gamma\), at least in non-neuronal cells, is now a widespread phenomenon. Cells express multiple PLC isoforms that have different regulatory features and it is unclear exactly which PLC isoforms are recruited to any given receptor. This may be important, as recruitment of different PLC isoforms may be responsible for the often-complex patterns of signalling following receptor activation. In particular the aim was to develop methods that allowed identification of the precise isoforms of signalling components (specifically PLC and protein kinase C (PKC)) recruited following receptor activation.

A transient elevation of intracellular [Ca\(^{2+}\)] ([Ca\(^{2+}\)]\(_i\)) was identified in response to PDGF-BB. Despite the recruitment and phosphorylation of PLC-\(\gamma\), the mechanism was apparently novel and Ins(1,4,5)P\(_3\)-independent. Muscarinic M\(_3\) receptor-dependent Ins(1,4,5)P\(_3\) and Ca\(^{2+}\) signalling was biphasic, consisting of a peak and plateau. Both agonist-occupied receptors relied on capacitative Ca\(^{2+}\) influx triggered by intracellular Ca\(^{2+}\) store release for maximum elevation of [Ca\(^{2+}\)], and initiated intracellular Ca\(^{2+}\) release from the same thapsigargin and Ins(1,4,5)P\(_3\)-sensitive intracellular store. This study demonstrated that activation of muscarinic M\(_3\) receptors abolished PDGF-mediated elevation of [Ca\(^{2+}\)], by depleting the intracellular store, whilst PDGF receptors inhibited subsequent muscarinic receptor-mediated elevation of [Ca\(^{2+}\)], possibly by inhibition of a PLC-\(\beta\) isoform. Furthermore, a PDGF-mediated elevation of [Ca\(^{2+}\)], was also identified in differentiated SH-SY5Y cells and hippocampal neurons.

This study identified five PLC (\(\beta_1, \beta_2, \beta_3, \gamma_1\) and \(\delta_2\)) and six PKC (\(\alpha, \gamma, \epsilon, \tau, \lambda\) and \(\zeta\)) isoforms in SH-SY5Y cells. Evidence implicated PLC-\(\beta_1, \beta_2, \beta_3\) and \(\delta_2\), and PKC\(\epsilon\) in muscarinic M\(_3\) receptor-mediated signalling, whilst the data indicated roles for PLC-\(\beta_1, \beta_2, \beta_3\) and \(\gamma_1\), and PKC\(\epsilon\) and \(\zeta\) in PDGF-mediated signalling.

Muscarinic M\(_3\) receptors, PDGF receptors and epidermal growth factor (EGF) receptors activated mitogen-activated protein (MAP) kinase in SH-SY5Y cells. However, only PDGF-mediated MAP kinase activation had a Ca\(^{2+}\)-dependent component suggesting that PDGF-mediated Ca\(^{2+}\) signalling is important in cell function. Clearly such Ca\(^{2+}\) signalling may play other roles and an understanding of these functions emphasises the need to understand the mechanism of [Ca\(^{2+}\)]\(_i\) regulation by growth factor receptors in neurons.
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Publications:


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1: INTRODUCTION

1.i. RECEPTOR SIGNALLING MECHANISMS

Signalling molecules binding to cell surface receptors and the subsequent activation of one or more intracellular signalling cascades, is a ubiquitous and fundamental mechanism employed by cells to respond to appropriate environmental signals. These agonists act in an endocrine, paracrine, or autocrine fashion to elicit a defined response from a target cell, that is often tissue-type dependent, following agonist binding to its cell surface receptor with high specificity. They employ a host of mechanisms and perform a variety of functions. Examples of such molecules include: steroids, opiates, growth factors, hormones, chemokines and neurotransmitters, and range from very large polypeptides (such as many members of the growth factor family) through small molecules (exemplified by acetylcholine and epinephrine (adrenalin)) to ions (such as Ca$^{2+}$).

Despite the huge variety of agonists, or ligands, there are relatively few classes of receptor. These are commonly grouped based on the tertiary structure of the receptor and are divided into four main families: the cytoplasmic or nuclear receptors that are activated as transcription factors upon ligand binding and are epitomized by the steroid receptors; growth factor receptors which span the plasma membrane and result in tyrosine-specific phosphorylation of intracellular proteins (receptor tyrosine kinases or RTKs); transmembrane receptors that stimulate the synthesis or release of second messengers which in turn mediate the cell response, typified by the adrenergic receptors and other GTP-binding protein (G-protein)-coupled receptors (GPCRs); and finally the transmembrane ion channels, which upon activation allow the flow of specific ions into or out of a cell, exemplified by the nicotinic acetylcholine receptor family. This thesis set out to investigate and compare several aspects of both growth factor and GPCR second messenger-mediated signalling in a neuronal context.

The pretext for this study was the possibility that these different classes of receptor could undergo heterologous regulation of downstream signalling following agonist stimulation. Also, RTKs are more commonly assigned to chronic effects within the cell, whereas recent evidence has defined several acute roles for PDGF in real neurons. Of primary interest was the phospholipase C (PLC)-mediated
generation of the ubiquitous second messenger inositol-1,4,5-trisphosphate (Ins(1,4,5)P$_3$), and the mechanisms for any subsequent elevation of intracellular calcium ([Ca$^{2+}$]). The complexity of receptor-mediated signalling is considered in greater detail below.

\[ \text{i.i.i. G-protein-coupled receptor signalling mechanisms} \]

GPCRs are the largest family of structurally related receptors, with over 1000 known members and are targets for a wide variety of extracellular stimuli such as: light, odorants, hormones and neurotransmitters. Whilst there is a great diversity in the primary structure of these receptors, there is some conservation in the known and predicted secondary and tertiary structures.

GPCRs are characterised by a single polypeptide chain comprising: an N-terminal extracellular domain, seven membrane-spanning $\alpha$-helices with associated intra- and extracellular loops, and a C-terminal intracellular domain [Dohlman et al., 1991]. Ligand binding usually involves residues in the trans-membrane $\alpha$-helices and/or extracellular domains [Strader et al., 1995], whilst the second and/or third intracellular loop and C-terminal tail are known to provide attachment sites for the G-proteins and potentially for other regulatory proteins. The variety of regulatory proteins, such as: regulators of GPCR signalling (RGS proteins) [reviewed in Dohlman and Thorner, 1997], GPCR kinases (GRKs) and $\beta$-arrestins [reviewed in Lefkowitz, 1998], as well as the G-protein and primary effector protein(s) in the microenvironment of the receptor likely determine the signalling specificity to downstream effectors and efficacy of any given GPCR. It remains unclear whether these proteins exist in a preformed signal transduction complex, which is stabilised by ligand binding, or whether ligand binding initiates the formation of the signalling complex de novo.

G-protein activation is the critical step for successful signal transduction following agonist binding to this class of receptors. G-proteins are grouped into four main families ($G_q$, $G_s$, $G_i$ and $G_{12}$) and composed of three heterologous sub-units termed $\alpha$ (17 known sub-unit isotypes excluding splice variants), $\beta$ (5 known sub-unit isotypes) and $\gamma$ (11 known sub-unit isotypes) [Simon et al., 1991; Exton, 1997a and references therein]. In the cell, the $\beta$ and $\gamma$ sub-units are constitutively
associated to form the Gβγ sub-unit. Receptor activation initiates the exchange of GDP (G-protein inactive when bound) for GTP (G-protein active when bound) by the trimeric G-protein. Agonist binding and subsequent GDP/GTP exchange promotes the dissociation of the Go and Gβγ sub-units which can then interact with primary effector molecules in a manner that can be either mutually independent, synergistic, antagonistic or exclusive (i.e. the effector binds only one of either Go or Gβγ).

G-proteins are intrinsic GTPases, and thus regulate the active lifetime of the signal by hydrolysis of the bound GTP. GTPase activating proteins (GAPs) are able to increase this GTPase activity, and thus decrease the lifetime of the active G-protein, modulating the agonist-mediated signalling. The agonist-bound receptor is a prime example of a class of proteins termed guanine-nucleotide-exchange factors, or GEFs. The function of a GEF protein is in direct opposition to the G-protein GTPase activity, acting to increase the dissociation rate of GDP and promote the binding of GTP, thus offsetting the action of GAP molecules. Activation of a G-protein is therefore a dynamic process, dependent on the combined effects of both GEF and GAP action.

Until recently, only two GAPs for heterotrimeric G-proteins were known, one for the Gq family of G-proteins and another for Gi. Reconstitution experiments with purified Gαq have shown that, at least in vitro, this Gα subunit has a poor intrinsic GTPase activity, but that its effector molecule, PLC-β1 acts as a GAP [Bernstein et al., 1992; Biddlecome et al., 1996] with up to a 60-fold increase of intrinsic GTPase activity. The GAP for Gi has also been identified as its effector protein, guanosine 3', 5'-cyclic monophosphate (cGMP) phosphodiesterase [Arshavsky and Bownds, 1992]. The recent discovery of the large RGS protein family (approximately 20 members) and the subsequent definition of their negative regulatory role in GPCR signalling may provide an almost ubiquitous mechanism for G-protein-mediated signal attenuation. RGS proteins have been identified as GAPs for the α sub-units of Gq and Gi families of G-proteins, and specifically for Gi, but not for the Gs or G12 families [Hepler et al., 1997; Dohlman and Thorner, 1997; Cavalli et al., 2000; Scheschonka et al., 2000; Skiba et al., 2000]. The return of the
Gα sub-unit to an inactive, GDP-bound form also allows for re-association of the Gα sub-unit with Gβγ [all reviewed in Morris and Scarlata, 1997; Exton, 1997a].

Upon activation, Gα and Gβγ sub-units are able to interact with specific target proteins within the cell, known as the effector proteins. Effector proteins are most often either enzymes, that produce intracellular second messengers and directly couple the GPCR to intracellular signalling pathways, or membrane-localised ion channels. Typical examples of second-messenger producing effector proteins include: ‘adenylyl cyclase’ which produces the second messenger adenosine 3', 5'-cyclic monophosphate (cAMP); ‘nitric oxide synthase’, producing nitric oxide (NO); ‘cGMP phosphodiesterase’ which degrades cGMP; and ‘phospholipase C’ which generates Ins(1,4,5)P3 and diacylglycerol (DAG) and is a major mechanism for the agonist-mediated elevation of [Ca2+]i, within most cells.

The direct interaction of G-protein sub-units has also been implicated in the inhibition of Ca2+ currents through membrane-bound, voltage-operated calcium channels [Caulfield et al., 1994; Maturana et al., 1999] and both the activation and inhibition of inwardly rectifying K+ channels [Fernandez-Fernandez et al., 1999; Jing et al., 1999]. Although it is clear that activated Gα or Gβγ sub-units can directly modulate ion channels, there is also evidence that channels can also be regulated in a G-protein-dependent manner that involves the generation of diffusible messengers subsequent to G-protein activation. G-protein βγ sub-units are also known to recruit other regulatory proteins to the receptor, such as members of the GRK family that are responsible for an agonist-specific loss of receptor responsiveness due to GPCR phosphorylation. A classic example of this function is the recruitment of β-adrenergic receptor kinase (βARK or GRK2), the first identified GRK, to the membrane by Gβγ sub-units following agonist stimulation of the β-adrenergic receptor [Pitcher et al., 1995]. Selective regulation of the Gαq sub-unit by an RGS domain in GRK2 has recently been reported which may suggest a role for GRK family members in the dual regulation of both receptor and G-protein [Carman et al., 1999].

The activation of a wide variety of classical GPCRs is also known to elicit a mitogenic response via the mitogen-activated protein kinase (MAPK) cascade. This pathway ultimately terminates within the cell nucleus with the activation of
transcription factors responsible for the generation of proteins that allow the cell to progress through the cell cycle. There are several known mitogenic pathways that follow this premise within most cells and they are distinguished and described by the final kinase of the pathway. Thus, the extracellular-regulated kinase (ERK), c-Jun NH₂-terminal Kinase-1 (JNK) and stress-activated protein kinase-2 (p38) cascades are all independent, yet similar, MAP kinase pathways that may be activated following mitogenic stimuli, with a high propensity for cross-talk and inter-/intra-regulation (discussed in greater detail in chapter 6) [reviewed in Cobb and Goldsmith, 1995; Kyriakis and Avruch, 1996].

Agonists such as: bombesin; bradykinin; substance P; endothelin; acetylcholine; prostaglandins; and lysophosphatidic acid (LPA) have all been shown to stimulate cell growth in a variety of cell types. This aspect of GPCR signalling commonly integrates with RTK-mediated signalling at the level of the small GTP-binding protein, Ras and is commonly transduced by Gβγ sub-units. More recently it has become established that GPCRs, in contrast to RTKs, mediate the activation of JNK via the Gβγ activation of the small GTP-binding proteins Rac and Cdc42, and that activation of this mitogenic pathway by GPCRs requires receptor endocytosis [Koch et al., 1994; Lefkowitz, 1998]. The favoured hypothesis for the endocytotic requirement is that the receptor recruits all the components of the pathway up to and including Raf into a multi-protein signalling complex at the membrane. Signal transduction however, appears to require the internalisation of active Raf in order to interact with downstream effectors [Daaka et al., 1997; reviewed in Gutkind, 1998]. More recently, evidence obtained from internalisation-deficient mutant M₃ receptors and endocytotic inhibitors in conjunction with wild-type M₃ receptors has demonstrated that endocytosis may not be a requirement for efficient MAP kinase activation by all muscarinic receptor subtypes [Budd et al., 1999].

Muscarinic cholinergic GPCRs are well-characterised examples of a family of GPCRs that couple to both G₉ and G₁ families of G-proteins. The muscarinic M₃ receptor is widely accepted to transmit ligand-mediated activation via the G₉ family of G-proteins and the subsequent PLC-mediated generation of multiple phosphoinositide and Ca²⁺ signalling mechanisms. The comparative study between classical RTK (represented by PDGF) and GPCR signalling presented in this thesis
utilised this muscarinic M₃ receptor as an archetypical example of PLC-coupled GPCRs.

1.i.ii. Muscarinic cholinoreceptor-mediated signalling

Cholinergic receptors, for which the endogenous ligand is the neurotransmitter acetylcholine, are divided into two distinct families. The nicotinic acetylcholine receptors are ligand-gated, membrane-bound ion (Na⁺) channels and the muscarinic acetylcholine receptors typify the seven transmembrane GPCR class. Therefore, in neuronal systems, acetylcholine can integrate both ionotropic and metabotropic signalling mechanisms.

The muscarinic cholinoreceptors are further sub-divided with 5 main isotypes cloned to date, M₁-M₅, which share a high degree of sequence homology. All subtypes share the common tertiary structure typical of GPCRs (detailed previously, Section 1.i.i.). With regard to the muscarinic receptors, the second intracellular loop (i2) has been proposed as the domain that governs G-protein binding [Burstein et al., 1998] along with the third intracellular loop (i3) that has been reported as the site of Gβγ binding in the absence of Ga [Wu et al., 1999]. Acute GPCR regulation involves receptor phosphorylation and the i3 loop is commonly the major site of phosphorylation that may be responsible for receptor desensitisation, G-protein ‘switching’ and/or internalisation by GRKs and other identified kinases such as PKC, protein kinase A (PKA) and casein kinase 1α [Debburman et al., 1995; Tobin et al., 1996; Tobin et al., 1997; Budd et al., 2000; reviewed in Tobin, 1997; Lefkowitz, 1998].

Both M₂ and M₄ subtypes couple predominantly to pertussis toxin-sensitive Gᵢ proteins resulting in inhibition of AC activity and the subsequent reduction of cAMP levels. The M₁, M₃ and M₅ receptors are predominantly coupled to PLC via the pertussis toxin-insensitive Gq/11 family of G-proteins [Burford et al., 1995; reviewed in Caulfield, 1993] with some evidence that M₁ and M₃ may also stimulate AC in some cell types. Thus, interactions between the muscarinic receptor subtypes and the effector proteins do not appear to be exclusive, especially where data was established from transfected cell lines where the receptor is expressed at relatively high level [Peralta et al., 1988; Richards, 1991; Burford et al., 1995].
Although generally accepted to play a stimulatory role in cell growth, the Gq-coupled muscarinic M3 receptor is also reported to play an inhibitory role in NIH 3T3 cell growth and mitogenesis and these roles were dependent on the stage of the cell cycle. Activation of the MAP kinase cascade, and subsequently ERK/JNK, may occur through a tyrosine-kinase-dependent mechanism that could involve Gβγ subunits, arrestins, the calcium-sensitive, Pyk-2 protein-mediated activation of Src family kinases, the phosphorylation of the adaptor protein Shc and the subsequent formation of Shc-GRB2 complexes that activate the low molecular mass G protein Ras [Luttrell et al., 1996; Wu et al., 1997]. Alternatively, mechanisms have been proposed through a Gβγ-mediated transactivation of the EGF receptor (see section 1.ii.iii. for a more detailed discussion) [Linseman et al., 1995; Daub et al., 1996; all reviewed in Lefkowitz, 1998; Gutkind, 1998]. Inhibition of cell growth was identified, only in actively growing cells, as occurring by a reduction in the level of cyclin D1 and hypophosphorylation of the retinoblastoma gene product, Rb [Nicke et al., 1999]. The actual mechanism employed is likely cell type specific and will rely on the relevant expression of other signalling components.

The muscarinic M3 receptor, upon which this present study concentrates, is predominantly studied as it stimulates the PLC-mediated hydrolysis of the minor membrane phospholipid, phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2) resulting in the production of Ins(1,4,5)P3 and DAG (see section 1.ii. and 1.iv. for a fuller discussion of these messengers). It is this role of the M3 muscarinic receptor that is best recognised and explored rather than any possible trophic effects. This pathway may not be the sole preserve of GPCRs however, as PLC-γ-recruiting RTKs may also signal via similar effector molecules to allow the regulation of [Ca2+]i (discussed in greater detail below) (see Fig 1.i.). The intention of this investigation was, initially, to compare and contrast muscarinic receptor PLC-mediated signalling with RTK-mediated PLC signalling (through methods that examined downstream components such as Ins(1,4,5)P3 and Ca2+).
1.1.iii. Growth Factor Signalling

Growth factors, in their more general sense, are responsible for a variety of responses in eukaryotic cells, including: proliferation, differentiation, apoptosis and cell survival. In many cases these signalling molecules are pleiotropic i.e. more than one growth factor may elicit the same response in a particular cell, and conversely the same growth factor may elicit different responses depending upon the cell type. The relevance of this statement is that it soon becomes apparent that not only is the presence of the receptor important but the cell type will determine the activation/regulation of relevant intracellular signalling pathways. This applies to all receptors.
Classical growth factors such as epidermal growth factor (EGF), fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF), to name some of the best understood examples, are large polypeptide signalling molecules (e.g. PDGF is 25-29 kDa) and their respective receptors are prototypic members of a large family of receptors characterised by an extracellular ligand-binding domain, a single transmembrane segment and a large intracellular catalytic domain. The catalytic domain exhibits protein tyrosine kinase activity or activates non-receptor protein tyrosine kinases (PTKs), thus receptors of this type are termed ‘receptor tyrosine kinases’ or ‘RTKs’ [Sprang and Bazan, 1993; Fantl et al., 1993]. The crucial step following activation of these receptors is dimerisation followed by autophosphorylation of the intracellular domain [Kazlauskas and Cooper, 1989; Fantl et al., 1993], although autophosphorylation may be a misleading term as the dimeric receptors actually cross-phosphorylate each other.

Tyrosine phosphorylation occurs upon specific tyrosine residues of the intracellular domain of the receptor and promotes the interaction of a host of proteins and enzymes with the receptor. These proteins contain Src homology-2 (SH-2) domains, which are 3-dimensional protein motifs, of ~100 amino acids, capable of binding to phosphorylated tyrosine residues [Pawson and Gish, 1992]. It has also been shown that the three amino acids C-terminal to the phosphorylated tyrosine are critical in determining the specificity of this interaction [Fantl et al., 1992]. Furthermore, tandem SH-2 domains may be a mechanism whereby not only the specificity, but also the affinity, of protein interactions can be increased [Ottinger et al., 1998].

Many of the recruited proteins also contain Src homology-3 (SH-3) domains, protein motifs of 50-75 amino acids, which function to mediate protein-protein interactions [Pawson and Gish, 1992]. These SH-3 domains target short amino acid sequences (about 10 amino acids long), rich in the amino acid proline, which may adopt a left-handed, polyproline type II helix conformation. Literary evidence also suggests that SH-3 domains may be responsible for the targeting of proteins to particular subcellular locations, including association with the plasma membrane [Wasenius et al., 1989; Bar-sagi et al., 1993]. Some of the specificity of the interactions of both SH-2 and SH-3 domains will undoubtedly be conferred by the sequence of the domain and not reliant purely on the existence of the domain.
Some of the proteins recruited following ligand-dependent activation of the receptor may themselves be targets for the intrinsic tyrosine kinase activity of the receptor. Tyrosine phosphorylation of these proteins, upon receptor binding, may result in a change in activity of the target molecule, in the case of an enzyme, or act as a regulatory, often localisation, signalling mechanism. Often the recruited proteins have no catalytic activity and act purely as 'adaptor' proteins whose function is to recruit other proteins to the activated receptor. As a further complication, the exact purpose of any phosphorylation of adaptor proteins is unknown but it may provide more binding sites for SH-2 domain containing proteins.

Once recruited to the receptor, these molecules activate a variety of second messenger systems and intracellular signalling cascades that may directly or indirectly mediate the cell response.

The complexity of the inter-regulation and cross-talk of multiple signalling pathways has long been studied as a method of determining the function behind the activation of a particular receptor. Often multiple signalling pathways converge in the nucleus, with the activation of transcription factors responsible for the expression of genes required by the cell to progress through the cell cycle.

The majority of growth factor signalling is studied in mesenchyme-derived cell lines, with the emphasis commonly on mitogenic and survival properties [Fantl et al., 1993; reviewed in Malarkey et al., 1995]. The pleiotropic nature of growth factors however, dictates that mechanisms established in one cell type may be specific and not applicable to all cells. It is likely therefore, that in specialised cell types, such as neurons, the mechanisms will be distinct. There has also been several reports published recently that implicate growth factors, and in particular PDGF, in the acute regulation of synaptic transmission and receptors involved in this process, such as the NMDA receptor [Valenzuela et al., 1995; Valenzuela et al., 1996; Hilborn et al., 1998; Lei et al., 1999] that are discussed in greater detail later (see section 1.v.).

One of the primary aims of this study was to compare several different aspects of acute 'classical' growth factor and GPCR signalling at the post-receptor level. Following the initial investigation into growth factor-mediated elevations of [Ca$^{2+}$], in the SH-SY5Y neuroblastoma cell (see Results Chapter 3, section 3.iii.), the PDGF receptor was chosen as a paradigm growth factor receptor for this study.
1.i.iv. Platelet-derived growth factor-mediated signalling

PDGF-mediated signalling is critical during embryogenesis for the correct development of kidneys, blood vessels, and lungs, and most relevantly to this study, the central nervous system (CNS). PDGF over-activity has been linked to several different disorders, e.g. atherosclerosis, kidney and lung fibrosis, and liver cirrhosis and the transforming potential of PDGF often aids in the progression of aggressive carcinomas by an autocrine signalling mechanism [reviewed in Heldin and Westermark, 1999].

PDGF, based on the known crystal structure of human PDGF, belongs to a class of growth factors termed the ‘cysteine knot’ family [Oefner et al., 1992; Sprang and Bazan, 1993]. Other members of this family include NGF and transforming growth factor-β (TGFβ) [Sprang and Bazan, 1993]. PDGF exists as two homologous isoforms, PDGF-A (14.5 kDa) and PDGF-B (12.5 kDa), but is biologically active only as a homo- or heterodimer. This growth factor therefore has three biologically active isotypes: PDGF-AA, PDGF-AB and PDGF-BB. The dimeric PDGF acts as a classical growth factor, inducing dimerisation and autophosphorylation of the PDGF receptor. The PDGF receptor also exists as two isotypes, α and β, and the cross-reactivity of these six molecules are summarised in Fig 1.ii.

PDGF-BB is the universal ligand as it leads to the activation of all possible PDGF receptor sub-unit combinations [Claesson-Welsh, 1994]. The formation of a particular signalling complex is governed not only by the PDGF dimer but also by the relative expression levels of the two PDGF receptor isoforms. Although displaying some similarities, the PDGF α and β chains exhibit a number of differences in the activation of signalling pathways and the active receptor-complex isotype will have an effect on the subsequent signal propagation [Gelderloos et al., 1998]. As an example, the degree of actin reorganisation in response to PDGF stimulation is specified by the nature of the receptor dimer [Hammacher et al., 1989].
Activation of the PDGF receptor is known to recruit a variety of proteins containing SH2 domains, including: PLC-\(\gamma\) [Meisenhelder et al., 1989; Kim et al., 1991]; the p85 sub-unit of phosphatidylinositol-3-kinase (PI-3-K) [Kazlauskas and Cooper, 1989]; Grb-2 [Lowenstein, 1992], the GTPase-activating protein (GAP) for the low molecular weight GTP-binding protein Ras [Kazlauskas et al., 1990]; SHP-2 (previously also known as Syp, SH-PTP2, PTP1D, SHPTP3 or PTP2C), a phosphotyrosine phosphatase (PTPase) [Lechleider et al., 1993]; and members of the Src family of non-receptor tyrosine kinases [Mori et al., 1993] [all reviewed in Claesson-Welsh, 1994; Malarkey et al., 1995; Heldin and Östman, 1998; Heldin and Westermark, 1999]. These proteins all bind to specific phosphotyrosine residues on the receptor, some of which are between the ‘split’ kinase domain and have been individually implicated in several functional responses. For example, PI-3K has been reported to regulate mitogenesis, inhibition of apoptosis, intracellular vesicle trafficking/secretion and regulation of actin and integrin function, in a cell type and...
agonist-dependent manner. Similarly, PLC-\(\gamma\) has been identified as playing a role in mitogenesis, chemotaxis, cell migration and cell transformation [reviewed in Carpenter and Cantley, 1996]. However, the definitive common theme for growth factor-mediated signalling is mitogenesis and transformation. Without exception, all of these proteins have been implicated in the activation of the mitogen-activated protein kinase (MAPK) pathway. The crucial consequence of MAPK activation is the transcription of genes required by the cell to progress through the cell cycle.

Recently evidence has shown that a small GTP-binding protein of the Ras superfamily of GTPases, most likely Rho and/or Rac1, can also associate with an activated PDGF-receptor \(\beta\) chain in 3T3 fibroblasts, despite the fact that it is not thought to contain any SH-2 domains that could mediate this interaction [Zubiaur et al., 1995]. Rho-family proteins have also been implicated in PDGF-receptor signalling in N1E-115 neuroblastoma cells [Zhang et al., 1996] and Rac is involved in JNK/p38 MAP kinase activation [reviewed in Gutkind, 1998]. The relevance of this is unclear, but Rho family GTPases are known regulators of phosphatidylinositol-4-phosphate 5-kinase, which is one of the principal mediators of PtdIns(4,5)P\(_2\) synthesis [Chong et al., 1994; Tolias et al., 1995]. Thus, they may exert their effect on the classical PLC-mediated signalling pathway in an indirect manner by regulation of the substrate required for second messenger generation.

It is interesting to note that activation of the PDGF-receptor in the absence of ligand (i.e. PDGF) has been shown during at least three cellular processes. Firstly, during the early stages of cell adhesion in AG1518 fibroblasts, PDGF-independent PDGF receptor dimer formation occurs and may suggest a new role for RTKs in integrin-mediated signalling [Sundberg and Rubin, 1996].

The second example involves a recent observation in Hs27 fibroblasts and Cos-7 cells. In this more peculiar instance, transactivation of the PDGF-receptor \(\beta\)-chain following EGF-receptor activation was evidenced by a rapid tyrosine phosphorylation and the recruitment of PI-3K. The PDGF \(\beta\)-chain was reported to be directly interacting with the EGF-receptor and this association was independent of PDGF receptor ligand in all cell lines tested [Habib et al., 1998]. A heterologous desensitisation of EGF-receptors following PDGF stimulation has also been reported [Wrann et al., 1980] and the mechanism includes a decrease in the affinity of the
EGF receptor for its ligand and a loss of intrinsic kinase activity [Bowen-Pope et al., 1983; Friedman et al., 1984; Hunter et al., 1984]. This effect can be reproduced by phorbol ester treatment of the cells and led to the hypothesis that PDGF receptor-activated PKC was the mediator of this regulation. However, more recent evidence would suggest otherwise and is discussed in more detail later (see section 1.iv.iii.).

Finally, PDGF receptor activation in the absence of ligand has been observed in response to the GPCR agonist LPA [Herrlich et al., 1998]. Although prior reports have demonstrated the ability of LPA to trans-activate the EGF receptor [Daub et al., 1996; Daub et al., 1997], PDGF receptor β-chain activation was observed here only in the absence of the EGF receptor. In contrast, PDGF receptor transactivation in preference to the EGF receptor has recently been demonstrated in primary mesangial cells [Goppelt-Struebe et al., 2000].

It is now speculated that the transactivation of certain growth factor receptors, in particular the EGF and PDGF receptors, may be a general feature of many GPCRs, especially those that are G\textsubscript{i}-coupled, mediate a mitogenic response and rely on the activation of the transcription factors via the various MAP kinase signalling cascades. Thus, the interactions between growth factor and GPCR-mediated signalling mechanisms may provide new paradigms in signal integration and will subsequently be discussed in greater detail (see section 1.ii.iii.).

Both the muscarinic M\textsubscript{3} and PDGF receptors are known to recruit and activate different isoforms of PLC (discussed in greater detail in section 1.ii.), whose catalytic commonality is the subsequent hydrolysis of a minor membrane component, the phospholipid PtdIns(4,5)P\textsubscript{2}, and the generation of intracellular second messengers (discussed in greater detail in section 1.iii.). It is important to understand therefore, the complexity of the regulation and role of PLC in agonist-driven cellular responses, in order to try and investigate, characterize and understand the downstream signalling events following the activation of these receptors.
**I.ii. PHOSPHOINOSITIDE-SPECIFIC PHOSPHOLIPASE C ISOFORMS**

To date there are at least 11 identified mammalian PLC isoforms (excluding splice variants) that are divided into three main families (β, γ and δ) [Rhee and Choi, 1989; Cockcroft and Thomas, 1992; reviewed in Lee and Rhee, 1995], and a recently proposed 4th family comprising the novel PLC-ε (discussed in Chapter 7) [Lopez *et al.*, 2001; Song *et al.*, 2001], based on primary and secondary structure homology. There are four PLC-β, two PLC-γ and four PLC-δ isoforms known, which are all single polypeptides containing two regions, X (~170 amino acids) and Y (~260 amino acids), of high sequence homology that compose the catalytic domain, whose function is the hydrolysis of PtdIns(4,5)P₂. They also have in common several other structural protein motifs such as EF-hand and C2 domains (both of which are calcium binding motifs that occur in a variety of unrelated proteins) and PH domains that generally play a key role in membrane attachment (see **Fig. I.iii.**). Until 1996, the modular design of the PLCs had been based on primary and secondary structure homology. Conformation of the suspected tertiary structure of PLCs was obtained with the determination of the three dimensional structure of PLC-δ₁ lacking the PH domain [Essen *et al.*, 1996]. To date this is still the only crystal structure of any of the PLC isoforms.

The PLC-δ isoforms are smaller (M₉ 85,000) than either the PLC-γ (M₉ 146,000) or PLC-β (M₉ 134,000-155,000) isoforms. All PLCs require calcium ions (Ca²⁺) for activity, with PLC-δ exhibiting the greatest calcium sensitivity *in vitro* [Allen *et al.*, 1997]. Calcium is required for either the function or orientation of the C2 domain [Essen *et al.*, 1996], and a Ca²⁺ ion is located at the active site where two histidine residues, equivalent to His¹¹¹ and His³⁵⁶, are universally conserved among all eukaryotic PLC isoforms. Point mutation of either histidine residue results in complete enzyme inactivation [Smith *et al.*, 1994].
The PLC-β and PLC-δ isoforms have short (50-70) amino acid sequences linking the X and Y regions of homology, whereas PLC-γ has ~400 that constitute two SH-2 domains, an SH-3 domain and a putative second ‘split’ PH domain. The PLC-β isoforms have a long C-terminal region following the C2 domain, whilst this region is almost non-existent on the PLC-δ isoforms. It is the difference in these regulatory regions that is responsible for linking different PLC isoforms to receptors with differing activation mechanisms. Thus, although the evidence shows a great deal of similarity in the structure of the various PLC isoforms, it is the differences between them that appear responsible for the differing regulatory mechanisms discussed below.
1.ii.i. G-protein-coupled receptors and the activation of PLC-β isoforms

GPCRs that transmit their signals via the Gq family of pertussis toxin-insensitive G proteins (Gq, G11, G14 and G16), are known to activate all four PLC-β isoforms and until recently were not thought to activate either PLC-γ or PLC-δ isotypes (see sections 1.ii.iii. and 1.ii.iv.). Stimulation of these seven transmembrane domain receptors, characteristic of hormones and neurotransmitters (e.g. certain receptor subtypes activated by acetylcholine, bradykinin, angiotensin II, bombesin, LPA, bombesin, vasopressin, endothelin and noradrenaline), results in the activation of a heterotrimeric G protein [Cockcroft and Thomas, 1992; Hepler and Gilman, 1992; Noh et al., 1995; Morris and Scarlata, 1997; Exton, 1997a].

Activated Gαq/11 sub-units stimulate PLC-β isoforms with differing potencies: PLC-β1 > PLC-β3 > PLC-β4 > PLC-β2. The Gβγ dimer, with a rank order of potency that differs from the Gα sub-unit, also activates PLC-β: PLC-β3 > PLC-β2 > PLC-β1, but PLC-β4 is unaffected by the Gβγ dimer [Lee and Rhee, 1995; Noh et al., 1995]. The potency of Gβγ-mediated PLC activation is considerably less (10-100-fold) than Gα-mediated effects. Thus, activation by Gβγ predominantly occurs following agonist-dependent activation of abundant G-proteins such as the pertussis toxin-sensitive Gø and Gi families, which can account for 1-2% of the total protein in a typical brain cell. Receptors capable of coupling to more than one family of G-proteins would therefore be capable of activating more than one PLC isoform. Although there are many examples of ‘promiscuous coupling’ of receptors to different G-proteins, a report recently provided a definitive example of a receptor that ‘switches’ G-protein coupling between the Gs and Gi G-protein families, following a PKA-dependent phosphorylation of the receptor [Daaka et al., 1997]. There are however, many reports of GPCR-mediated activation of PLC isoforms and PLC enzyme activity that are only partially pertussis toxin-sensitive. This suggests that activation of two or more PLC isoforms is possibly a more general mechanism for some receptors in specific cell types.

It is likely therefore, that both Gα and Gβγ sub-units, generated from the same or more often, different heterotrimeric G-proteins, may play a role in signalling through PLC. In some cases, the efficient activation of a given PLC isoform may require the independent action of both Gα and Gβγ sub-units. The sites of
interaction with the PLC under these circumstances must therefore be distinct. This is known to be true for PLC-\(\beta_1\) and PLC-\(\beta_2\). In this example, \(G\alpha_q\) contacts the C2 domain downstream of the Y region of homology and the \(G\beta\gamma\) sub-unit contact has been localised to the region including, and flanking, the small insert between the X and Y regions [Park et al., 1993; Kuang et al., 1996; Wang et al., 1999a; reviewed in Morris and Scarlata, 1997]. This suggests that if \(G\alpha\) and \(G\beta\gamma\) can act independently on the effector molecule they may also act additively/synergistically or even antagonistically.

There is now however, direct evidence that the N-terminal PH domains of both PLC-\(\beta_1\) and PLC-\(\beta_2\) also interact with \(G\beta\gamma\) sub-units, activating and recruiting the PLC to the membrane. The use of PLC-\(\beta_2\) and PLC-\(\delta_1\) chimeras demonstrated a \(G\beta\gamma\)-dependent recruitment and activation of PLC-\(\delta_1\) when the PI I domain was replaced with that of PLC-\(\beta_2\) [Wang et al., 2000]. Also, the isolated PH domain of PLC-\(\beta_1\) has been demonstrated to be \(G\beta\gamma\) and PI-3K-dependent for activation and membrane localisation, possibly recruited by one of the products of PI-3K activation, phosphatidylinositol 3-phosphate (PtdIns(3)P) [Razzini et al., 2000]. It is also possible that the lower sensitivity of this isoform to \(G\beta\gamma\) activation, than either PLC-\(\beta_3\) or PLC-\(\beta_2\), may be due to the relative levels of the required phosphoinositides rather than any differential \(G\beta\gamma\) regulation. Whether this PH domain-mediated effect precludes the previously reported site of interaction of PLC-\(\beta\) with \(G\beta\gamma\), or is specific to a particular \(G\beta\gamma\) sub-unit combination is unclear. Both mechanisms of \(G\beta\gamma\) recruitment support the hypothesis that PLC-\(\beta_1\) and PLC-\(\beta_2\) bind to membranes in a PtdIns(4,5)P\(_2\) and Ca\(^{2+}\)-independent manner [Runnels et al., 1996; Jenco et al., 1997].

1.ii.ii. Mechanisms for the activation of PLC-\(\gamma\) isoforms

PLC-\(\gamma\) is of particular interest to this study as it is the potential link between the ligand-activated PDGF receptor and phosphoinositide/Ca\(^{2+}\) signalling. PLC-\(\gamma\) is typically activated by RTKs and upon recruitment to, for example, the activated PDGF-receptor, is phosphorylated on at least three tyrosine residues: tyrosine 771, 783 and 1254 [Kim et al., 1991]. This has the effect of increasing its intrinsic phosphatidylinositol-specific lipase activity. A point mutation in Tyr\(^{783}\) is sufficient
to prevent the activation of PLC-γ by PDGF in NIH 3T3 cells [Kim et al., 1991; Cockcroft and Thomas, 1992]. Catalytically inactive mutants of PLC-γ are still capable of eliciting a mitogenic response however [Smith et al., 1994], as long as they are still recruited to the membrane. This activity was localised to the SH-2 domains, and more recently it has been shown that PLC-γ1 can act as an adaptor for Grb-2, a key protein in the activation of the MAPK cascade [Pei et al., 1997]. Importantly, for the study of the role of this isozyme in the cell cycle, mice carrying a disruption within the PLC-γ1 gene died during mid-gestation, at about embryonic day 9 [Ji et al., 1997].

The efficient activation of PLC-γ, and indeed the RTK, requires highly reactive oxygen free radicals, most commonly in the form of hydrogen peroxide, $\mathrm{H}_2\mathrm{O}_2$, due to their action as inhibitors of PTPases [Knebel et al., 1996]. It is important to realise that tyrosine phosphorylation is a dynamic process within the cell, and the regulation of signalling through tyrosine phosphorylation-dependent pathways is the result of the opposing action of RTKs/PTK (protein tyrosine kinases) and PTPases [Sun and Tonks, 1994; Weiss and Schlessinger, 1998]. Contrary to this statement, the action of PTPases may also act in a positive manner to promote signalling in vivo. For example, the efficient activation of Src family PTKs requires phosphorylation by RTKs/PTKs simultaneous with dephosphorylation by PTPases at an inhibitory site near the C-terminus (Tyr527) [Courtneidge, 1994; Thomas and Brugge, 1997]. One of these PTPases, SHP-2, like PLC-γ, has been shown to act as an adaptor for Grb-2 [Bennet et al., 1994]. It is clear that activation of the MAPK cascade must be a primary function of activated RTKs as any one receptor can employ multiple mechanisms to activate this signal relay.

Following activation, the SH-3 domain targets PLC-γ to the actin microfilament network [Bar-Sagi et al., 1993]. This may be in order to promote another protein-protein interaction, or to localise the enzyme with its substrate, $\text{PtdIns}(4,5)\mathrm{P}_2$, which is known to cluster at focal adhesion plaques [Lee and Rhee, 1995; Rebecchi and Pentyala, 2000].

It is possible for PLC-γ to be activated independently of RTK activation by a variety of mechanisms. Some cell surface receptors (e.g. several cytokine receptors and the T-cell antigen receptor) recruit non-receptor tyrosine kinases of the Src,
Syk, and Jak/Tyk families. These PTKs commonly phosphorylate the receptor, in the absence of any intrinsic tyrosine kinase activity on the receptor, providing targets for SH-2 domain-containing proteins. Following the recruitment of PLC-γ to the receptor, it is subsequently phosphorylated by the PTK [reviewed in Lee and Rhee, 1995].

Receptors that activate: phospholipase D (PLD), which produces phosphatidic acid (PA); cytosolic phospholipase A₂ (cPLA₂), producing arachidonic acid (AA); or PI-3-K, producing PtdIns(3,4,5)P₃ may activate PLC-γ indirectly and secondary to receptor-mediated activation of these enzymes through the production of lipid-derived second messengers [reviewed in Lee and Rhee, 1995; Rhee and Bae, 1997]. This mechanism may occur in the absence of any tyrosine phosphorylation and thus raises the question of whether localisation is a major regulatory factor for this isoform. PLC-γ has been reported to bind specifically to membranes containing PtdIns(3,4,5)P₃ via an interaction with the PH domain [Falasca et al., 1998] and an interaction of PtdIns(3,4,5)P₃ with its SH-2 domains has also been proposed [Bae et al., 1998]. PLC-γ has been reported to be mainly cytosolic in a resting cell and primarily actin fibre-associated [McBride et al., 1991], whilst its’ substrate is a membrane component.

Following PDGF stimulation, PLC-γ₁ activation is required prior to PLD activation [Lee et al., 1994], but PA (a product of PLD activation) is generally acknowledged as an allosteric enhancer of PLC-γ activity, capable of activating both tyrosine-phosphorylated and non-phosphorylated forms of PLC-γ to a similar extent [Jones and Carpenter, 1993]. Arachidonic acid, in the presence of tau proteins (or an unusually large ~700kDa protein called ‘AHNAK’, meaning ‘giant’ in Hebrew, in non-neuronal cells [Sekiya et al., 1999]), which are exclusively expressed in neuronal cells and associate with microtubulcs [Lee, 1990], is capable of activating PLC-γ₁ [Hwang et al., 1996]. In fact, the addition of exogenous AA is sufficient to mimic growth factor activation in Balb-c 3T3 fibroblasts [Munaron et al., 1997].
1.ii.iii. ‘Cross-talk’ between GPCRs and PLC-γ

The recruitment, activation and/or tyrosine phosphorylation of PLC-γ1 has been observed in response to stimulation by a number of classical GPCRs, including those for platelet-activating factor (PAF), thrombin, purinergic receptors, muscarinic M5 receptor and the angiotensin II type 1 receptor [Gusovsky et al., 1993; Dhar and Shukla, 1994; Linseman et al., 1995; Rao et al., 1995; Vassort and Puccat, 1996; Di Salvo et al., 1997]. There are several reports of the ligand-independent transactivation of RTKs occurring in response to a range of GPCR agonists in a variety of cell types [Linseman et al., 1995; Rao et al., 1995; Daub et al., 1996; Daub et al., 1997]. One of the better-documented examples of this is the transactivation of both EGF receptors and PDGF-β receptors by angiotensin II [Linseman et al., 1995; Pei et al., 1997; Murasawa et al., 1998]. Such interactions could suggest a possible mechanism for the recruitment of PLC-γ, often thought of as the reserve of RTKs, into the GPCR signalling mechanisms.

Following angiotensin II stimulation of primary rat vascular smooth muscle cells, several signalling pathways are activated that resemble those induced following EGF or PDGF-β receptor activation [Berk et al., 1986]. There is an angiotensin II-mediated, Ca2+-insensitive phosphorylation of both growth factor receptors which, in the case of the PDGF-β receptor, has been suggested to be a separate population of receptors than those activated by the endogenous PDGF receptor ligand [Heeneman et al., 2000]. This suggestion of compartmentalisation infers that the PLC-γ isoform that has been demonstrated to associate and co-immunoprecipitate with the angiotensin II receptor [Venema et al., 1998], may also be from a different population to the PLC-γ recruited following ligand-dependent PDGF receptor activation. Activation of PLC-γ following angiotensin II stimulation results in the formation of a PLC-γ-Grb2 complex [Pei et al., 1997], which may or may not require further adaptor proteins with SH-2 domains, and binds to a YIPP motif in the C-terminal tail of the angiotensin II receptor. This is the same site required by the PTPase, SHP-2, which acts as an adaptor for JAK2, a protein tyrosine kinase [Marrero et al., 1998] and has also been identified as an adaptor for Grb-2 [Bennet et al., 1994]. Interestingly, despite the number of known specialized adaptor proteins for growth factor receptor SH-2 domain docking, the suggested adaptor
proteins, in both cases just mentioned, are unusual as they both modulate growth factor signalling pathways.

Members of the Src family of PTKs may facilitate the tyrosine phosphorylation of PLC-γ1 following the stimulation of some GPCRs. A mechanism has been hypothesised through Pyk-2 (a proline-rich PTK), which is activated in response to agonists for several GPCRs that elevate [Ca^{2+}], [Lev et al., 1995; Yu et al., 1996; Eguchi et al., 1999], and binds the activated GPCRs using SHP-2 as an adaptor [Tang et al., 2000]. Pyk-2 activation, which has been suggested to be NO-sensitive [Wang et al., 1999], following the activation of GPCRs that couple to the G_{i} or G_{q} families of G proteins, subsequently binds to and activates Src family kinases [Dikic et al., 1996; Tang et al., 2000]. A Gα_{13}-mediated Pyk-2 activation has also been reported in response to muscarinic M_{1} receptor activation, triggering an SRE (serum response element) reporter gene activation through a RhoA-dependent pathway [Shi et al., 2000]. Src family PTKs are also implicated in the tyrosine phosphorylation of Gα_{q} and Gα_{11} sub-units following muscarinic receptor stimulation. This suggested that tyrosine phosphorylation of Gα sub-units plays a positive regulatory role in the interaction of the G protein with its receptor [Umemori et al., 1997]. Transactivation of the EGF receptor, dimerisation in the absence of ligand, has also been demonstrated to play a regulatory role in the modulation of the delayed rectifier and Kv1.2 potassium channels in response to muscarinic M_{1} receptor activation [Huang et al., 1993; Tsai et al., 1997].

That localisation may be a major regulatory feature of PLC has raised the possibility of another mechanism for the activation of PLC-γ by GPCRs. Pleckstrin homology domains are commonly thought to mediate protein-membrane interactions, but have also been reported to interact with the Gβγ sub-units of G proteins [Pitcher et al., 1995; DebBurman et al., 1996]. The activation of a GPCR may recruit cytosolic PLC isozymes to the membrane, via this mechanism, as all PLCs contain PII domains. A summary of the PLC-γ activation mechanisms for which there is evidence can be seen in Fig 1.iv.
Fig 1.iv. A schematic showing the variety of mechanisms that are currently known to activate and/or translocate PLC-γ₁ upon RTK, cytokine or GPCR stimulation; or any receptor that activates cPLA₂, PI-3K or PLD.

1.ii.iv. Regulation and activation of PLC-δ isoforms

Of all the PLC isoforms, the elucidation of the activation and regulatory mechanisms for PLC-δ is proving the most difficult despite the determination of the crystal structure of this enzyme [Essen et al., 1996]. It is also the only PLC for which there is a known crystal structure and this did suggest a role for the C2 domain, a Ca²⁺ and phospholipid binding domain, in the orientation of the PLC-δ₁ catalytic site to its membrane-localised substrate, PtdIns(4,5)P₂ [Essen et al., 1996; reviewed in Rizo and Südhof, 1998].

The pleckstrin homology domain of PLC-δ is known to bind PtdIns(4,5)P₂ and Ins(1,4,5)P₃ with affinities comparable to the intact protein [Rebecchi et al., 1992; Cifuentes et al., 1993], and plays an essential role on the activity, when bound
to PtdIns(4,5)P$_2$ [Lomasney et al., 1996], and membrane localisation of this enzyme. Evidence from *in vitro* studies on calcium sensitivity has reported PLC-δ as the most calcium-sensitive of all the PLC isoforms [Allen et al., 1997]. This led to the proposal of a mechanism whereby receptor activation followed by an elevation of [Ca$^{2+}$], may lead to PLC-δ activation secondary to activation of a different isoform. This has been demonstrated in PLC-δ$_1$ over-expressing CHO and PC12 cells, where activation of PLC-δ$_1$ by capacitative calcium entry occurred subsequent to PLC-β activation by thrombin or bradykinin [Kim et al., 1999].

*In vitro* evidence has elucidated two proteins that interact with and may regulate PLC-δ$_1$. One is the GAP for the low molecular mass GTP-binding protein Rho, RhoGAP, and the other is a novel G protein, G$_{\text{h}}$ (that also has an intrinsic transglutaminase activity) originally identified associated with the agonist-occupied α$_1$-adrenergic receptor [Homma and Emori, 1995; Feng et al., 1996].

In summary, the hydrolysis of PtdIns(4,5)P$_2$, with the subsequent generation of Ins(1,4,5)P$_3$ and DAG has been observed in many cell types following stimulation of either muscarinic M$_3$ or PDGF receptors. The production of these second messengers in response to agonist-dependent, membrane-bound receptor activation is attributed to phosphoinositide-specific PLC [Berridge, 1987; Lopez-Rivas et al., 1987; Kim et al., 1991; Renard et al., 1992; Mathias et al., 1997]. Ins(1,4,5)P$_3$ and DAG result in the release of calcium from intracellular stores and the activation of conventional (Ca$^{2+}$ and DAG-sensitive) protein kinase C (PKC) isoforms, respectively [reviewed in Berridge, 1993; Mikoshiba, 1997]. Often downstream signals generated by activated PLC isoforms are used as a direct measure of agonist-mediated PLC activation, but, what this fails to impart however, is exactly which isoforms are activated within a particular cell and their relative contribution to these downstream signalling events. The important question therefore, is not ‘is PLC activated?’ but rather, ‘which PLC isoforms are activated?’
One of the primary interests of this study was to determine if the differential activation of PLC isoforms could be measured in response to RTK and GPCR activation, and whether this information could be used to assess their roles in the generation of downstream messengers such as Ins(1,4,5)P₃ and calcium.
This signalling pathway, the PLC-mediated hydrolysis of PtdIns(4,5)P$_2$, is a ubiquitous mechanism, utilised by more than 100 known extracellular signalling molecules that transmit signals predominantly via GPCRs [Cockcroft and Thomas, 1992; Berridge, 1993; Lee and Rhee, 1995; Berridge et al., 2000]. The Ins(1,4,5)P$_3$ and DAG generated are commonly assigned the major second messenger roles although the subsequent metabolism of these molecules does provide the potential for the production of many other polyphosphoinositide and lipid-derived second messengers.

PtdIns(4,5)P$_2$ itself has been reported to modulate the activity of several key signalling proteins such as PKC, GRK and including the β and δ isoforms of PLC [Ferguson et al., 1995; James et al., 1995], by acting as a membrane attachment site for proteins with PH domains [Lee and Rhee, 1995; Toker, 1998]. The evidence for PLC-β binding PtdIns(4,5)P$_2$ was obtained from avian isoforms and may be species specific as more recent evidence has shown that, in vitro, neither mammalian PLC-β$_1$ nor PLC-β$_2$ bind to membranes in a PtdIns(4,5)P$_2$-specific manner [Jenco et al., 1997]. In fact, PtdIns(4,5)P$_2$ has been reported to inhibit the GAP activity of PLC-β$_1$ and may be able to directly modulate signal transduction in this manner [Berstein et al., 1992]. In vitro however, the PH domain of PLC-δ$_1$ does bind tightly to PtdIns(4,5)P$_2$ [Lemmon et al., 1995] and the hydrolysis of this anchoring lipid may provide an indirect means of regulation for the PLC-δ isoforms.

Both PtdIns(4,5)P$_2$ and the related phospholipid PtdIns(3,4,5)P$_3$ have been recently proposed as 'lipid second messengers' due to their ability to recruit PH domain-containing proteins, alone or in concert with other factors. Examples of proteins other than PLC recruited in this manner include: Bruton's tyrosine kinase (Btk), GAP, GRP1, GRKs, members of the ADP ribosylation factor (ARF) exchanger family and the serine/threonine kinase Akt/PKB [Jiang et al., 1998; Venkateswarlu et al., 1998; Razzini et al., 2000; and references therein]. Furthermore, PtdIns(4,5)P$_2$, and in some systems PtdIns(3,4)P$_2$ and PtdIns(3,4,5)P$_3$, plays an integral role in maintaining and altering cell morphology by providing membrane anchoring sites for a variety of actin-binding proteins, such as gelsolin and profilin. Many reports now indicate that fluctuations in the cellular level of
PtdIns(4,5)P$_2$ correlate with the degree of actin assembly or decreases in number of actin stress fibres [all reviewed in Janmey, 1994; Toker, 1998 and references therein].

The modulation of PLD activity (*in vitro* evidence suggested potent activation [Liscovitch *et al*., 1994]) and ARF1 by PtdIns(4,5)P$_2$ has also implicated this multi-functional phospholipid in a number of other cellular processes such as secretion, phagocytosis and vesicle trafficking [reviewed in Toker, 1998]. Furthermore, PtdIns(4,5)P$_2$ has recently been reported to inhibit capacitive Ca$^{2+}$ entry via an unknown mechanism of interaction with plasma membrane Ca$^{2+}$ channels upon intracellular Ca$^{2+}$ store depletion [Kaznacheyeva *et al*., 2000].

Despite only being a minor membrane component, the rapid hydrolysis of PtdIns(4,5)P$_2$ and its subsequent depletion under conditions of agonist stimulation do not appear rate limiting for signal transduction [Willars *et al*., 1998]. The identification of the phosphatidylinositol transfer protein isoforms, α and β, have recently provided a molecular mechanism for the rapid transfer of PtdIns(4,5)P$_2$ from the site of synthesis to the plasma membrane [reviewed in Cockcroft, 1997].

The level of cellular PtdIns(4,5)P$_2$ can change dramatically during second messenger generation and thus may directly play a role in signal generation [Cockcroft, 1997; Willars *et al*., 1998]. The mechanism of PtdIns(4,5)P$_2$ involvement apparently involves its depletion under conditions of agonist stimulation but remains unclear. Of primary interest to this study however, is the characteristic Ins(1,4,5)P$_3$-mediated release of Ca$^{2+}$ from intracellular calcium stores and subsequent Ca$^{2+}$ influx across the membrane. This is the first signal transduction pathway to be discussed of two major signalling pathways directly activated following the hydrolysis of PtdIns(4,5)P$_2$, the second being the generation of DAG and subsequent PKC activation which will be addressed shortly (see section 1.iv.).
1.iii.i. Ins(1,4,5)P₃ and Ca²⁺ signalling

The ability of Ca²⁺ to play such a pivotal role in intracellular signalling results from the ability of cells to shape Ca²⁺ signals in the dimensions of space, time and amplitude. In order to generate the wide variety of reported Ca²⁺ signals, different cell types employ components selected from a Ca²⁺ signalling 'toolkit' which is composed of signalling, homeostatic and sensory mechanisms [Berridge, 1998; Taylor, 1998; Berridge et al., 2000]. The Ca²⁺ ions that contribute toward agonist-mediated elevations of [Ca²⁺], can accumulate within the cytoplasm from a variety of sources following the activation of a wide variety of membrane-bound receptors. This includes Ca²⁺ entry from the extracellular space either via Ca²⁺ channels gated directly by agonist binding (receptor operated Ca²⁺ channels or ROCCs), through second messenger-operated Ca²⁺ channels that open as a consequence of second messenger production following agonist binding (SMOCCs) or through voltage operated Ca²⁺ channels (VOCCs) that open due to changes in the membrane potential of the cell [Hofmann et al., 1994].

However, in response to agonist-mediated activation of RTKs and GPCRs, the initial elevation of [Ca²⁺], occurs most often following agonist-mediated release of Ca²⁺ from intracellular Ca²⁺ stores. These Ca²⁺ stores are generally accepted to be specialised components of the endoplasmic reticulum and their possible compartmentalisation has been the topic of debate for some years, and may, again, be a cell type-specific phenomenon. Calcium is released from these stores following the activation of intracellular Ca²⁺ release channels that are gated by the second messengers Ins(1,4,5)P₃, Ca²⁺ [Berridge, 1993; Mikoshiba, 1997; Taylor, 1998; Berridge et al., 2000]; sphingosine-1-phosphate [Mao et al., 1996; Olivera et al., 1999; Young et al., 1999]; cyclic ADP ribose [Lee, 1993; Lee, 2001]; and NAADP [Genazzini and Galione, 1997; Peterson and Cancela, 1999; Lee, 2001]. Furthermore, within the cell, both Ca²⁺ [Berridge et al., 2000] and reactive oxygen species [Hu et al., 2000] are known to increase the sensitivity of the Ins(1,4,5)P₃-operated channel for its ligand and the Ins(1,4,5)P₃ receptor has also been proposed as a coincidence detector for Ca²⁺ elevation and Ins(1,4,5)P₃ generation in neurons [Nakamura et al., 1999]. Of these channels, the two most widely recognised are the Ins(1,4,5)P₃ receptor and ryanodine receptor (RYR) that are accepted to be predominantly gated by Ins(1,4,5)P₃ and Ca²⁺, respectively, and whilst identification
of a sphingolipid-gated, endoplasmic reticulum Ca\(^{2+}\) store receptor (SCaMPER) has been reported [Mao et al., 1996], the receptor for NAADP remains unknown.

In non-excitable cells, the subsequent entry of Ca\(^{2+}\) into the cell across the plasma membrane is ascribed to a ‘capacitative mechanism’ in which the Ca\(^{2+}\) entry pathway is specifically dependent on the receptor-mediated depletion of the agonist-sensitive intracellular Ca\(^{2+}\) store [Putney, 1986; Berridge, 1995; Mikoshiba, 1997]. Calcium flowing into a cell from the extracellular space creates a measurable current that has been identified in many cell types and is termed \(I_{\text{CRAC}}\) (Calcium Release-Activated Calcium current), and was originally measured in RBL cells [Hoth and Penner, 1992]. The initiation of this process has been suggested to be an all-or-nothing phenomenon in some, but not all instances [Huang and Putney, 1998]. Some of the inconsistency surrounding capacitative Ca\(^{2+}\) entry may be explained by recent evidence that suggests the existence, in both excitable and non-excitable cells, of an antagonistic non-capacitative Ca\(^{2+}\) entry mechanism involving the gating of SMOCCs by polyunsaturated fatty acids (PUFA), dominated by arachidonic acid [Shuttleworth, 1996; Shuttleworth and Thompson, 1999; Mignen and Shuttleworth, 2000; Luo et al., 2001]. The direct action of phospholipase A\(_2\) on membrane lipids can generate PUFA, but more recent evidence has implicated DAG lipase action on DAG as a primary source and provides a direct link between PtdIns(4,5)P\(_2\) hydrolysis and the production of PUFAs [Broad et al., 1999].

The mechanism by which the Ca\(^{2+}\) store transmits a signal to the membrane-bound Ca\(^{2+}\) channels to initiate capacitative Ca\(^{2+}\) entry and store refilling has however, long been a subject for debate and still remains unclear [Putney and Bird, 1993; Berridge, 1995; Mikoshiba, 1997; Berridge et al., 2000]. There are two favoured mechanisms; one involves the release and/or generation of a soluble, diffusible factor within the cell that activates the plasma membrane Ca\(^{2+}\) channels. This factor is known as ‘CIF’ (calcium influx factor) and within the parameters of this capacitative model, CIF is generated independently of the actual mechanism used to empty the store i.e. agonist or receptor-independent. Alternatively, the second proposed mechanism hypothesised a molecular coupling between the Ca\(^{2+}\) release channels on the endoplasmic reticulum and the Ca\(^{2+}\) entry channels on the plasma membrane [Berridge, 1995; Berridge et al., 2000]. The recent discovery of the ‘Homer’ protein, which provides a molecular link between the Group 1
metabotropic glutamatergic receptors and the Ins(1,4,5)P$_3$ receptor, strongly supports the second theory but does not discount the first [Tu et al., 1998]. Several homologues of the drosophila trp (transient receptor potential) gene family have now been identified in mammalian cells and have been proposed to encode the store operated capacitative Ca$^{2+}$ influx channels [Boulay et al., 1999]. In non-excitable cells recent reports have suggested Trp1 encodes the primary store operated Ca$^{2+}$ influx channels in both HEK293 cells and salivary gland cells [Lui et al., 2000; Wu et al., 2000], and hTrp3 has also been proposed as a candidate protein for a direct molecular coupling mechanism in HEK293 cells [Kiselyov et al., 1998]. DAG regulation has been suggested for both Trp3 and Trp6 [Hofmann et al., 1999], which links this mechanism to PtdIns(4,5)P$_2$ hydrolysis or the activation of PLD. In any event the mechanisms are not held to be mutually exclusive and have both been demonstrated in a variety of cell types, but again may be activated in either an agonist or cell type-specific manner.

1.iii.ii. **Muscarinic and PDGF receptor Ca$^{2+}$ signalling**

Agonist occupancy of muscarinic M$_3$ cholinergic receptors in a variety of cell types elicits a biphasic Ins(1,4,5)P$_3$ elevation, which alongside the accumulation of [H]-InsP$_3$ against a Li$^+$ block has been interpreted as indicative of a rapid, partial desensitisation [Wojcikiewicz et al., 1993; Willars and Nahorski, 1995a]. Although the temporal profile of Ins(1,4,5)P$_3$ generation is reflected in the subsequent elevation of [Ca$^{2+}$], [Fisher et al., 1994; Wojcikiewicz et al., 1994b; Burford et al., 1995; Willars and Nahorski, 1995a] this cannot be taken as evidence for the role of any muscarinic receptor desensitisation in the downstream signalling. It is generally accepted that Ins(1,4,5)P$_3$ generation is the cause rather than the effect of an elevation of [Ca$^{2+}$], and it is clear that Ca$^{2+}$ can modulate the generation of this second messenger as all PLC isoforms are reported to be Ca$^{2+}$-sensitive to differing degrees [Cockcroft and Thomas, 1992; Rhee and Choi, 1992; Allen et al., 1997].

Muscarinic M$_3$ receptor-mediated subsequent elevations of [Ca$^{2+}$], are also generally accepted to be biphasic, with the rapid 'peak' and sustained 'plateau' phases consisting of intracellular and extracellular Ca$^{2+}$ sources, respectively [Berridge, 1993; Tobin et al., 1993; Willars and Nahorski, 1995a]. In transfected CHO (Chinese hamster ovary) cells the M$_3$ muscarinic receptor is reported to
differentially regulate the Ca$^{2+}$ entry component of the response at higher agonist concentrations (carbachol, > 10 μM) by co-activating monovalent cation channels [Carroll and Peralta, 1998]. At depolarising membrane potentials there was also an agonist concentration-dependent decrease in the influx but not Ca$^{2+}$ store release component of the intracellular Ca$^{2+}$ signal [Carroll and Peralta, 1998], which may be relevant to muscarinic receptor signalling in neuronal systems. The magnitude, as well as the spatial (3-dimensional distribution within the environs of the cell) and temporal profiles, of a Ca$^{2+}$ response undoubtedly all contribute to specify the downstream responses and this may provide a mechanism whereby the cell can respond differentially to the agonist concentration.

The sensitivity of the cell to prolonged muscarinic receptor agonist exposure may be modulated by the internalisation and degradation of the receptor [Mullaney et al., 1993], or alternatively by a similar regulation of other components of the signalling pathway such as the G-protein [Sorensen et al., 1997], effector molecule (in this case PLC) [Sorenson et al., 1998] and the Ins(1,4,5)P$_3$ receptor [Wojcikiewicz et al., 1994a]. However, the attenuation of the muscarinic receptor-mediated downstream signals, such as Ins(1,4,5)P$_3$ and Ca$^{2+}$ also occurs very rapidly. The mechanisms underlying this desensitisation are unclear, but the currently accepted model is the phosphorylation of the receptor by receptor-specific kinases [Wojcikiewicz et al., 1993; Tobin and Nahorski, 1993; Tobin, 1997; Pitcher et al., 1998].

Elevation of [Ca$^{2+}$], by activated PDGF receptors is often assumed to be dependent upon the generation of the second messenger, Ins(1,4,5)P$_3$ following the recruitment of PLC-$\gamma$. Although this second messenger has been measured in response to PDGF in a number of cell types, most often mesenchymal in origin [Estacion and Mordan, 1993a; Miyakawa et al., 1998], there is also evidence in the N1E-115 neuroblastoma cell [Zhang et al., 1996] and in several different glial cell lines [Fatatis and Miller, 1997; Fatatis and Miller, 1999; Saqr et al., 1999]. The subsequent elevation of [Ca$^{2+}$], is often reported as involving both Ca$^{2+}$ release from intracellular stores and Ca$^{2+}$ entry across the plasma membrane. However, in some cases the mechanism for Ca$^{2+}$ entry has been argued to be independent of capacitative Ca$^{2+}$ entry following Ca$^{2+}$ store depletion [Ma et al., 1996; Mathias et al., 1997] and contrasting reports identify the plasma membrane-bound channels
opened during PDGF-mediated Ca\(^{2+}\) entry as not L-type and voltage-insensitive in A172 glioblastoma cells [Szollosi et al., 1991], but L-type in Swiss 3T3 fibroblasts [Miyakawa et al., 1998]. Furthermore, PDGF-activated T-type Ca\(^{2+}\) channels and non-specific cation channels have also been identified in fibroblasts and glioma cells [Huang et al., 1991; Estacion and Mordan, 1993; Saqr et al., 1999]. Throughout the literature on PDGF-mediated Ca\(^{2+}\) signalling there is disparity and a lack of correlation between any measurement of second messenger and its putative role in the elevation of \([\text{Ca}^{2+}]\).

More recently, the Ins(1,4,5)P\(_3\) receptor competitive inhibitor, heparin, has been shown to completely block PDGF-mediated Ca\(^{2+}\) store release in myoblasts [Mathias et al., 1998] and cultured hippocampal neurons, but, unexpectedly, in the latter case, the heparin increased rather than blocked depressions in NMDA-evoked currents that were reported to be both PLC-\(\gamma\)- and Ca\(^{2+}\)-dependent [Lei et al., 1999]. In contrast, heparin-sensitive Ins(1,4,5)P\(_3\) receptors were not required for PDGF-mediated Ca\(^{2+}\) signalling in rat vascular muscle cells despite a complete block of the signal by antibodies to PtdIns(4,5)P\(_2\) [Huang et al., 1991]. In general, evidence for PDGF-mediated Ca\(^{2+}\) signalling is contrasting in nature and no doubt there will be multiple mechanisms dependent upon the cell type.

So, although Ins(1,4,5)P\(_3\) may, in some cell types, contribute to PDGF and muscarinic receptor-mediated signalling, it is clear from the literature that the other product of PLC activation, DAG, is also implicated in the signalling of both class of receptor. The major role of DAG, the next topic of discussion, is the activation of several of the PKC isoforms, which themselves may play multiple roles within the cell. Due to the high number of PKC isoforms, their contribution to receptor signalling is doubtless dependent upon selective expression and differential means of regulation.
With the second major consequence of PtdIns(4,5)P$_2$ hydrolysis being the generation of DAG and the subsequent activation of several isoforms of the PKC family of serine/threonine kinases, the predominant interest of this study is the differential activation of PKC by the PDGF and muscarinic receptor agonists.

The PKC protein family comprises 11 known isoforms, which are subdivided into the ‘conventional’ PKC proteins (α, β and γ) that are Ca$^{2+}$-sensitive and are modulated by lipid cofactors such as, phosphatidyl-L-serine and DAG. Both the ‘novel’ (δ, ε, η, and θ) and ‘atypical’ (ζ, τ and λ) PKC isoform subfamilies are acknowledged to be Ca$^{2+}$ independent, but the ‘novel’ PKCs may still rely on lipid cofactors such as DAG and phosphatidyl-L-serine. In addition to phosphatidyl-L-serine, other anionic lipids have been implicated in the membrane localisation of some PKC isoforms, most notably, arachidonic acid and some of the polyphosphoinositides [all reviewed in Newton, 1997]. The DAG-dependent isoforms can also be activated, in vitro, by phorbol esters, tumour-promoting agents that are analogues of DAG [Huang et al., 1986]. PKC is a prolific kinase and has crucial roles in cell growth and differentiation, neurotransmitter release and receptor regulation, ion channel modulation and gene expression [reviewed in Newton, 1997]. However, the regulatory mechanisms for the ‘atypical’ PKC isoatypes are still not clearly established.

An unusual member of this kinase family is human PKCµ as it shares a great deal of homology with the murine protein kinase D (PKD) and the kinase core more closely resembles that of calmodulin-dependent kinases [Newton, 1997]. In A431 cells, the activation of PKD by PDGF, in vivo, has demonstrated that prior activation of PKC is required and thus further removes PKCµ from the family of PKC [van Lint et al., 1998].

The regulation of the PKC isoforms is itself a huge area of study, due to the number of PKC isoforms and the massive number of PKC target proteins, so this discussion has been confined to PKC and its relationship with RTK and GPCRs. It is interesting to note that autophosphorylation of PKC appears to play a role in both homologous and heterologous desensitisation and down-regulation and is essential
for the return of PKC to the cytoplasm following agonist-mediated membrane recruitment [Ohno et al., 1990; Feng et al., 2000; reviewed in Newton, 1997].

Evidence from immunocytochemical studies has clearly established that different PKC isoforms localize to different subcellular compartments [Jaken, 1996], and this may provide a method of regulation that allows PKC to respond efficiently to second messenger production by specific receptors. Negative feedback by PKC (and PKA) following GPCR activation has also long been established as a mechanism for the homologous or heterologous desensitisation of GPCRs and their downstream signalling. Indeed, short-term phorbol ester activation of PKC (all isoforms except PKCζ and PKCλ are phorbol ester-sensitive) has been implicated in the desensitisation of phosphoinositide-coupled GPCRs by inhibition of agonist-induced generation of PtdIns(4,5)P2-derived second messengers [Huwiler et al., 1991; Pavenstadt et al., 1992; Willars et al., 1996]. A direct or indirect phosphorylation of PKC phosphorylation targets including PLC and the receptor [Herrero et al., 1994], which may result in subsequent internalisation in the latter case [Pearce et al., 1988], has also been observed. A PKCa/c-mediated phosphorylation of PLC-β3 occurs concomitant with agonist and phorbol ester-induced desensitisation of the platelet-activating factor receptor-coupled inositol lipid response in RBL-2H3 cells [Ozawa et al., 1993]. A decrease in the catalytic activity of both avian PLC-β and a partially purified PLC-β1 has also been reported [Cunningham et al., 1999; Filtz et al., 1999] subsequent to PKC-mediated phosphorylation. To date there is no evidence to support a direct interaction of PKC with G-proteins of the Gq and Gi/o families that provide a link between these receptors and effectors, although the PKC-mediated phosphorylation of Gαz has been reported [Glick et al., 1998] and disruption of G-protein-PLC coupling is often hypothesised.

PDGF has been identified as activating several different PKC isoforms, as measured by translocation, phosphorylation, down-regulation and PKC-dependent transcriptional activation, depending upon the cell type. It has been suggested that the DAG required for activation may be phosphatidylcholine (PC)-derived rather than generated from the hydrolysis of PtdIns(4,5)P2, implying PLD and not PLC is responsible for the agonist-mediated activation [Ha and Exton, 1993; Exton, 1997a].
The implicated functions of this RTK-mediated PKC activation include: expression of intermediate-early genes such as \textit{c-myc}, \textit{c-fos}, \textit{egr-1}, \textit{junB} and \textit{fra-1}; \(\alpha_2\)-integrin expression; activation of the Na\(^+\)/H\(^+\) exchanger; cell differentiation and mitogenic signalling. The Ca\(^{2+}\)-independent activation of PKC\(\delta\) and \(\varepsilon\) are both reported in response to this growth factor. Recent evidence has suggested that both PLC-\(\gamma\) and PI-3K may activate PKC\(\varepsilon\), which is known to bind to 14-3-3 proteins, actin and cytoskeletal elements, through compensatory, independent pathways [Moriya \textit{et al.}, 1996]. Again, specificity will rely on several factors and may well be a consequence of agonist and/or cell type, as PI-3K is not involved in the transduction of hepatocyte growth factor-mediated PKC\(\varepsilon\) activation in neocortical cells [Machide \textit{et al.}, 1998].

PKC\(\delta\) activation has also been implicated in the up-regulation of L-type calcium channels in neuronal cells following exposure to ethanol [Gerstein \textit{et al.}, 1998] and the over-expression of this isoform completely inhibited cell growth in both NIH 3T3 and C166 cells [Akita \textit{et al.}, 1990; Ha and Exton, 1993; Ohno \textit{et al.}, 1994].

In addition, PDGF also activates the atypical PKC isoform, PKC\(\zeta\), known to be necessary for mitogenic signalling. Both MAP kinase and MEK are activated by PKC\(\zeta\) in response to serum and tumour necrosis factor-\(\alpha\), binding to and directly activating Ras [Diaz-Meco \textit{et al.}, 1994; Sanz \textit{et al.}, 1994; Berra \textit{et al.}, 1995].

The phosphoinositide-coupled muscarinic M\(_3\) receptors also activate PKC, which has long been implicated in the partial desensitisation of receptor-mediated activation of PLC and the subsequent generation of Ins(1,4,5)P\(_3\) and Ca\(^{2+}\). This negative feedback mechanism is generally acknowledged to be predominantly at the level of PLC or the G protein-PLC coupling and there is some evidence to suggest a mechanism involving a direct PKC-mediated phosphorylation of PLC-\(\beta_1\) and PLC-\(\beta_3\) [Kopp \textit{et al.}, 1990; Ozawa \textit{et al.}, 1993]. Further, PKC has also been implicated in the muscarinic M\(_3\) receptor-mediated mechanisms for both noradrenaline release and elevation of [Ca\(^{2+}\)], in SH-SY5Y cells where it has been reported to play both positive and inhibitory roles respectively [Murphy \textit{et al.}, 1992]. Several PKC isoforms, including PKC\(\alpha\), PKC\(\varepsilon\) and PKC\(\zeta\) are reported to translocate or activate following muscarinic receptor activation [Willars \textit{et al.}, 1996; Guizzetti \textit{et al.}, 1998;
Guizetti and Costa, 2000], and inhibition of either PI-3K or PLD have been shown to block the agonist-mediated translocation of PKCζ [Guizetti and Costa, 2000].

What is clear from this, is that both PDGF and muscarinic M₃ receptors are capable of activating more than one isoform of PKC. However, the definitive roles of the specific PKC isoforms in agonist-mediated cellular responses is proving far more difficult to elucidate (also see Appendix A), due to the inability to selectively inhibit or down-regulate PKC. In this thesis we sought to determine which isoforms were involved in mediating the PDGF and muscarinic receptor-mediated responses within the SH-SY5Y neuroblastoma cell line. As with other proteins that exist as multiple isoforms, the selective expression of PKC in a neuronal cell type may prove an indirect means of regulation and it was therefore, important to identify which PKC isoforms were present prior to any further investigations.
1.v. NEURONAL SIGNALLING

The SH-SY5Y human neuroblastoma cell line is a commonly utilised neuronal model system. This thesis aimed to utilise this model system in preparation for working in primary cultures of real neurons. It is therefore, not only important to define PDGF and muscarinic M3 receptor-mediated signalling in the SH-SY5Y cell, but to define PDGF and muscarinic M3 receptor-mediated signalling in a neuronal context.

Communication between neuronal cells occurs via a juxtacrine signalling mechanism that is more commonly referred to as ‘neurotransmission’. This highly specialized form of cell-cell signalling occurs at synapses. The two cells involved in generating and receiving the signal are known as the pre-synaptic and post-synaptic cells, respectively, and are separated from each other by a small gap called the synaptic cleft. In order for a signal to pass from the pre-synaptic cell to the post-synaptic cell, signalling molecules must cross the cleft and contact specific receptors on the post-synaptic cell surface. These signalling molecules are given the general term ‘neurotransmitters’ and examples include molecules such as acetylcholine, L-glutamate and nucleotides such as ATP and UTP.

Intracellular neuronal signalling mechanisms can be sub-divided into two distinct mechanisms. ‘Ionotropic’ and ‘metabotropic’ signalling mechanisms deal with fast and slow synaptic transmission respectively, and are initiated following the activation of specific receptors. Ionotropic mechanisms commence upon the activation of receptors that are intrinsic ion channels, such as the GABA\(_A\) receptors, nicotinic acetylcholine receptors, and NMDA and AMPA glutamatergic receptors. The initiation of metabotropic mechanisms follows the activation of receptors coupled to second messenger production (and thus tend to be slower) such as the muscarinic acetylcholine receptors, metabotropic glutamate receptors and bradykinin receptors, and has long been implicated in several forms of synaptic plasticity and neurotransmitter release.

Both the PDGF and muscarinic receptors would be defined as metabotropic in a neuronal system and may possibly be considered as unusual choices to investigate acute signalling characteristics in this context. However, this investigation is not without precedent as both receptors have been implicated in several aspects of neuronal signalling.
1.v.i. Muscarinic and PDGF Receptor signalling in neurons

Activation of the muscarinic receptors in the SH-SY5Y cell (discussed in more detail in section 1.vi.) is well characterised as eliciting a phosphoinositide-dependent elevation of $[\text{Ca}^{2+}]$, [Lambert and Nahorski, 1990; Tobin et al., 1993; Willars and Nahorski, 1995a] (see section 1.iii.). Furthermore, PLC-coupled muscarinic receptors have been implicated in a variety of neuron-specific functions such as synaptic plasticity, memory and learning [Nathanson, 2000].

The $\text{Ins}(1,4,5)\text{P}_3$ receptor is a major intracellular $\text{Ca}^{2+}$ release channel in most cell types [reviewed in Taylor and Richardson, 1991; Berridge, 1998; Taylor, 1998; Berridge et al., 2000]. Furthermore, excitable cells, such as neurons, may also contain intracellular $\text{Ca}^{2+}$ stores that are sensitive to the ryanodine receptor (RYR) ligands caffeine and ryanodine [McPherson and Campbell, 1993; Berridge, 1998]. One of the major roles of the ryanodine receptor appears to be in the process of 'calcium-induced $\text{Ca}^{2+}$ release' (CICR) and this receptor can be gated by both $\text{Ca}^{2+}$ [Stern, 1992] and another putative second messenger, cADP-ribose [Galione, 1993; Genazzini and Galione, 1997]. Interestingly, small elevations of $\text{Ins}(1,4,5)\text{P}_3$, which may not be able to directly release intracellular $\text{Ca}^{2+}$ by gating the $\text{Ins}(1,4,5)\text{P}_3$ receptor, have been suggested to sensitise this receptor to $\text{Ca}^{2+}$, thus supporting the role of coincidence detection for the $\text{Ins}(1,4,5)\text{P}_3$ receptor and converting the cytoplasm into an excitable medium [reviewed in Berridge, 1998 and references therein].

The potential role of VOCCs and other $\text{Ca}^{2+}$ entry channels following either receptor stimulation or $\text{Ca}^{2+}$ store depletion provides a second mechanism for the integration of both ionotropic and metabotropic mechanisms. The presence of more than one, possibly distinct, major intracellular $\text{Ca}^{2+}$ store may also provide a mechanism for the integration of ionotropic and metabotropic signalling and is likely to play an important role in the integration of synaptic signals [Simpson et al., 1995; Berridge, 1998]. An alternative mechanism, whereby another agonist regulates the size of the intracellular $\text{Ca}^{2+}$ pool available to phosphoinositide-linked receptors has also been proposed. This hypothesis would also suggest the existence of discrete $\text{Ca}^{2+}$ stores within the cell, access to which may be facilitated by the agonist-mediated translocation of $\text{Ca}^{2+}$ between such stores [Short et al., 2000; Short and Taylor, 2000]. Finally, the concept of a 'neuron within a neuron', proposed by
Berridge, 1998, allows for the rapid integration of a wide variety of signals by the plasma membrane bound receptors, which in turn translates these many signals into forms understood by the endoplasmic reticulum. Thus, agonist stimulation resulting in an elevation of \([Ca^{2+}]\), initiates a complex pattern of receptor-mediated \(Ca^{2+}\) increases within the cell and may potentially draw on \(Ca^{2+}\) from a variety of intra- or extracellular sources.

The physiological role of PDGF-receptor activation is diverse in both the embryo and the adult. Agonist stimulation is known to be responsible for mitogenicity, chemotaxis, and actin reorganisation, differentiation and to play an anti-apoptotic role. Although the classical target cells for PDGF are fibroblasts and smooth muscle cells, the receptors are also found in many areas of the CNS, including the brain. Within the brain, PDGF is known to play a neurotrophic and neuroprotective role in mature neurons, in the regulation of synapses, the enhancement of myelination and neurite formation and in the regeneration, prevention and minimisation of ischaemia-induced neuronal injury [all reviewed in Heldin and Westermark, 1999 and references therein]. Although astrocytes were originally thought to be the main or only source of PDGF within the CNS, it is now clear that several cell types, including neurons, synthesize PDGF.

Despite the extensive study of PDGF signal transduction in mesenchymal-derived cells, very little is known about PDGF-mediated phosphoinositide and calcium signals and how these relate to the function of PDGF in neuronal systems (see section 1.iii.ii. for a fuller discussion of PDGF-mediated \(Ca^{2+}\) signalling).

Recent, potentially important observations, are that PDGF signalling has been implicated in the inhibition of type \(\gamma\)-aminobutyrate receptors, the inhibition of neuronal sodium channels, the long-term inhibition of NMDA receptors and inhibition of the excitatory transmission between CA1 hippocampal neurons, through a Src and calcium-dependent mechanism [Valenzuela et al., 1995; Valenzuela et al., 1996; Hilborn et al., 1998; Lei et al., 1999]. Furthermore, a very recent observation has suggested that PDGF may facilitate its neuroprotective role by the rapid increase in activity and cell surface number of \(Na^+\)-dependent glutamate transporters, that are the primary mechanism for the removal of excitatory amino acids from the extracellular space of the CNS [Sims et al., 2000]. Following reports that in the
CA1 pyramidal neurons, both PDGF and muscarinic receptors have been implicated in a c-Src-dependent modulation of the NMDA receptor [Lei et al., 1999; Lu et al., 1999]. An investigation was undertaken into the elevation of \([Ca^{2+}]_i\) in response to PDGF in the SH-SY5Y cells.

Acute PDGF-mediated elevations of \([Ca^{2+}]_i\) may therefore be important within neurons. With the previous reports of \(Ca^{2+}\)- and phosphoinositide-mediated PDGF receptor signalling already established in mesenchyme-derived cells, specialised cell types - such as neurons - may have distinct and as yet undefined mechanisms. These aspects of growth factor signalling mechanisms are considered here in a neuronal setting using the SH-SY5Y human neuroblastoma cell line and primary cultures of rat hippocampal cells.
The SH-SY5Y HUMAN NEUROBLASTOMA CELL LINE

This investigation utilised the SH-SY5Y neuroblastoma cell line, which displays many of the characteristics of human foetal sympathetic ganglion cells [Ross et al., 1983]. SH-SY5Y cells have been used extensively to study phosphoinositide and Ca$^{2+}$ signalling and its regulation in a neuronal-like context [Lambert and Nahorski, 1990; Lambert and Nahorski, 1992; Willars and Nahorski, 1995a]. Prior studies have shown that these cells express several endogenous classical RTKs, including: the bFGF-receptor, the EGF-receptor, the NGF-receptor, the insulin-like receptor (both IGF1-R and IGF2-R) and the PDGF-receptor (both α- and β-chains) [Jensen et al., 1992; Pahlman et al., 1992; Loret et al., 1992; Janet et al., 1995], as well as several endogenous G-protein coupled receptors (GPCRs), including PLC-coupled muscarinic cholinoreceptors of predominantly the $M_3$ isotype [Lambert et al., 1989].

The G-protein coupled bradykinin (B$_2$) and LPA receptors have also been identified in the SH-SY5Y cells and although activation of either receptor results in a rapid, yet transient, elevation of [Ca$^{2+}$], they are thought to utilise different mechanisms. The bradykinin receptor is reported as PLC-linked following activation and increases cytosolic Ca$^{2+}$ following Ins(1,4,5)P$_3$-mediated intracellular Ca$^{2+}$ store gating [McDonald et al., 1994; Willars and Nahorski, 1995a; Willars and Nahorski, 1995b]. In contrast, LPA-mediated Ca$^{2+}$ signalling has been demonstrated as Ins(1,4,5)P$_3$-independent and utilises instead the novel sphingolipid second messenger, sphingosine-1-phosphate [Young et al., 1999; Young et al., 2000a]. Both bradykinin and LPA receptors were useful tools within the context of this study by providing endogenous internal controls for the muscarinic M$_3$ receptor. Interestingly, in HSWP (human foreskin fibroblast) cells, bradykinin-mediated Ca$^{2+}$ signalling is partially dependent on a tyrosine kinase mechanism [Lee et al., 1993a]. However, in these HSWP cells bradykinin exhibits a peak and plateau profile of elevation of [Ca$^{2+}$], and it is the plateau phase that has been shown to be composed of Ca$^{2+}$ entry via a tyrosine kinase activity. In the SH-SY5Y cells, this plateau phase is not apparent and thus may be cell type-specific phenomena.

The expression of several endogenous GPCRs, RTKs, and in the case of the acetylcholine receptors, both nicotinic and muscarinic sub-types, lends the SH-SY5Y cell to be a suitable neuronal-like cell to study the integration of multiple signalling...
pathways and the heterologous desensitisation of shared signalling pathways. In addition to the receptors, the SH-SY5Y cells also express many other signalling components that may not be directly involved in phosphoinositide signal transduction following receptor activation, but may play important regulatory roles once the pathway is activated e.g. various G proteins, and several PLC and PKC isoforms.

Neuroblastoma and neuronal-like cell types are commonly utilised when the focus is on signalling systems that directly relate to nerve function. In general, the receptors under investigation in these circumstances are either fast ligand-gated ion channels or GPCRs. To date there are relatively few reports of growth factor-mediated second messenger-initiated signalling in neuronal or neuronal-like cells. Whether or not growth factor-mediated elevations of $[\text{Ca}^{2+}]$, influence or modulate the physiological role of the cell was, until recently, unknown and may depend upon the cell function and stage of development. For example, growth factor-mediated signalling is unlikely to play a mitogenic or differentiation role in terminally differentiated nerve cells, but may instead act as a survival factor [Jensen et al., 1992].

This study aimed to determine the mechanism for PDGF-mediated elevations of $[\text{Ca}^{2+}]$, in the SH-SY5Y cell and to define the components of the signalling pathway. To fully understand the similarities and differences between RTK and GPCR signalling it was therefore necessary to equally define the muscarinic $M_3$ receptor-activated signalling molecules. Following this, this study hoped to determine the effect of $\text{Ca}^{2+}$ on functional aspects of the SH-SY5Y cell before moving into primary neuronal cells.
1.vii. AIMS

The aims of this study were to investigate signal transduction mechanisms following the stimulation of both GPCRs and RTKs, typified by the muscarinic and PDGF receptors respectively (both presumed to be PLC-linked for the elevation of \([\text{Ca}^{2+}]_i\)). The SH-SY5Y neuroblastoma cell line was utilised as a model neuronal system, one of its major strengths being the endogenous expression of both receptors, with the eventual aim of repeating the work in primary neurons.

The ultimate goal of these studies was to characterise, compare and contrast the phosphoinositide signalling, via both classes of receptor, and to develop methodology that would allow the dissection of the complex signalling through proteins such as PLC and PKC, which exist as multiple isoforms within most cells. The eventual aim therefore, was to state which receptor utilised which PLC and PKC isoforms, and to relate these differences to contrasting second messenger generation and subsequent functional responses of the SH-SY5Y neuroblastoma cells.

The initial aim of this project was to identify and characterise an RTK-mediated elevation of \([\text{Ca}^{2+}]_i\). To achieve this required the determination of the answers to such questions as: what was the source of the \(\text{Ca}^{2+}\) i.e. was the \(\text{Ca}^{2+}\) from an intracellular or extracellular source?; was PLC-mediated \(\text{Ins}(1,4,5)\text{P}_3\) generation the mediator of this signal?; and if so, could we define which PLC isoforms were activated following agonist occupation of the receptor?

As a direct comparison, the G-protein- and PLC-coupled, phosphoinositide signalling pathway activated following stimulation of muscarinic \(M_3\) cholinergic receptors in SH-SY5Y cells [Baird and Nahorski, 1989; Lambert et al., 1989; Wojcikiewicz et al., 1994; Willars and Nahorski, 1995a; Willars and Nahorski, 1995b; Willars et al., 1998] was characterised under serum starved conditions.

Although PLC-\(\gamma\) is thought to mediate responses to RTKs, and PLC-\(\beta\) to activation of GPCRs, it is unclear whether other isoforms contribute to the ligand-activated, receptor-mediated responses. However, what is becoming clear from the literature is that there are an increasing number of reports regarding GPCR transactivation of not only RTKs, but also the PLC-\(\gamma\) isoform once thought to be the sole province of RTKs. There are no current reports that identify RTK-mediated
activation of the PLC-β isoforms that remain the territory of GPCRs. But, the activation mechanisms of the PLC isoforms allows for differential regulatory processes and this study aimed to delineate the different PLC isoforms responsible for particular phases of an agonist-induced elevation of $[\text{Ca}^{2+}]$.

Measurements of Ins(1,4,5)P$_3$ and $[^{3}H]\text{-InsP}_x$ were used as an index of total PLC activation, and agonist-induced translocation of both PLC and PKC investigated. Strategies that down-regulated, inhibited or sensitised the Ins(1,4,5)P$_3$ receptor were employed to determine its role in receptor signalling. Finally, with a view to future studies in primary neurons, agonist-mediated elevations of $[\text{Ca}^{2+}]$, were identified in both differentiated SH-SY5Y cells and also in rat hippocampal neurons.

Ultimately this study aimed to define the function of cytosolic elevations of $[\text{Ca}^{2+}]$, following RTK activation. A functional consequence was identified when cytosolic elevations of $[\text{Ca}^{2+}]$, were blocked by BAPTA loading of the cells and a significant reduction in ERK activation was observed.
2: MATERIALS AND METHODS

2.i. TISSUE CULTURE

2.i.i. SH-SY5Y cell culture

Undifferentiated human neuroblastoma SH-SY5Y cells (passages 15-30, originally from ECACC) were grown in 175cm² culture flasks containing 50ml of medium. Culture medium consisted of Minimum Essential Medium (MEM) with Earle’s salts supplemented with 50 IU.ml⁻¹ penicillin, 50 µg.ml⁻¹ streptomycin, 2.5 µg.ml⁻¹ amphotericin B, 2mM L-glutamine, 5% (v/v) new-born calf serum and 5% (v/v) foetal calf serum. Stock cultures were maintained at 37°C in 5% CO₂/humidified air, re-fed every three days and passaged weekly. Confluent cultures were harvested in sterile harvesting buffer (10mM HEPES, 154mM NaCl, 0.54mM EDTA, pH 7.4) and reseeded at a 1:3 - 1:6 ratio as required.

2.i.ii. SH-SY5Y serum starvation

PDGF is greatly enriched in platelets and the presence of serum in the cell culture could desensitise this, and other, classical growth factor/mitogenic signalling pathways. Consideration of the effect of serum on agonist-mediated elevations of [Ca²⁺], are discussed later in more detail (see Chapter 4). Thus, to investigate PDGF and muscarinic receptor-mediated signalling in the SH-SY5Y cells, all cultures were serum-starved for 20 hours (unless otherwise stated), in MEM supplemented with 50 IU.ml⁻¹ penicillin, 50 µg.ml⁻¹ streptomycin, 2.5 µg.ml⁻¹ amphotericin B, 2mM L-glutamine, prior to any experimental manipulation.

2.i.iii. Cell preparation

Confluent cultures of SH-SY5Y cells were harvested and reseeded in 6ml of media at an appropriate density (see individual methods) in 6-well multidishes. For the D-Ins(1,4,5)P₃ mass assay, generation of [³H]-inositol phosphates and saturation binding of [³H]-NMS, cells were reseeded in 24-well multidishes to obtain confluent monolayers. After leaving overnight to allow for cell attachment, the growth media was replaced with 4ml or 1ml (6 and 24-well plates, respectively) of serum starvation media (see section 2.i.ii) and incubated at 37°C in 5% CO₂/humidified air for 20 hours.
2.i.iv. Differentiation of the SH-SY5Y cells

Confluent cultures of SH-SY5Y cells were harvested and reseeded in 6ml of media at a low density (20-30% confluent) (on 22mm diameter coverslips for single-cell imaging) in 6-well multidishes. Following an overnight incubation to allow for cell attachment, differentiation was initiated by replacing the growth media with one containing 10μM all-trans retinoic acid and 1% heat-inactivated foetal calf serum (v/v) rather than the 5% new-born / 5% foetal calf serum (as described in section 2.i.i.). Cells were cultured for a further 6 days prior to use (herein referred to as SH-SY5Y_{RA6} cells) with medium changed every two days and re-supplemented with fresh retinoic acid. Control (undifferentiated) SH-SY5Y cells were grown in media containing the vehicle for retinoic acid (0.1% ethanol, v/v) for 24 hours prior to use.

2.i.v. Preparation of Hippocampal cells

Hippocampal neurons were isolated from the brains of four postnatal day 1 rats (Wistar, female) and prepared for single-cell imaging of Ca^{2+} signalling as described previously [Forsythe and Westbrook, 1988; Brewer et al., 1993; Brewer, 1995]. Dissected regions were treated with papain (20 IU.ml\(^{-1}\), 20 min, 37°C). Tissue was triturated 10-15 times through the fire-polished tip of a nine-inch Pasteur pipette. After undispersed tissue settled for 3 min, the supernatant was centrifuged (3 min, 200g). The supernatant was discarded and the pellet of cells was resuspended in 2ml of Neurobasal medium (NB) supplemented with B27 growth supplement (see section 2.i.vi.) and plated onto 5 glass coverslips per hippocampi.

2.i.vi. Hippocampal Cell Culture

Hippocampal cells were maintained at 37°C in 5% CO\(_2\)/humidified air and re-fed every three days. Cells were grown in a 3ml volume of NB media supplemented with B27 growth supplement, additional pyruvate (1.0mM), 50 IU.ml\(^{-1}\) penicillin, 50 μg.ml\(^{-1}\) streptomycin and 2.5 μg.ml\(^{-1}\) amphotericin B. NB media and the B27 supplement have been specifically developed for the maintenance of neuronal cultures [Brewer et al., 1993]. NB was derived from Dulbecco modified Eagle’s medium (DMEM) but contains less NaCl and NaHCO\(_3\) resulting in lower osmolarity.
and less cysteine and glutamine to reduce glial cell proliferation. This base medium also contains amino acids and a vitamin missing from DMEM: alanine, asparagine, proline and vitamin B12. The B27 supplement contains optimised concentrations of Bottenstein’s N2 (insulin, transferrin, progesterone, putrescine and selenium) as well as thyroid hormone T3, fatty acids, vitamin E, and other anti-oxidants. For the first 3 days of growth, medium also included 10% foetal calf serum and 25μM each of 2-mercaptoethanol and L-glutamate. Cytosine arabinoside (3μM), to inhibit glial cell growth, was present between 1 and 3 days after plating. This protocol produces cultures with a distinctly neuronal morphology [Brewer, 1995].
2.ii. WESTERN BLOTTING AND IMMUNOPRECIPITATION

2.ii.i. Preparation of whole cell extracts

Confluent monolayers of serum starved SH-SY5Y cells in 6-well multidishes were washed once with 1ml of Kreb's/HEPES buffer ((KHB) 10mM HEPES, 4.2mM NaHCO₃, 11.7mM D-glucose, 1.2mM MgSO₄, 1.2mM KH₂PO₄, 4.7mM KCl, 118mM NaCl, and 1.3mM CaCl₂) before the cells were solubilised with 200μl of solubilization buffer (9.2mM Tris, 0.92mM EGTA (TE), 150mM NaCl, 0.1% (v/v) sodium-dodecyl-sulphate (SDS), 1% (v/v) ethylphenylpolyethylene glycol (Nonidet P40), 0.5% (v/v) deoxycholate, 0.5mM phenylmethylsulphonyl fluoride (PMSF), 10μM benzamidine hydrochloride, 5μM iodoacetamide). The cell extract was centrifuged to remove insoluble cell debris (20,800 g, 5 min, 4°C) and 150-

2.ii.ii. Cell fractionation

For the translocation of PLC and PKC isoforms, used as an index of activation, separation of SH-SY5Y cells into cytosolic and membrane fractions was performed using the following method.

Subsequent to agonist stimulation at 37°C, confluent monolayers of SH-SY5Y cells were lysed in 200μl of ice-cold lysis buffer (20mM Tris-HCl, 5mM EGTA, 2mM EDTA, 1mM DTT, 0.5mM PMSF, 10μM benzamidine hydrochloride, 5μM iodoacetamide, pH 7.4) and extracted on ice for 30 min. Samples were then centrifuged (20,800 g, 15 min, 4°C) to separate cytosolic and membrane fractions. A volume of 150-185μl of the supernatant (cytosolic fraction) was removed to a fresh microfuge tube and the insoluble pellet (membrane fraction) was resuspended in a similar volume of solubilization buffer (see section 2.ii.i.).

2.ii.iii. Western Blotting (Immunoblotting)

Cell extract or fractionate (see section 2.ii.i. or 2.ii.ii., respectively), with equivalent concentrations of protein, were combined with an equal volume of 2x sample buffer (100mM Tris, 2% SDS, 0.1% bromophenol blue, 10% (v/v) glycerol, 200mM dithiothreitol (DTT)). Aliquots were denatured by placing in a Grant Boekal heating block at 90°C for 5 min, and proteins were separated by SDS/PAGE
(Bio-Rad Mini-PROTEAN II Electrophoresis Cell) using an 8% running gel (4.6ml dH₂O, 2.7ml 30% acrylamide mix, 2.5ml 1.5M Tris (pH 8.8), 0.1ml 10% SDS (w/v), 0.1ml 10% ammonium persulphate (w/v) and 6μl TEMED in a total volume of 10ml) and 5% stacking gel (6.8ml dH₂O, 1.7ml 30% acrylamide mix, 1.25ml 1M Tris (pH 6.8), 0.1ml 10% SDS (w/v), 0.1ml 10% ammonium persulphate (w/v) and 10μl TEMED in a total volume of 10ml) at 200V for 45 min submerged in running buffer (25mM Tris-base, 250mM glycine, 0.1% SDS (w/v)).

Proteins were transferred to nitrocellulose (0.45μm pore size), pre-soaked in blotting buffer (39mM glycine, 48mM Tris-base, 0.037% SDS (w/v), 20% methanol (v/v), pH 8.3) using a semi-dry blotter (60 min, 0.65mA per cm²) and blocked (either overnight at 4°C or 30 min at 20°C) with 5% (w/v) dry milk in TBST (20mM Tris-HCl, 150mM NaCl, 0.05% (v/v) Tween-20, pH 7.5). The nitrocellulose was then incubated at room temperature with primary antibody, diluted in TBST/5% dry milk, for 1 hour (see Table I for antibody concentrations). After washing in TBST (2 x 1 min, 1 x 15 min, 2 x 5 min), the nitrocellulose was incubated, again at room temperature, with an anti-mouse or anti-rabbit horseradish peroxidase (HRPO)-conjugated secondary IgG antibody for 1 hour (1:1000, in TBST/5% milk). Immunoreactive bands were detected with enhanced chemiluminescence (ECL) reagents (2 min incubation) and exposure to Hyperfilm-ECL.
Table I: Antibody working dilutions

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Supplier</th>
<th>Working Dilution (µg.ml(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLC β1</td>
<td>rabbit</td>
<td>Santa Cruz</td>
<td>0.10</td>
</tr>
<tr>
<td>PLC β2</td>
<td>rabbit</td>
<td>Santa Cruz</td>
<td>0.40</td>
</tr>
<tr>
<td>PLC β3</td>
<td>rabbit</td>
<td>Santa Cruz</td>
<td>0.20</td>
</tr>
<tr>
<td>PLC β4</td>
<td>rabbit</td>
<td>Santa Cruz</td>
<td>0.20</td>
</tr>
<tr>
<td>PLC γ1</td>
<td>rabbit</td>
<td>Santa Cruz</td>
<td>0.20</td>
</tr>
<tr>
<td>PLC γ2</td>
<td>rabbit</td>
<td>Santa Cruz</td>
<td>0.20</td>
</tr>
<tr>
<td>PLC δ1</td>
<td>mouse</td>
<td>Upstate Biotechnology</td>
<td>1.0</td>
</tr>
<tr>
<td>PLC δ2</td>
<td>rabbit</td>
<td>Santa Cruz</td>
<td>0.40</td>
</tr>
<tr>
<td>PKC(\alpha)</td>
<td>mouse</td>
<td>Transduction Laboratories</td>
<td>0.10</td>
</tr>
<tr>
<td>PKC(\beta)</td>
<td>mouse</td>
<td>Transduction Laboratories</td>
<td>0.10</td>
</tr>
<tr>
<td>PKC(\gamma)</td>
<td>mouse</td>
<td>Transduction Laboratories</td>
<td>0.250</td>
</tr>
<tr>
<td>PKC(\delta)</td>
<td>mouse</td>
<td>Transduction Laboratories</td>
<td>0.250</td>
</tr>
<tr>
<td>PKC(\epsilon)</td>
<td>mouse</td>
<td>Transduction Laboratories</td>
<td>0.50</td>
</tr>
<tr>
<td>PKC(\iota)</td>
<td>mouse</td>
<td>Transduction Laboratories</td>
<td>0.50</td>
</tr>
<tr>
<td>PKC(\lambda)</td>
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<td>Transduction Laboratories</td>
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<td>PKC(\mu)</td>
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<td>Transduction Laboratories</td>
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<tr>
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<td>Transduction Laboratories</td>
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</tr>
<tr>
<td>PKC(\zeta)</td>
<td>rabbit</td>
<td>Sigma</td>
<td>5.0</td>
</tr>
<tr>
<td>Phospho-ERK</td>
<td>mouse</td>
<td>Santa Cruz</td>
<td>0.20</td>
</tr>
<tr>
<td>PDGFR (\beta) chain</td>
<td>mouse</td>
<td>Upstate Biotechnology</td>
<td>0.10</td>
</tr>
<tr>
<td>(\alpha_{q11})</td>
<td>rabbit</td>
<td>Santa Cruz</td>
<td>0.20</td>
</tr>
</tbody>
</table>

Immunoblots were analysed by densitometer where appropriate, using a Biorad densitometer (model: GS-670) and Molecular Analyst software (v1.2) also from Biorad.
2.ii.iv. Immunoprecipitation of PLC γ1

Individual wells were washed with 1ml of KHB, and lysed in 1ml of solubilization buffer (see section 2.ii.i.). Following extraction on ice for 30 min, samples were centrifuged (20,800 g, 3 min, 4°C). To 850μl of the supernatant, removed to a fresh microfuge tube, 5μl of anti-PLC-γ1 antibody was added and left on ice for 1 hour.

Immunocomplexes were captured with 150μl of a 4.5 mg.ml⁻¹ suspension of protein-A-sepharose and placed on rollers for 30 min at 4°C. The beads were washed twice with 1ml volumes of ice-cold TTBS (100mM Tris base, 1.5M NaCl, 0.05% Tween-20, pH 7.4), twice with 1ml volumes of ice-cold TE buffer (10mM Tris, 1mM EGTA, pH 7.2), and then denatured by placing in a Grant Boekal heating block at 90°C for 5 min following the addition of 20μl of 2x sample buffer (see section 2.ii.iii). The samples were then subjected to 8% SDS/PAGE and immunoblotting was used to determine the extent of PLC γ1 immunoprecipitation (see section 2.ii.iii.).

2.ii.v. Down regulation of PLC isoforms

Confluent monolayers of SH-SY5Y cells were harvested and reseeded in 6ml of growth media at an equivalent density in 6-well multidishes. After leaving overnight to allow for cell attachment, the growth media was replaced with 4 ml of serum starvation media and simultaneously incubated with either vehicle (dH₂O), methacholine (1mM) or PDGF (200 ng.ml⁻¹) for 24 hours. Agonists were added to the well in volumes of 20 μl.

Whole cell extract from vehicle (dH₂O), methacholine and PDGF treated wells was immunoblotted for the PLC isoforms (see section 2.ii.i. and 2.ii.iii., respectively).
2.ii.vi. Phosphorylation of PLC isoforms

Confluent monolayers of SH-SY5Y cells in 6-well multidishes were washed once with 1ml of phosphate-free KHB (10mM HEPES, 4.2mM NaHCO$_3$, 11.7mM D-glucose, 1.2mM MgSO$_4$, 4.7mM KCl, 118mM NaCl, and 1.3mM CaCl$_2$) and then incubated at 37°C for 1 hour in 1ml of phosphate-free KHB supplemented with 5μl of [32P]-labelled orthophosphate (50μCi).

Following agonist stimulation, wells were rapidly aspirated and the cell monolayers solubilised with 500μl of solubilization buffer (see section 2.ii.i.) and extracted on ice for 30 min. Samples were centrifuged (20,800 g, 3 min, 4°C) and 450μl of the supernatant removed to a fresh microfuge tube. To this, 3μl of anti-PLC antibody was added and incubated on ice for 1 hour. Immunocomplexes were captured with 100μl of a 5.4 mg.ml$^{-1}$ suspension of protein-A-sepharose and placed on rollers for 30 min at 4°C. The beads were washed twice with 1ml volumes of ice-cold TTBS and at least twice with 1ml volumes of ice-cold TE buffer (see section 2.ii.iv.). Following the addition of 25μl of 2x sample buffer the samples were then subjected to 8% SDS/PAGE (see section 2.ii.iii.) and the gels vacuum dried before exposure to hyperfilm at -80°C for 48-96 hours.
2.iii. MEASUREMENT OF INTRACELLULAR CALCIUM AND PHOSPHOINOSITIDES

2.iii.i. Population calcium measurements in suspension

Determinations of [Ca\(^{2+}\)], in suspensions of fura-2-loaded cells utilised a previously described method [Willars et al., 1998]. In brief, serum starved confluent SH-SY5Y cells were harvested, washed once with 10ml of KHB, and resuspended in 2.5ml of the same buffer. A 0.5ml aliquot was removed and treated exactly as described below with the exclusion of fura-2-acetoxymethylester (fura-2-AM), allowing the determination of auto fluorescence. Fura-2-AM was added to the remaining cells, at 5μM, and the cells left in the dark with gentle stirring for 1 hour at room temperature. Supernatant containing extracellular fura-2-AM was removed following centrifugation (1,400g, 40 s, 20°C) of 0.5ml aliquots. The cells were washed twice with 1ml volumes of KHB before re-suspension in 3ml of the same buffer and placed within a Perkin-Elmer spectrofluorimeter at 37°C (model: LS-50B). Agonist was added in 20μl aliquots, and the 340:380 ratio (R) converted to [Ca\(^{2+}\)], as described in Grynkiewicz et al., 1985. Briefly, the following equation is used to derive the intracellular calcium concentration:

\[
[Ca^{2+}]_i = K_d \times Sfb \times \left(\frac{(R_{\text{max}} - R)}{(R - R_{\text{mn}})}\right)
\]

The \(K_d\) refers to the \(K_d\) of fura-2 for Ca\(^{2+}\) (135nM at 22°C, 224nM at 37°C), and the Sfb \((380_{\text{max}}/380_{\text{mn}})\) is derived in Grynkiewicz et al., 1985. \(R_{\text{max}}\) was determined following the addition of 0.1% Triton X-100 in the presence of 1.3mM extracellular calcium and refers to the maximum measurable 340:380 ratio. The subsequent addition of 6.7mM EGTA was used to obtain \(R_{\text{mn}}\), the minimum measurable 340:380 ratio.

2.iii.ii. Population calcium measurements on adherent cells

Confluent cultures of SH-SY5Y cells were harvested and re-seeded at an equivalent density on 11 x 22 mm glass coverslips in 6-well multidishes (1 coverslip per well). Following serum starvation, the coverslips were washed in 1ml of KHB and the cells loaded with fura-2-AM (3.3μM), in the presence of pluronic acid F-127
(0.05%, v/v) for 1 hour at room temperature. The adherent SH-SY5Y monolayers appeared resistant to fura-2-AM uptake and the pluronic F-127, which is a very mild detergent, was included to enhance dye loading of the cells. Following two 3ml washes with KHB at 37°C, the coverslip was then incubated for a further 10 minutes at 37°C to facilitate hydrolysis of fura-2-AM. The coverslip was then transferred to a coverslip holder and placed within a Perkin-Elmer spectrofluorimeter at 37°C (model: LS-50B) in a final volume of 2ml. Agonists and inhibitors were added in 20μl aliquots.

With emission at 509 nm, the 340/380 nm excitation ratio was recorded every 1 s as an index of [Ca²⁺]. These values were used to convert all population 340:380 nm ratios to [Ca²⁺], [Grynkiewicz et al., 1985] (see section 2.iii.i.). The determination of the auto-fluorescence and the calibrations to derive Rₘₐₓ and Rₘᵦ were performed each day in separate experiments. In brief, from each 6 well plate, one coverslip was used to determine the auto-fluorescence by the method above but omitting the fura-2-AM. For the loaded cells, the Rₘₐₓ of fluorescence was determined by the addition of ionomycin (10μM) and Rₘᵦ by the addition of EGTA (8mM).

2.iii.iii. Single cell imaging of agonist-mediated elevations of [Ca²⁺]ᵢ

Cells (SH-SY5Y, SH-SY5Y₉₆ and hippocampal cells) were cultured on sterilised glass coverslips (22 mm diameter), until 60-70% confluent, in appropriate growth medium (see section 2.i.).

The coverslips were washed once with KHB and then incubated in the dark, at room temperature, for 1 h in 2ml KIIB supplemented with fura-2-AM (5μM). The coverslips were washed twice in 1ml KHB and incubated for a further 30 min to allow for complete de-esterification of the dye, before being mounted on the stage of a Nikon Diaphot inverted epifluorescence microscope. KHB was continuously perfused over the cells at the rate of 5 ml.min⁻¹. Agonists were applied to the bath, and perfusion terminated until re-initiated to wash the agonist from the cells. After subtraction of background fluorescence, images at wavelengths above 510 nm, excitation at 340 and 380 nm (40 ms at each wavelength), were collected with an intensified charge-coupled device camera (Photonic Science). Experiments were
conducted on a Quanticell 700 Imaging system (VisiTech International) with the temperature maintained at 37°C.

Ratiometric values were converted to approximate $[\text{Ca}^{2+}]_i$ using the previously described method of Grynkiewicz [Grynkiewicz et al., 1985]. Calibrations to define $R_{\text{max}}$, $R_{\text{min}}$ and the Sfb were obtained as described previously (see section 2.iii.ii.).

2.iii.iv. Preparation of D-Ins(1,4,5)P$_3$ binding protein from bovine adrenal cortex

The fresh bovine adrenal glands were de-medullated and the cortex removed and placed on ice. The cortex was homogenised in 8 x 50ml volumes of ice-cold homogenisation buffer (20mM NaHCO$_3$, 1mM dithiothreitol (DTT), pH 8.0) using a Polytron tissue disruptor.

The homogenate was centrifuged at 3,000g for 10 min at 4°C and the supernatant removed. The pellet was washed (resuspended and re-homogenised) in the same buffer, and then re-centrifuged as above. The pooled supernatant fraction was centrifuged at 38,000g for 20 min at 4°C. The supernatant was removed and discarded and the pellet washed with buffer as above. The washed pellet was resuspended in homogenisation buffer at a protein concentration of 20 mg.ml$^{-1}$ and frozen in 5ml batches for subsequent use. This preparation has been described previously [Challiss et al., 1990].

2.iii.v. Measurement of D-Ins(1,4,5)P$_3$ mass

Generation and measurement of D-Ins(1,4,5)P$_3$ was done using a modification of a previously described method [Willars and Nahorski, 1995a].

In brief, the confluent monolayers of cells were washed once with 1ml of KHB at 37°C and incubated for a further 15 min at 37°C with 200µl of KHB per well. A 50µl aliquot of buffer containing a 5x concentration of agonist was then added to the well. Reactions were stopped by the addition of 250µl of ice-cold 1M trichloroacetic acid (TCA). For the zero time point, TCA was added prior to the addition of agonist.
Multidishes were incubated on ice for at least 15 min and 40μl of 10mM EDTA added to 160μl aliquots of sample, followed by 200μl of a freshly prepared 1:1 (v:v) mixture of tri-n-octyl-amine and 1,1,2-trichloro-trifluoro-ethane. After vortexing, samples were left at room temperature for 15 min, then centrifuged (20,800g, 2 min), and 100μl of the aqueous upper phase taken and added to 50μl of 25mM NaHCO₃. Samples were stored at 4°C until assayed for D-Ins(1,4,5)P₃.

D-Ins(1,4,5)P₃ was quantified in single 30μl aliquots of duplicate generations in a final volume of 120μl (25mM Tris-HCl, 1mM EDTA, pH 8.0) at 4°C using bovine adrenal cortical binding protein and D-myo-[³H]-inositol 1,4,5-trisphosphate (specific activity 44 Ci.mmol⁻¹, 3.6nCi per tube) as the radioligand. Authentic D-Ins(1,4,5)P₃ (0.1nM - 3μM) was used as standard, with non-specific binding defined by 10μM Ins(1,4,5)P₃. Following a 30 min incubation on ice, protein was collected with 2ml of ice-cold buffer (25mM Tris-HCl, 1mM EDTA, 5mM NaHCO₃, pH 8.0) by rapid filtration (Brandel Cell Harvester) onto Whatman GF/B glass fibre filters. After two rapid 5ml washes with the same buffer, the filters were counted by liquid-scintillation spectrometry in 4ml of Emulsifier-Safe scintillation cocktail.

Protein content of the wells (typically 100-200μg) was determined in 0.1M NaOH digests of at least two wells per multidish by the method of Lowry.

The isolation and assay protocols for the determination of D-Ins(1,4,5)P₃ mass were exactly as described previously [Willars and Nahorski, 1995a] using a radioreceptor method assessed for stereospecificity and positional specificity [Challiss et al., 1990]. When n is given, this represents the number of duplicate generations of D-Ins(1,4,5)P₃.

2.iii.vi. Measurement of [³H]-inositol phosphate accumulation

Cells were prelabelled with 3μCi.ml⁻¹ of myo-[³H]-inositol (86 Ci.mmol⁻¹) in a 0.5ml volume of growth media for 48 hours at 37°C to ensure equilibrium labelling [Willars et al., 1998]. The cells were also serum-starved for the last 24 hours of labelling.

Following a 10 min preincubation at 37°C in 250μl of KIIB containing 10mM Li⁺, a further 250μl of KHB (+Li⁺) containing agonist was added. Reactions were halted by the addition of 500μl of ice-cold 1M TCA. After a 15 min extraction
on ice, an 800μl aliquot from each well was removed and 200μl of 10mM EDTA added. After vortexing with 1ml of a freshly prepared 1:1 (v:v) mixture of tri-n-octyl-amine and 1,1,2-trichloro-trifluoro-ethane, 50μl of 250mM NaHCO₃ was added to 700μl of the aqueous phase.

This was applied to a Dowex AG1-X8 formate column, which was then washed with 10ml of 25mM ammonium formate. Inositol phosphates were eluted from the column with 10ml of 1M ammonium formate/0.1M formic acid. A 2ml aliquot of this eluant was counted by liquid scintillation spectrometry following the addition of 7-8ml of Emulsifier-Safe Scintillation Cocktail [Baird et al., 1989].

2.iii.vii. Saturation binding of [³H]-N-methyl-scopolamine ([³H]-NMS)

Cell monolayers in 24-well plates were washed once with 1ml of KHB before incubation for 1 hour at 37°C in a 1ml volume of KHB containing [³H]-NMS (0.1-100nM). At each of the seven concentrations of [³H]-NMS used, a duplicate measurement of total binding and a single measurement of non-specific binding (by the addition of 2μM atropine) was determined. Following incubation, the cells were washed with two 1ml volumes of ice-cold KHB before being digested with 0.5ml of 0.1M NaOH. This was neutralised with 0.5ml of 0.1M HCl and [³H] determined by liquid scintillation spectrometry following the addition of 4.5ml Emulsifier-Safe scintillation cocktail.

2.iii.viii. Release of ⁴⁵Ca²⁺ from permeabilised cells

A confluent 175cm² flask of SH-SY5Y cells was harvested and washed once in 5ml of ice-cold 'cytosol-like' buffer ((CLB) KCl 120mM, KH₂PO₄ 2mM, (H₂COONa)₂.6H₂O 5mM, MgCl₂.6H₂O 2.4mM, HEPES 20mM, Na₂ATP 2mM, pH 7.2). CLB was prepared in plastic labware to avoid Ca²⁺ contamination and the free calcium concentration was adjusted to ~100nM using EGTA (stock concentration 10mM). Cells were resuspended in 2.8ml of the same buffer and permeabilization initialised by the addition of 2.5 μg.ml⁻¹ of the detergent β-escin.

Intracellular Ca²⁺ stores were loaded with ⁴⁵Ca²⁺ by the addition of 0.5 mCi.ml⁻¹ of ⁴⁵CaCl₂ (1000 Ci.mmol⁻¹). The suspensions were mixed gently and left
for 15 min at room temperature with periodic mixing to allow for $^{45}$Ca$^{2+}$ uptake to reach equilibrium [Wojcikiewicz et al., 1990].

Reactions were initiated by the addition of 50μl of cell suspension to 50μl of CLB containing a 2x concentration of agonist in 1.5ml microfuge tubes. After 2 min (variable times for PDGF) the tubes were centrifuged (20,800g, 2 min) followed by the addition of 400μl of a silicone oil mixture (Dow Corning 550/556, 1:1 (v:v)) to each tube. The tubes were immediately re-centrifuged (20,800g, 2 min) to separate cells from the supernatant. The aqueous phase and most of the silicone oil was carefully removed and the tubes inverted for 30-60 min to remove the remaining silicone oil from the pellet.

Emulsifier-Safe scintillation cocktail (1.1ml) was added to each tube that was then capped and vortex mixed. The tubes were left overnight to allow full dispersion of the radioactivity before being measured by scintillation spectrometry.

2.iii ix. Down regulation of the Ins(1,4,5)P$_3$ receptor

Confluent cultures of SH-SY5Y cells were harvested and reseeded in 8ml of growth media at an equivalent density in 6-well multidishes. After leaving overnight to allow for cell attachment, the cells were then serum starved for 20 hours and simultaneously incubated with 100μM methacholine for the final 7 hours of this process.
2.iv. ACTIVATION OF THE MAPK PATHWAY

2.iv.i. Measurement of ERK activation

Prior to the addition of agonist, confluent cell monolayers in 6-well multidishes were incubated for 30 min at 37°C in KHB, in the absence or presence of 30µM BAPTA-AM (the pentaacetoxymethyl ester of 1,2-bis(2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid). Following agonist stimulation for 0–60 min, reactions were halted by rapid aspiration and the addition of 200µl of solubilization buffer (see section 2.ii.i.). The multidishes were incubated on ice for 15 min before the cell extract was transferred to a fresh 1.5ml microfuge tube and centrifuged (20,800g, 4°C, 15 min). 100µl of the supernatant was removed to a fresh tube and combined with 100µl of 2x sample buffer.

Aliquots, equilibrated for protein (~30 µg sample⁻¹), were denatured by placing the samples in a Grant Boekal heating block for 5 min at 90°C, and subjected to 8% SDS/PAGE and transfer of the proteins to nitrocellulose (see section 2.ii.iii.). The nitrocellulose was then incubated with a phospho-ERK antibody (reacts with phosphorylated human ERK 1 and 2), in TBST/5% milk for 1 hour at room temperature. After washing in TBST (2 x 1 min, 1 x 15 min, 2 x 5 min), the nitrocellulose was incubated with an HRPO-conjugated anti-mouse secondary IgG antibody for 1 hour (1:1000, in TBST/5% milk) at room temperature. Immunoreactive bands were detected with ECL reagents and exposure to Hyperfilm-ECL.

Immunoreactive bands were quantified by densitometry as described previously (see Table I).
2.v. DETERMINATION OF PROTEIN CONTENT

Protein concentrations were determined using the method of Lowry [Lowry et al., 1951]. Samples were prepared from 6 and 24-well multidishes by the addition of 1ml per well of 0.1M NaOH. Protein standards were prepared using bovine serum albumin (BSA, lyophilised fraction IV) diluted in 0.1M NaOH to give a range of concentrations of 0–400 µg.ml⁻¹.

To 500µl of both sample and standards, 1ml of a freshly prepared mixture containing 98% of solution A (2% Na₂CO₃ in 0.1M NaOH), 1% of solution B (1% CuSO₄) and 1% of solution C (2% Na⁺K⁺tartrate) was added. Tubes were vortexed and allowed to stand at room temperature for 10 min. 100µl of Folin reagent was added to each tube, followed by vortex mixing and a further 20 min incubation at room temperature. Finally, 1ml of dH₂O was added to the tubes, which were then vortexed, transferred to cuvettes and the absorbance readings determined at 750 nm.

Protein concentrations of samples were determined using a standard curve constructed from the absorbance values of the protein standards.
2.vi. CHEMICALS AND MATERIALS

Standard chemical reagents of analytical grade were obtained from Sigma (Poole, Dorset, UK) and Elgastat purified water was used throughout. MEM, NB medium, B27 supplement, penicillin/streptomycin, L-glutamine, newborn calf serum, foetal calf serum and all tissue culture materials were from Life Technologies Ltd. (Inchinan, Scotland). The papain used for the dissociation of hippocampal cells was supplied by Worthington Biochemical Corporation (Lakewood, New Jersey 08701, USA).

EGF, NGF, Fura-2-AM, fura-2 free acid, Ro-318220, pluronic acid F-127, thapsigargin, DMS, PDBu, BAPTA-AM, ionomycin (calcium salt) and ionomycin (free acid) were from Calbiochem (Nottingham, UK).

Methacholine, rhPDGF-AA, rhPDGF-BB, Bradykinin, atropine (sulphate salt), retinoic acid, DTT, pre-stained molecular protein markers (SDS-7B), BSA, ATP, β-escin, low-molecular-mass heparin (4-6 kDa), LPA, thimerosal, caffeine, and HRPO-conjugated anti-mouse/anti-rabbit (whole molecule) IgG antibodies were from Sigma (Poole, Dorset, UK).

Glass coverslips, both 11 x 22 mm and 22 mm diameter, were obtained from Chance Proper (UK) and the cuvettes used for spectrofluorimetry were obtained from Sarstedt (Leicester, UK). Dow Corning (High Wycombe, Berks, UK) supplied both Dow Corning 550 and 556 oils.

For details of all primary antibodies, please refer to Table I (see section 2.ii.iii). Santa Cruz Biotechnology Inc., Upstate Biotechnology Inc. and Transduction Laboratories are all based in the USA (Santa Cruz, CA; Lake Placid, NY 12946, and Lexington, KY 40511-2624, respectively).

The Mini-PROTEAN II electrophoresis gel equipment was all from Bio-Rad (Hercules, CA 94547, USA). Nitrocellulose (0.45µm pore size) for protein transfer was supplied by Schleicher and Schuell (London, UK). Amersham Pharmacia Biotech UK Ltd. (Little Chalfont, Bucks, UK) supplied the [32P]-orthophosphate, [3H]-NMS, 45CaCl2, hyperfilm-ECL, protein-A-sepharose and ECL reagents. NEN Life Science Products (Stevenage, UK) supplied the myo-[3H]-inositol (117 Ci.mmol-1) and [3H]-Ins(1,4,5)P3 (58.4 Ci.mmol-1). Emulsifier-Safe scintillation cocktail was supplied by Packard (Pangbourne, Berks, UK).

bFGF II was a kind gift of Dr. Helen Burns (University of Birmingham).
2.vii. DATA ANALYSIS

Concentration-response curves were fitted by Graph-PAD (Academic Press Inc. 1986) using a standard four-parameter logistic equation. EC\textsubscript{50} mean values and standard errors of the mean were generated from the mean of values generated from separate curves. All data given in text are ‘mean ± S.E.M.’. All statistical analysis between mean data was by unpaired, two-tailed, Student’s \textit{t}-test assuming unequal variance. Significance of temporal spatial redistribution of both PLC and PKC isoforms was determined by single factor analysis of variance (ANOVA). Two-way ANOVA was used to compare [\textsuperscript{3}H]-NMS binding and Ins(1,4,5)P\textsubscript{3} production in the presence of PKC and sphingosine kinase inhibitors. Acceptance of significance for all tests was at \( p<0.05 \).
3: IDENTIFICATION OF A GROWTH FACTOR-MEDIATED ELEVATION OF $[\text{Ca}^{2+}]_i$ AND ELEMENTARY CHARACTERISATION.

3.i. INTRODUCTION

This results chapter concentrates on the identification of the PDGF receptor as a suitable paradigm RTK and subsequently characterises the agonist-mediated elevation of $[\text{Ca}^{2+}]_i$ in SH-SY5Y cells. A parallel study investigates the muscarinic receptor-mediated elevation of $[\text{Ca}^{2+}]_i$, and initiates the primary aim of this study which was to investigate and compare GPCR and RTK-mediated signalling within the SH-SY5Y cell with regard to signal transduction mechanisms that evoked an elevation of $[\text{Ca}^{2+}]_i$.

The SH-SY5Y human neuroblastoma cell endogenously expresses several types of membrane-bound classical growth factor receptors that are assumed to play primarily trophic roles within the cell. In addition these cells also express a number of seven-transmembrane, G protein-coupled receptors (see section 1.vi.). Previous reports had already provided a suitable phosphoinositide-coupled GPCR in the form of the muscarinic acetylcholine receptor [Baird et al., 1989; Willars and Nahorski, 1995a; Willars and Nahorski, 1995b], which in the SH-SY5Y cell, is predominantly of the M$_3$ isotype [Lambert et al., 1989].

The initial RTK screening followed reports that EGF, bFGF, NGF and PDGF receptors were all endogenously expressed in the SH-SY5Y cells [Jensen et al., 1992; Pahlman et al., 1992; Janet et al., 1995]. Despite the reported presence of IGF receptors (I and II) [Loret et al., 1992], these were not investigated. Classical RTK-mediated signalling occurs following phosphorylation of the cytoplasmic domain of the receptor, resulting in the recruitment of intermediary signalling components. In contrast, IGF receptors recruit insulin receptor substrate (IRS) proteins that are heavily phosphorylated in place of the cytoplasmic domain of the receptor [reviewed in Myers et al., 1994].

Many growth factors are active only as dimers, and induce dimerisation and autophosphorylation of their respective RTK. Growth factor receptors have intrinsic tyrosine kinase activity and following receptor activation, autophosphorylation of the cytoplasmic domain of the receptor recruits a variety of signalling molecules often...
leading to multiple effects on the target cell [Hammacher et al., 1989; Claesson-Welsh, 1994; reviewed in Malarkey et al., 1995; Heldin et al., 1998] (see section 1.i.iii. for a fuller discussion). As both PDGF receptor α and β chains are expressed in the SH-SY5Y cell [Pahlman et al., 1992], and have similar but distinct signalling characteristics yet can both recruit PLC-γ [Kim et al., 1991; Gelderloos et al., 1998], it was essential to determine which PDGF receptor was involved in the agonist-mediated elevation of \([\text{Ca}^{2+}]_i\).

In contrast, GPCR-mediated elevations of \([\text{Ca}^{2+}]_i\), are often documented as dependent upon G-protein Go and Gβγ subunits, with Goq-coupled receptors activating PLC-β isoforms and subsequent Ins(1,4,5)P3 generation [Smrcka et al., 1991; Taylor et al., 1991; Camps et al., 1992; reviewed in Lee and Rhee, 1995].

If GPCRs and RTKs that elevate \([\text{Ca}^{2+}]_i\), within the cell rely on the same \(\text{Ca}^{2+}\) source, do they utilise similar mechanisms? If this is the case, some heterologous desensitisation may be expected to occur at the level of \(\text{Ca}^{2+}\) signalling or some other shared signalling intermediary protein [see Willars and Nahorski, 1995b].

One of the major drawbacks of the SH-SY5Y human neuroblastoma as a model neuronal cell line is the lack of differentiation. In an attempt to use a more ‘neuronal-like’ cell in some experiments, the SH-SY5Y cells were differentiated using retinoic acid (10 μM) for 6 consecutive days [Pahlman et al., 1984; Martin et al., 1999] (see Chapter 2, section 2.i.iv). The cell morphology changes in this time and the SH-SY5Y_RA6 cell is reported to be more neuronal-like and would provide a superior model for a neuronal cell than its undifferentiated counterpart [Pahlman et al., 1984]. Undifferentiated neuroblastoma cells have a short dividing time and may exhibit some neuronal-like features, which are generally at very low levels compared to differentiated neurons, and become more apparent upon differentiation [reviewed in Prasad, 1975; Temple, 1990].

This first results chapter begins to answer some of the questions raised at the onset of this study. Such as: which PDGF receptor subtype is responsible for the elevation of \([\text{Ca}^{2+}]_i\); was the \(\text{Ca}^{2+}\) source intra- or extracellular; do PDGF and methacholine result in \(\text{Ca}^{2+}\) release from the same intracellular \(\text{Ca}^{2+}\) store; is there
any heterologous desensitisation between these two distinct receptor families?; and finally, is a PDGF-mediated elevation of \([\text{Ca}^{2+}]\), relevant to signal transduction in terminally differentiated neurons?
3.ii. RESULTS

Identification of the PDGF-mediated elevation of $[Ca^{2+}]_i$.

Initially adherent populations of serum starved SH-SY5Y cells were stimulated with four different classical RTK ligands: basic FGF; EGF; NGF; and PDGF-BB; receptors for which are reported in SH-SY5Y cells. Concentrations approximate to those reported for maximal mitogenic ability were used to assess their ability to elevate $[Ca^{2+}]_i$. Neither basic FGF, EGF nor NGF stimulation resulted in an elevation of $[Ca^{2+}]_i$. However, stimulation with PDGF-BB at a concentration of 200 ng.ml$^{-1}$ in the presence of 1.3 mM extracellular Ca$^{2+}$ ($[Ca^{2+}]_e$) resulted in a marked elevation of $[Ca^{2+}]_i$ to a peak height of 260 ± 7 nM, $n = 6$ from a basal 64 ± 6 nM, $n = 6$ (see Fig 3.i.).

![Graph showing calcium ion concentration over time for different agonists](image)

**Fig 3.i.** $[Ca^{2+}]_i$ in adherent SH-SY5Y cells following basic FGF, EGF, NGF and PDGF-BB at the concentrations indicated. Data are representative of at least three independent experiments.
PDGF and methacholine-mediated elevation of $[Ca^{2+}]_i$ in the absence or presence of 1.3 mM $[Ca^{2+}]_e$ and identification of the PDGF receptor subtype.

Stimulation of SH-SY5Y cells with PDGF-BB (200 ng ml$^{-1}$) resulted in a slow, transient elevation in $[Ca^{2+}]_i$, following a 'lag' phase that varied between 20-60 seconds (see Fig 3.i). Reducing the $[Ca^{2+}]_e$ to nominally Ca$^{2+}$ free (i.e. with no addition of Ca$^{2+}$ to the buffer) 50s prior to agonist addition, resulted in a reproducible, but significantly reduced elevation of $[Ca^{2+}]_i$ in response to the same concentration of PDGF-BB. The maximum elevation was reduced (108 ± 3 nM, n = 6; compared to 260 ± 7 nM, n = 6, in the presence of 1.3 mM $[Ca^{2+}]_e$, p<0.001) and basal levels were re-established within 3-4 minutes compared to 5-10 minutes in the presence of 1.3 mM $[Ca^{2+}]_e$ (Fig. 3.ii. A).

Muscarinic receptor activation with a maximal concentration of methacholine (1 mM) resulted in a biphasic $[Ca^{2+}]_i$ elevation. A rapid (<10 seconds) increase in $[Ca^{2+}]_i$, to a peak of 978 ± 80 nM, n = 4; was followed by a slow decline to a lower, but sustained, elevated $[Ca^{2+}]_i$, of 220 ± 15 nM, n = 3. In nominally Ca$^{2+}$ free buffer, the muscarinic receptor-mediated elevation in $[Ca^{2+}]_i$ exhibited a significantly reduced peak height (640 ± 28 nM, n = 3; compared to 978 ± 80 nM, n = 4, in the presence of 1.3 mM $[Ca^{2+}]_e$, p<0.05) and the sustained phase was abolished (Fig. 3.ii. B).

The PDGF receptor exists as two isoforms, α and β (for a discussion of their cross reactivity with the PDGF A and B ligands, see Chapter 1, Fig 1.i.). As PDGF-BB also activates PDGF receptor α-chains, the potential role of this receptor isotype in the PDGF-mediated increase in $[Ca^{2+}]_i$, was investigated. Addition of PDGF-AA, which interacts only with the PDGF receptor α-chains, failed to produce any elevation in $[Ca^{2+}]_i$, and the subsequent addition of PDGF-BB elicited a normal elevation of $[Ca^{2+}]_i$, 254 ± 10 nM, n = 3 (Fig. 3.ii. C).
Fig 3.i. Agonist-mediated elevations in $[\text{Ca}^{2+}]_i$, in adherent SH-SYSY cells following: A) PDGF-BB (200 ng ml$^{-1}$) and B) methacholine (1 mM), in the presence (solid lines) or absence (dotted lines) of 1.3 mM $[\text{Ca}^{2+}]_e$. C) Stimulation with PDGF-AA followed by PDGF-BB (both 200 ng ml$^{-1}$). Bars indicate presence of agonist. Data are representative of at least three independent experiments.
**Concentration-dependence of the PDGF-mediated elevation of \([\text{Ca}^{2+}]_i\).**

Using populations of SH-SY5Y cells, the [PDGF]-dependence of the elevation of \([\text{Ca}^{2+}]_i\) was examined over the concentration range 0.5 ng.ml\(^{-1}\) to 266 ng.ml\(^{-1}\) (20 pM to 10.64 nM). The magnitude of the PDGF-mediated elevation in \([\text{Ca}^{2+}]_i\) was concentration-dependent with an estimated EC\(_{50}\) of 14 ng.ml\(^{-1}\) (-9.27 (\(\log_{10}\) M) or 0.54 nM) and no elevation below 1 ng.ml\(^{-1}\) (40 pM) (Fig. 3.iii.).

![Graph showing concentration dependence of PDGF on [Ca\(^{2+}\)]_i](image)

**Fig 3.iii.** The [PDGF]-response relationship for PDGF-BB-mediated elevations of \([\text{Ca}^{2+}]_i\). Data are plotted against both [PDGF] (ng.ml\(^{-1}\)) and \(\log_{10}\) [PDGF] (M), see inset. Data are mean ± S.E.M. (n ≥ 3).
Effect of intracellular Ca\(^{2+}\) store depletion on a subsequent PDGF-mediated elevation of \([Ca^{2+}]_i\).

Further experiments were designed to determine the identity of the intracellular Ca\(^{2+}\) store released by PDGF, and whether Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) stores was a prerequisite for Ca\(^{2+}\) influx. Non-mitochondrial Ca\(^{2+}\) stores were depleted by the addition of 2 \(\mu\)M thapsigargin for 11 minutes in the presence of 1.3 mM \([Ca^{2+}]_e\) [Wojcikiewicz et al., 1994b]. This abolished subsequent \([Ca^{2+}]_i\) responses to both PDGF (200 ng.ml\(^{-1}\)) and methacholine (1 mM) (Fig 3.iv. A).

Stimulation with a maximal concentration of methacholine (1 mM) and its continued presence also completely blocked the response to a subsequent addition of PDGF either in the presence (Fig 3.iv. B) or absence (data not shown) of 1.3 mM \([Ca^{2+}]_e\). Following challenge with methacholine, a normal response (253 ± 6 nM, \(n = 4\)) to a maximal concentration of PDGF (200 ng.ml\(^{-1}\)) could be measured 5 min after the addition of atropine (2 \(\mu\)M), an antagonist of the muscarinic receptors (Fig 3.iv. C) in the presence of 1.3 mM \([Ca^{2+}]_e\). The magnitude of the PDGF response increased in a time-dependent manner following the addition of atropine, with no measurable response after only 1 min and a significantly reduced response (152 ± 19 nM, \(n = 3\); 60% of control; \(p<0.05\)) after 2 min (data not shown).
Fig 3.iv. PDGF fails to elevate \([Ca^{2+}]_i\) in SH-SY5Y cells following: A) thapsigargin (2 μM) and B) Methacholine (1 mM). C) Antagonism of the muscarinic response with atropine (2 μM) allows the time-dependent return of the PDGF-mediated elevation of \([Ca^{2+}]_i\). Data are representative of at least three separate experiments.
**PDGF attenuates muscarinic receptor-mediated Ca\(^{2+}\) signalling.**

In the presence of 1.3 mM \([Ca^{2+}]_e\), prestimulation with a maximal concentration of PDGF (200 ng.ml\(^{-1}\)) significantly reduced a subsequent muscarinic receptor-mediated response given 5 min after and in the continued presence of PDGF (285 ± 27 nM, n = 3; compared to 978 ± 80 nM, n = 3, in the absence of PDGF; p<0.005) (Fig 3.v. A).

Repeating the experiment in nominally Ca\(^{2+}\) free buffer, the elevation in \([Ca^{2+}]_e\), due to PDGF stimulation had, within 3-4 min, returned to basal levels prior to stimulation by methacholine (see Fig 3.ii. A). Prestimulation with PDGF under these conditions resulted in a significant reduction in the response to methacholine given 5 minutes after PDGF (156 ± 21 nM, n = 4; compared to 640 ± 28 nM, n = 3; p<0.001) (Fig 3.v. B).

Was this heterologous interaction at the level of the intracellular Ca\(^{2+}\) store?
Fig 3.v. A) Prestimulation with PDGF-BB (200 ng.ml\(^{-1}\)) in the presence of 1.3 mM \([Ca^{2+}]_e\) significantly reduced \((p<0.005)\) the subsequent methacholine-mediated elevation of \([Ca^{2+}]_e\). B) Following PDGF prestimulation in low \([Ca^{2+}]_e\), the subsequent muscarinic receptor-mediated response was even more significantly reduced \((p<0.001)\). Data shown are representative of at least three separate experiments.
**PDGF attenuation of the muscarinic receptor-mediated elevation of \([Ca^{2+}]_i\) is not due to \(Ca^{2+}\) store depletion.**

A previous report has shown that in the absence of extracellular \(Ca^{2+}\) (EGTA-buffered to \(-100\) nM), the \(Ca^{2+}\) ionophore, ionomycin, releases \(Ca^{2+}\) from the rapidly releasable, agonist-sensitive, intracellular \(Ca^{2+}\) store in SH-SY5Y cells. This release is very rapid (<5 sec), takes the form of a \(Ca^{2+}\) spike and can, therefore, be used to monitor the \(Ca^{2+}\) content of the store [Purkiss and Willars, 1996]. Thus, by determining the content of the intracellular \(Ca^{2+}\) store, in the absence of extracellular \(Ca^{2+}\) and in the absence or presence of agonist prestimulation, the relative abilities of PDGF and methacholine to deplete the intracellular \(Ca^{2+}\) store were investigated.

The elevation of \([Ca^{2+}]_i\) by ionomycin (10 \(\mu\)M) following PDGF stimulation (200 ng.ml\(^{-1}\)) was not significantly different to the \(Ca^{2+}\) release by ionomycin in cells not exposed to PDGF (388 ± 16 nM, \(n = 5\); compared to 438 ± 84 nM, \(n = 6\), respectively) (Fig. 3.vi. A). In contrast, the elevation of \([Ca^{2+}]_i\), following stimulation with a maximal concentration of methacholine (1 mM) was significantly less than that in the absence of methacholine stimulation (132 ± 22 nM, \(n = 4\); compared to 598 ± 108 nM, \(n = 8\), respectively, \(p<0.001\)) (Fig. 3.vi. B).

During these experiments it was observed that the size of the intracellular ionomycin-sensitive \(Ca^{2+}\) pool significantly decreased in a time-dependent manner when the cells were maintained in \(Ca^{2+}\)-free buffer (388 ± 16 nM, \(n = 5\), at 400 sec compared to 707 ± 48 nM, \(n = 4\), at 50 sec, \(p<0.001\)) (Fig. 3.vi. C). In order to compare ionomycin-mediated elevations of \([Ca^{2+}]_i\), in the presence or absence of agonist prestimulation, ionomycin was added at the same time point (different for each agonist) irrespective of whether the cells had been exposed to agonist (see Fig. 3.vi. A and B).
Fig 3.vi. Determination of the (ionomycin-sensitive) intracellular Ca\(^{2+}\) store content following the addition of A) PDGF (200 ng ml\(^{-1}\)) or B) methacholine (1 mM). Solid lines represent measurements of ionomycin release following agonist and broken lines indicate the application of ionomycin alone. C) Adherent SH-SY5Y cells were incubated in low [Ca\(^{2+}\)]\(_{e}\) for varying times and the content of the intracellular store determined by the addition of ionomycin. Data are mean ± S.E.M. of at least four independent experiments.
Effect of serum starvation on the PDGF and muscarinic receptor-mediated elevation of \([Ca^{2+}]_i\).

Using single-cell Ca\(^{2+}\) imaging on cells maintained in serum: a maximal concentration of PDGF (200 ng.ml\(^{-1}\)) elicited an elevation of \([Ca^{2+}]_i\), with a peak height of 370 ± 18 nM, \(n = 27\) (from 81 cells) in 30% of the cell population measured (see Fig 3.vii. A); and that following removal of PDGF by washing with buffer, 98% of the cells responded to a subsequent challenge with methacholine (1 mM), elevating the \([Ca^{2+}]_i\), to a peak height of 642 ± 21 nM, \(n = 79\) (from 81 cells) (see Fig 3.vii. B).

In contrast, following a 20-hour serum starvation: there was an increase in both the number of cells responding to PDGF (80% compared to 30%) and the magnitude of the peak height of the elevation of \([Ca^{2+}]_i\), (564 ± 24 nM, \(n = 52\) (from 65 cells); compared to 370 ± 18 nM, \(n = 27\) (from 81 cells) maintained in serum, \(p<0.05\)) (see Fig 3.vii. C); and all cells (100%) responded to the subsequent challenge with methacholine, also exhibiting a significant increase in the magnitude of the peak height of the elevation of \([Ca^{2+}]_i\), (771 ± 16 nM, \(n = 65\) (from 65 cells) compared to 642 ± 21 nM, \(n = 79\) (from 81 cells) maintained in serum, \(p<0.05\)) (see Fig 3.vii. D). (\(n\) derived from individual cells in 4 independent experiments under each condition).
**PDGF and muscarinic receptor-mediated elevation of [Ca^{2+}] in the SH-SY5Y\textsubscript{RA6} cell.**

Differentiation of the SH-SY5Y cells with retinoic acid (10 μM) resulted in a much lower cell density as cell growth was inhibited by cell differentiation. Cell morphology alters to a more neuronal phenotype: differentiated SH-SY5Y cells had a smaller, elongated cell body and longer, more numerous neurite outgrowths. The effect of differentiation was then investigated with regard to the PDGF and muscarinic receptor-mediated elevations of [Ca^{2+}], using single cell imaging. This data was compared to that derived from single cell imaging of serum-starved, undifferentiated SH-SY5Y cells.

The muscarinic receptor agonist methacholine (1 mM) evoked an elevation of [Ca^{2+}], in 90% of the SH-SY5Y\textsubscript{RA6} cell population compared to 100% of the serum starved, undifferentiated SH-SY5Y cells. However, the peak height of the methacholine-mediated elevation of [Ca^{2+}], was significantly reduced (492 ± 26 nM, \(n = 36\) (from 40 cells) compared to 771 ± 16 nM, \(n = 65\) (from 65 cells) in undifferentiated SH-SY5Y cells; \(p<0.001\)) (see Fig 3.viii. A).

PDGF-BB (200 ng.mL\(^{-1}\)) elicited a significantly reduced elevation of [Ca^{2+}], and in a significantly reduced cell population. Only 50% of SH-SY5Y\textsubscript{RA6} cells responded compared to 80% of serum starved, undifferentiated counterparts, with a significantly reduced peak height (171 ± 12 nM, \(n = 20\) (from 40 cells) compared to 564 ± 24 nM, \(n = 52\) (from 65 cells) in undifferentiated SH-SY5Y cells; \(p<0.001\)) (see Fig 3.viii. B) (\(n\) derived from individual cells in 3 independent experiments under each condition).
**PDGF and muscarinic receptor-mediated elevation of \([Ca^{2+}]_i\); in hippocampal neurons from 1 day old rats.**

One of the primary goals of this thesis was to investigate and characterise growth factor-mediated signalling in a neuronal context. A recurring question was: how relevant would this be to growth factor-mediated elevations of \([Ca^{2+}]_i\) in a culture of primary neurones? Several attempts to use sympathetic ganglia cells (on the basis that the SH-SY5Y neuroblastoma cell is most like foetal sympathetic ganglia [Ross et al., 1983]) were unsuccessful. The low cell numbers obtained, even from adult rats, were insufficient and unsuitable for either single cell imaging or cell population experiments. The study then turned to young (1 day old post-natal rats), hippocampal cells following a report of a PDGF-mediated elevation of \([Ca^{2+}]_i\) in these primary neurons in culture [Lei et al., 1999]. Due to the heterogeneous nature of neuronal cell preparations, neurons were identified by their ability to depolarise when challenged with 60 mM KCl subsequent to agonist stimulation. Only cells that could depolarise were used to determine the agonist-mediated response.

Both PDGF and muscarinic receptor-mediated elevations of \([Ca^{2+}]_i\) were identified in the rat hippocampal neurones. Methacholine (1 mM) evoked a rapid (<10 sec) and transient elevation of \([Ca^{2+}]_i\), in 76% of the neuron population examined with a peak height of 140 ± 9 nM, n = 19 (from 25 cells) (Fig. 3.ix. A). PDGF-BB (200 ng.ml\(^{-1}\)) stimulation also resulted in a rapid (<10 sec) and transient elevation of \([Ca^{2+}]_i\), in 100% of the neuron population examined with a peak height of 219 ± 16 nM, n = 27 (from 27 cells) (Fig. 3.ix. B) (n derived from individual cells in 2 independent preparations).
Fig 3.vii. Changes in $[\text{Ca}^{2+}]_i$ in SH-SY5Y cells either maintained in serum (A and B) or serum starved for 20 h (C and D) following challenge with methacholine (1 mM: B and D) and PDGF (200 ng.ml$^{-1}$: A and C). Images of fura-2 fluorescence (sample rate, 1.6s) were converted to approximate $[\text{Ca}^{2+}]_i$ as described in Methods, section 2.iii.ii. and 2.iii.iii. Images are peak elevation of $[\text{Ca}^{2+}]_i$, representative of 3 independent experiments.
Fig 3.viii. Changes in $[\text{Ca}^{2+}]_i$ in SH-SY5Y_{R46} cells following challenge with A) methacholine (1 mM) and B) PDGF (200 ng.ml$^{-1}$).

Images of fura-2 fluorescence (sample rate, 1.6s: left hand panels) were converted to approximate $[\text{Ca}^{2+}]_i$ (right hand panels) as described in Methods, section 2.iii.ii. and 2.iii.iii. Images are peak elevation of $[\text{Ca}^{2+}]_i$, representative of 3 independent experiments. Data are mean ± S.E.M. of 36 and 20 cells, respectively, obtained from 3 independent experiments.
Fig 3.ix. Changes in $[\text{Ca}^{2+}]_i$ in hippocampal cell preparations following challenge with A) methacholine (1 mM) and B) PDGF (200 ng.ml$^{-1}$) - lines indicate period of agonist exposure. Images of fura-2 fluorescence (sample rate, 1.6s) were converted to approximate $[\text{Ca}^{2+}]_i$ as described in Methods, section 2.iii.ii. and 2.iii.iii. and representative of 2 independent experiments. Data are mean ± S.E.M. of 25 and 27 cells, respectively, obtained from 2 independent experiments.
3.iii. DISCUSSION

This study demonstrated that an agonist-mediated elevation of [Ca$^{2+}$], was not a general feature of RTK signalling. PDGF-BB stimulation, but not NGF, EGF, bFGF or PDGF-AA, evoked an elevation of [Ca$^{2+}$], that was concentration-dependent, with an apparent EC$_{50}$ of 14 ng.ml$^{-1}$ (0.54 nM). The muscarinic receptor agonist methacholine, also demonstrated an elevation of [Ca$^{2+}$]. Both PDGF-BB and methacholine Ca$^{2+}$ responses were composed of intracellular Ca$^{2+}$ store release and Ca$^{2+}$ influx across the plasma membrane. A significant elevation of the Ca$^{2+}$ response to both agonists occurred when the cells were serum starved rather than maintained in serum.

Protocols resulting in depletion of the intracellular Ca$^{2+}$ store, such as thapsigargin and methacholine prestimulation completely inhibited the elevation of [Ca$^{2+}$], to a subsequent challenge with PDGF-BB. Antagonism of muscarinic receptor signalling with atropine allowed the PDGF-mediated elevation of [Ca$^{2+}$], to return in a time-dependent manner. In contrast, prestimulation with PDGF was unable to ablate a subsequent Ca$^{2+}$ response when challenged with methacholine, although a significant reduction did occur. This heterologous desensitisation was even more apparent in a nominally Ca$^{2+}$-free buffer and did not occur as a consequence of intracellular Ca$^{2+}$ store depletion.

Differentiation of the SH-SY5Y cell resulted in reduced peak elevations of [Ca$^{2+}$], to both PDGF-BB and methacholine. Finally, in answer to the question: how relevant is the mechanism for PDGF-mediated Ca$^{2+}$ signalling in a neuronal context? a PDGF-mediated (and methacholine-mediated) elevation of [Ca$^{2+}$], was identified in primary cultures of rat (1 day old) hippocampal cells.

Initially populations of SH-SY5Y cells in suspension were used but the investigation quickly turned to adherent populations as several lines of evidence suggested the modulation of RTK signalling by integrins. Integrins appear to play a role in mediating proliferative, migratory and anti-apoptotic RTK responses within the cell [Assoian, 1997]. Furthermore, the substrate to which the cells are anchored has been shown to alter the response of the PDGF receptor due to the differential recruitment of signal relay enzymes following ligand-dependent activation [DeMali et al., 1999]. Adherent and suspension populations exhibited no apparent alteration in the profile or magnitude of the [Ca$^{2+}$], response to either PDGF or methacholine (data not shown).
The cells were also serum starved prior to any experimental manipulations as the growth media included growth factor-containing serum that may have desensitised the signalling systems under investigation. The theoretical effect of serum starvation is the synchronisation of all the cells in the G1/G0 phase of the cell cycle as they are prevented from progressing through the cell cycle in the absence of growth factors. The signal transduction mechanisms of trophic factors are likely dependent upon the stage of the cell cycle, and therefore measuring a population of cells in different stages of the cell cycle may produce complicated and complex results.

A possible drawback of this process is that prolonged serum starvation may force the cells into quiescence. Under these conditions not only will the cells respond asynchronously as they re-enter the cell cycle, but also it is rare to achieve 100% return from the quiescent state following subsequent growth factor stimulation, i.e. some cells become 'stuck' in the G1/G0 phase. The SH-SY5Y human neuroblastoma cells are, by definition 'transformed cells' and may not be reliant on growth factors for progression from the G1/G0 phase of growth to the next stage of the cell cycle, however, desensitisation of RTK signalling pathways may still occur. Single cell imaging of \([\text{Ca}^{2+}]_i\) was utilised to determine the relevance of serum starvation in this context.

Although PDGF receptor activation has been previously studied in neuronal cell types, research was often centred on trophic effects due to the potential uses of growth factors in the treatment of neuro-degenerative diseases of the CNS [Lindsay et al., 1994; reviewed in Heldin and Westermark, 1999].

This thesis demonstrates a PDGF-mediated elevation of \([\text{Ca}^{2+}]_i\) in the SH-SY5Y human neuroblastoma cell line and emphasizes the principle of acute intracellular \(\text{Ca}^{2+}\) signalling by PDGF in a neuronal-like context. It also demonstrates that \(\text{Ca}^{2+}\) signalling was not a general feature of classical RTK-mediated signalling in these cells and suggests that the PDGF-mediated elevation of \([\text{Ca}^{2+}]_i\) plays a specific role. Despite the reported expression of the PDGF receptor \(\alpha\)-chain in these cells [Pahlman et al., 1992], the addition of PDGF-AA, which interacts only with this receptor isotype but still recruits PLC\(\gamma\)1 [Gelderloos et al., 1998], failed to produce any elevation in \([\text{Ca}^{2+}]_i\). The subsequent addition of PDGF-BB elicited a normal elevation of \([\text{Ca}^{2+}]_i\), demonstrating that only the
activation of the PDGF receptor β-chain is responsible for the PDGF-mediated elevation of [Ca\(^{2+}\)].

The data presented here are generally consistent with previous reports of muscarinic receptor-mediated elevations in [Ca\(^{2+}\)], elicited via the predominantly muscarinic M\(_3\) receptor isotype [Lambert and Nahorski, 1990; Willars and Nahorski, 1995a; Willars and Nahorski, 1995b]. In contrast to this prior work, the current study used serum starved cells to enable comparisons between the RTK and GPCR signalling, and there is some evidence of an effect of serum starvation on muscarinic receptor-mediated Ca\(^{2+}\) signalling within these experimental paradigms (discussed later).

Muscarinic and PDGF receptor-mediated elevations in [Ca\(^{2+}\)], were composed of both Ca\(^{2+}\) release from an intracellular store and Ca\(^{2+}\) influx from the extracellular medium. The intracellular Ca\(^{2+}\) release component of the response to PDGF originated from a thapsigargin-sensitive intracellular Ca\(^{2+}\) store that is also mobilized by activation of G\(\alpha_q\)-coupled muscarinic receptors. Similar observations have been made with regard to combinations of G\(\alpha_q\)-coupled GPCRs that are co-expressed on individual cells [Willars and Nahorski, 1995b].

The PDGF-mediated elevation of [Ca\(^{2+}\)], was also examined following antagonism of the muscarinic receptor-mediated elevation of [Ca\(^{2+}\)], with atropine. The magnitude of the PDGF-mediated elevation of [Ca\(^{2+}\)], under these conditions was temporally relative to the interval between atropine and PDGF addition and is consistent with the model of capacitative Ca entry [Putney, 1986; Irvine, 1990; Berridge, 1995; Mikoshiba, 1997]. Muscarinic receptor prestimulation most likely inhibits subsequent PDGF challenge at the level of Ca\(^{2+}\) as a consequence of the depletion of intracellular Ca\(^{2+}\) stores. Time is required for the refilling of the rapidly releasable intracellular Ca\(^{2+}\) store following antagonism of the muscarinic receptor-mediated response with atropine. Under conditions of store depletion, agonist-induced elevations in [Ca\(^{2+}\)], are, therefore, dependent on the rate of store refilling [Putney and Bird, 1993; Willars and Nahorski, 1995b].

Given that muscarinic receptor prestimulation abolishes PDGF-mediated elevation of [Ca\(^{2+}\)], we also would predict that muscarinic receptor-mediated signalling would be attenuated if initiated during an ongoing PDGF-mediated response. In the presence of 1.3 mM [Ca\(^{2+}\)], the addition of methacholine was at
the peak of the PDGF response, at which point the shared intracellular Ca\(^{2+}\) store could be expected to be maximally depleted by PDGF. This may account for the significantly reduced elevation of [Ca\(^{2+}\)], in response to a subsequent challenge with methacholine. This interpretation was further supported by repeating the experiment in a nominally Ca\(^{2+}\) free buffer. There has been a report of mutual inhibition between muscarinic and EGF receptors in the SH-SY5Y cell which suggested a novel mechanism for the integration of multiple signals in a neuronal context. However, this mutual inhibition was not at the level of a shared signalling component, but as a result of distinct receptor-mediated mechanisms and subsequent effects on the tyrosine phosphorylation of receptor-specific proteins [Zhang and Jope, 1999].

These results suggested that PDGF-mediated increases in [Ca\(^{2+}\)], originated from a thapsigargin-sensitive, non-mitochondrial, intracellular Ca\(^{2+}\) store that is also released by activation of muscarinic receptors. They also highlight that in SH-SY5Y cells, PDGF-induced Ca\(^{2+}\) release and entry mechanisms were unlikely to be independent mechanisms as under conditions of prior Ca\(^{2+}\) store depletion, in the presence of 1.3 mM [Ca\(^{2+}\)], PDGF was unable to elevate [Ca\(^{2+}\)]. These data are in contrast to previous reports, in other cell types, which have suggested PDGF-mediated Ca\(^{2+}\) entry mechanisms that are independent of associated store release [Huang et al., 1991; Estacion and Mordan, 1993b; Ma et al., 1996; Mathias et al., 1997].

Based on the results of this study, the current hypothesis would be: in the SH-SY5Y cell, intracellular Ca\(^{2+}\) store release is a prerequisite for Ca\(^{2+}\) entry across the plasma membrane following PDGF stimulation. Methacholine blocks any subsequent PDGF mediated elevation in [Ca\(^{2+}\)], presumably by fully depleting a shared intracellular Ca\(^{2+}\) store, whereas PDGF attenuates but does not abolish muscarinic receptor-mediated signalling most likely because it is unable to fully deplete this Ca\(^{2+}\) pool.

The ability of PDGF to reduce the methacholine-mediated elevation of [Ca\(^{2+}\)], appeared disproportionate to its limited ability to release intracellular Ca\(^{2+}\) if depletion of a shared Ca\(^{2+}\) store were responsible. However, this argument assumes that the relatively small PDGF-mediated [Ca\(^{2+}\)], increase accurately reflected limited Ca\(^{2+}\) mobilization. Thus, elevation of [Ca\(^{2+}\)], is a net balance between influx to and efflux from the cytosol. If the slower release by PDGF can be dealt with
more effectively than the rapid release by methacholine, or PDGF is able to activate extrusion mechanisms more effectively than methacholine, measuring global elevation of \([\text{Ca}^{2+}]\), may underestimate the true (or relative) extent of PDGF-mediated Ca\(^{2+}\) release from the Ca\(^{2+}\) store.

Whilst the single-cell Ca\(^{2+}\) imaging experiments demonstrated more asynchronous responses to PDGF than the uniform rapid responses to methacholine, these data also suggested that methacholine (1 mM) markedly depletes the intracellular Ca\(^{2+}\) store whereas PDGF did not. This was in agreement with ionomycin-mediated release of the agonist-sensitive intracellular Ca\(^{2+}\) store that also suggested activated muscarinic receptors abolish the PDGF-mediated elevation of [Ca\(^{2+}\)], by depleting a shared intracellular Ca\(^{2+}\) pool.

However, this mechanism did not appear responsible for the significant reduction of the muscarinic receptor-mediated elevation of [Ca\(^{2+}\)], following PDGF prestimulation, as PDGF-mediated Ca\(^{2+}\) store release has no significant effect on Ca\(^{2+}\) store content. The precise mechanisms underlying this inhibitory GPCR-RTK cross talk remains to be established. However, these results imply regulation of a protein upstream of Ca\(^{2+}\) release that is either common to both GPCR and RTK Ca\(^{2+}\) signalling pathways, or undergoes heterologous desensitisation following PDGF stimulation. Indeed, based on results contained within this thesis, a possible explanation is discussed later (see Chapter 5: Discussion).

The PDGF-mediated elevation of [Ca\(^{2+}\)]\(i\) was asynchronous in both serum maintained and serum starved cells, in agreement with a previous report [Renard et al., 1992]. However, the significant increase in the number of cells responding following serum starvation was suggestive of a desensitised signalling pathway in serum maintained cells. Alternatively, the PDGF-mediated elevation of [Ca\(^{2+}\)]\(i\) may be cell cycle-dependent and alignment in the Gi/G0 growth phase was responsible for the increased cell number that responded to agonist.

Serum starvation had an unexpected effect on the muscarinic receptor-mediated Ca\(^{2+}\) signalling and resulted in a significant increase in the magnitude of the methacholine-mediated elevation of [Ca\(^{2+}\)]\(i\), as compared to cells maintained in serum. This suggested a component of serum was desensitising muscarinic receptor-mediated Ca\(^{2+}\) signalling. Was this another example of cross talk between RTKs and GPCRs? If desensitisation was not occurring at the level of receptor, were serum and prestimulation with PDGF recruiting the same mechanism to
desensitise muscarinic receptor signalling? Alternatively, if the mechanisms were
different, why would serum desensitise Ca\textsuperscript{2+} signalling via this GPCR?

Muscarinic receptor activation has a known mitogenic effect in the SH-
SY5Y cells [Pam White – personal communication]. Serum-dependent
desensitisation may therefore occur to inhibit the trophic effects of this receptor.

In an effort to investigate the PDGF signalling in a more neuronal-like cell,
the SH-SY5Y cells were differentiated with retinoic acid. This produces a cell that
has a more neuronal phenotype, but unfortunately, both the methacholine and
PDGF-mediated elevations of [Ca\textsuperscript{2+}], were significantly diminished following this
treatment.

In contrast to the results of this study, a comparative study between SH-
SY5Y and SH-SY5Y\textsubscript{RA6} cells reported no change in the methacholine-mediated
elevation of [Ca\textsuperscript{2+}], following differentiation. These results were based on cell
population measurements, not single cells, and in spite of an approximate doubling
of muscarinic receptor and an associated elevation of Ins(1,4,5)P\textsubscript{3} generation
[Martin \textit{et al.}, 1999].

PDGF-dependent PI-3K signalling, as measured by the phosphorylation and
activation of Akt isoforms has also been reported as suppressed following 3T3-L1
adipocyte differentiation [Summers \textit{et al.}, 1999], but whether this is relevant to
other signalling pathways recruited by the PDGF receptor is unclear. PI-3K
activation results in PtdIns(3,4,5)P\textsubscript{3} generation, an attachment site for PLC-\gamma
and whilst the existence of PI-3K is well documented in SH-SY5Y cells [Lavie and
Agranoff, 1996; Encinas \textit{et al.}, 1999; Kurihara \textit{et al.}, 2000], this may only be
relevant in the context of this study if the PDGF-mediated elevation of [Ca\textsuperscript{2+}], is
dependent upon PLC-\gamma1.

There are a multitude of possible explanations and mechanisms, such as:
reduced receptor numbers following differentiation and the alteration in the
expression levels of one or more integral signal transduction components. The
definition of the exact mechanism is beyond the scope of this study and may be
attributable to a combined effect following differentiation. For example, expression
levels of both PLC and PKC appeared to alter in the SH-SY5Y\textsubscript{RA6} cell (see
Appendix A) and in conjunction with a reduced level of PDGF receptor expression,
which was reported in the 3T3-L1 cells following differentiation [Vaziri and Faller, 1996], may be entirely responsible for these findings.

Whether this effect of retinoic acid was indicative that, in a ‘real’ neuron, the PDGF-mediated elevation of [Ca$^{2+}$], would be less significant, was examined by investigating the PDGF response in a primary culture of neurons. Stimulation of both PDGF and muscarinic receptors in primary cultures of rat hippocampal neurons evoked rapid elevations of [Ca$^{2+}$]. Glial cell population was originally a matter for concern as these cells also express PDGF receptors and are inseparable from the neuronal cells during culture. Their numbers were severely limited however by adding mitogenic inhibitors simultaneous with cell plating. These data confirm the recent observation that PDGF can elevate [Ca$^{2+}$] in these cells [Lei et al., 1999].

Having identified a PDGF-mediated elevation of [Ca$^{2+}$], in the SH-SY5Y human neuroblastoma cell line, the next logical step was to characterise the mechanism for the Ca$^{2+}$ release from the intracellular Ca$^{2+}$ store. The proposed second messenger-mediated signalling pathway, based primarily on studies in cells of mesenchymal origin [Renard et al., 1992; Zhang et al., 1996; Miyakawa et al., 1998], was Ins(1,4,5)P$_3$ generation (following PLC-γ1 recruitment), acting via the Ins(1,4,5)P$_3$ receptor.
4: CHARACTERISATION OF THE MECHANISM FOR THE PDGF-MEDIATED ELEVATION OF [Ca$^{2+}$].

4.i. INTRODUCTION

With an increasing interest not only in the well-reported chronic effects of RTK action, but also in the recently identified acute effects of ligand-activated RTKs, particularly in neuronal systems, the understanding of RTK-mediated acute Ca$^{2+}$ signalling may be of greater import than was previously assumed. Thus, following the identification of a PDGF-mediated elevation of [Ca$^{2+}$], in the SH-SY5Y cell line (see Chapter 3), characterisation of the mechanism was the subsequent goal and is the subject of this second results chapter.

A PDGF-mediated elevation in [Ca$^{2+}$] is generally attributed to the activation of PLC-$\gamma$ [Lopez-Rivas et al., 1987; Kim et al., 1991]. PDGF-mediated Ins(1,4,5)P$_3$ generation or inositol monophosphate accumulation against a Li$^+$ block has been successfully demonstrated in a variety of cell types, most often mesenchyme-derived and including: HepG2 cells [Rameh et al., 1998]; Swiss 3T3 fibroblasts [Miyakawa et al., 1998]; WFB fibroblasts [Hasegawa-Sasaki et al., 1988]; Cos-1 cells [Falasca et al., 1998], NIH 3T3 fibroblasts [Bae et al., 1997]; N1E-115 neuroblastoma cells [Zhang et al., 1996]; and mouse embryo fibroblasts [Hess et al., 1998].

In contrast, a muscarinic receptor-mediated elevation in [Ca$^{2+}$] is attributed to PLC-$\beta$ activation via pertussis toxin-insensitive G proteins of the $G_{q/11}$ family [Lambert and Nahorski, 1990; Rhee and Choi, 1992; Lee and Rhee, 1995] and Ins(1,4,5)P$_3$-mediated Ca$^{2+}$ signalling is a well characterised general feature of these receptors, not predominating in any one cell type.

PLC activation results in the hydrolysis of the membrane lipid PtdIns(4,5)P$_2$, producing diacylglycerol, an activator of many PKC isoforms, and Ins(1,4,5)P$_3$ which diffuses through the cell to interact with the Ins(1,4,5)P$_3$-sensitive calcium release channels [Berridge et al., 1987; Putney and Bird, 1993]. Activation of PKC isoforms results in phosphorylation events within the cell that are involved in the regulation of many cellular processes [Newton, 1997]. If PLC action is indeed responsible for Ca$^{2+}$ signalling following ligand-dependent activation of neuronal PDGF and muscarinic receptors, then it is reasonable to assume that the generation of second messengers could be identified.
During the course of this study, it became increasingly apparent that the mechanism utilised by PDGF in elevating intracellular Ca\textsuperscript{2+} in the SH-SY5Y cell was not as obvious or easily defined as the literature would suggest. Strategies that investigated second messenger production and possible inhibition or enhancement were initially employed. As the wealth of evidence in the current literature suggested an Ins(1,4,5)P\textsubscript{3}-mediated mechanism for PDGF, alternative strategies relied on the down regulation, inhibition or sensitisation of the second messenger target, the Ins(1,4,5)P\textsubscript{3} receptor.

The results of all these investigations are presented and discussed within this chapter and together indicate that, in contrast to the muscarinic receptor, the PDGF receptor-mediated mechanism for the elevation of [Ca\textsuperscript{2+}] is novel and independent of Ins(1,4,5)P\textsubscript{3}.

The G-protein coupled bradykinin (B\textsubscript{2}) and LPA receptors have also been identified in the SH-SY5Y cells and although activation of either receptor results in a rapid, yet transient, elevation of [Ca\textsuperscript{2+}], they are thought to utilise different mechanisms. The bradykinin receptor is reported as PLC-linked following activation and increases cytosolic Ca\textsuperscript{2+} following Ins(1,4,5)P\textsubscript{3}-mediated intracellular Ca\textsuperscript{2+} store gating [McDonald et al., 1994; Willars and Nahorski, 1995a; Willars and Nahorski, 1995b]. Compared to the muscarinic receptor, bradykinin-mediated Ins(1,4,5)P\textsubscript{3} generation exhibits a significantly reduced peak height and no sustained phase in SH-SY5Y cells [Willars and Nahorski, 1995a]. In contrast, LPA has been demonstrated as Ins(1,4,5)P\textsubscript{3}-independent and may utilise instead the novel sphingolipid second messenger, sphingosine-1-phosphate (S-1-P) [Young et al., 1999; Young et al., 2000a]. Both bradykinin and LPA receptors were useful tools within the context of this study by providing endogenous internal controls for the muscarinic receptor population of SH-SY5Y cells that evoke a robust, and sustained, elevation of global Ins(1,4,5)P\textsubscript{3}.

It is important that we thoroughly understand the mechanism of the PDGF-mediated elevation in [Ca\textsuperscript{2+}], within a neuronal system if this rapidly released messenger is to play a role in modulating acute responses within the cell (see section 1.v.i.). However, the nature of signalling mechanisms may indicate that phosphoinositide and Ca\textsuperscript{2+} signalling mechanisms already established in mesenchyme-
derived cells may be specific, and therefore specialised cell types - such as neurons - may have distinct and as yet undefined mechanisms.
4.ii. RESULTS

*Activation of muscarinic receptors but not PDGF receptors results in enhanced Ins(1,4,5)P_3 generation.*

Initially it was determined whether PDGF receptor stimulation of SH-SY5Y cells was associated with enhanced phosphoinositide metabolism.

The PDGF-mediated (200 ng.ml⁻¹) effect on Ins(1,4,5)P₃ generation was similar to that of vehicle-treated cells, demonstrating no PDGF-mediated increase in global Ins(1,4,5)P₃ (determined by two way ANOVA). In contrast, stimulation with methacholine (1 mM) caused a robust and reproducible elevation in Ins(1,4,5)P₃. Muscarinic receptor activation evoked a peak at 10-20 seconds (peak: 159 ± 8 pmol.mg protein⁻¹, n = 3; basal: 39 ± 5 pmol.mg protein⁻¹, n = 3), followed by a sharp decline, then a second rising phase (peaking at 40-120 seconds) before a gradual decline to a sustained elevated phase (97 ± 11 pmol.mg protein⁻¹, n = 3) (Fig. 4.i.A).

Although the shape of this profile was consistent between individual experiments, the time of the second rising phase varied slightly between experiments. Thus mean data from a number of experiments do not accurately reflect the profile of the response.

Repeating the experiments without serum starvation verified that this protocol was the probable source of these dissimilarities. In two separate experiments where the cells were maintained in serum, Ins(1,4,5)P₃ generation peaked at 10-20 seconds (461 and 564 pmol.mg protein⁻¹) and declined to a sustained elevated plateau (198 and 158 pmol.mg protein⁻¹, respectively) (Fig. 4.i.B).
Fig. 4.i. A) Temporal profile of Ins(1,4,5)P\(_3\) generation following 1 mM methacholine (□), 200 ng.m\(^{-1}\) PDGF (▲), or vehicle (■) in serum-starved SH-SY5Y cells. For PDGF and vehicle, data are mean ± S.E.M. (n = 3). The muscarinic receptor-mediated response is representative of 4 independent experiments. B) Ins(1,4,5)P\(_3\) generation following 1 mM methacholine in cells either maintained in serum (■) or serum starved (□). Data are representative of 2 and 4 independent experiments respectively.
Serum starvation does not alter the number of cell surface muscarinic receptors.

The number of cell surface muscarinic receptors was investigated in cells that were exposed to serum or serum starved, using the radiolabelled muscarinic antagonist, [³H]-N-methylscopolamine (NMS). The maximum binding of [³H]-NMS to SH-SY5Y cells was not significantly different in cells that had been maintained in serum or serum starved for 20 hours (335 ± 17 fmol.mg protein⁻¹, n = 4; and 318 ± 70 fmol.mg protein⁻¹, n = 4, respectively). There was no significant difference in the Kd values (-9.75 ± 0.33, log_{10} M, n = 4; compared to -9.88 ± 0.08, log_{10} M, n = 4, respectively) (Fig.4.ii.).

![Graph showing specific binding of [³H]-NMS to SH-SY5Y cells](image)

**Fig. 4.ii.** Specific binding of [³H]-NMS to SH-SY5Y cells either serum starved for 20 hours (□) or maintained in serum (■). Data are mean ± S.E.M. (n = 4).
The effect of PKC and sphingosine kinase inhibitors on muscarinic and PDGF receptor-mediated elevations of Ins(1,4,5)P$_3$.

PDGF and muscarinic receptor-mediated production of Ins(1,4,5)P$_3$ was examined in the presence of the Roche compound (Ro31-8220, 10 μM), to inhibit PKC, or DMS (30μM) to inhibit sphingosine kinase (10 minute pre-treatment).

The profile of PDGF-mediated (200 ng.ml$^{-1}$) Ins(1,4,5)P$_3$ production was similar to that of Ro31-8220, DMS and vehicle-treated cells, indicating no significant PDGF-mediated elevation of global Ins(1,4,5)P$_3$, as determined by two way ANOVA (Fig. 4.iii.A).

In contrast, stimulation with methacholine (1 mM) caused a robust and reproducible elevation in Ins(1,4,5)P$_3$. Muscarinic receptor activation evoked a peak of Ins(1,4,5)P$_3$ production at 10-20 seconds (peak: 131 ± 7 pmol.mg protein$^{-1}$, n = 3) that exhibited a small, yet significant, depression following either Ro31-8220 (98 ± 5 pmol.mg protein$^{-1}$, n = 3, p<0.05) or DMS (91 ± 6 pmol.mg protein$^{-1}$, n = 3, p<0.05) pre-treatment. However, there were no significant differences in the temporal profiles of Ins(1,4,5)P$_3$ generation in either the presence or absence of the inhibitors (determined by two way ANOVA) (Fig. 4.iii.B).
Fig 4.iii. Temporal profile of Ins(1,4,5)P$_3$ generation following either PDGF (200 ng ml$^{-1}$) or methacholine (1 mM) in the absence or presence of a 10 minute pre-incubation with either Ro31-8220 (10 μM), A) and B) or DMS (30 μM), C) and D), respectively. All data are mean ± S.E.M. (n = 3).
Activation of muscarinic receptors but not PDGF receptors results in enhanced phosphoinositide metabolism.

In response to either vehicle (KHB) or PDGF (200 ng.ml\(^{-1}\)) there was no increase in \(^{3}\text{H}\)-InsP\(_x\) over a 20 min time period in the presence of Li\(^+\) (10 mM, see methods section 2.iii.vi.). In contrast, there was a robust response to methacholine (1 mM) (2756 ± 179%, \(n = 6\); over basal by 20 min) (Fig. 4.iv.A).

The agonist potency for [Ca\(^{2+}\)]\(_{e}\) elevation is often greater than that for Ins(1,4,5)P\(_3\) [Willars and Nahorski, 1995a]. Therefore, the accumulation of \(^{3}\text{H}\)-InsP\(_x\) was repeated with a concentration of PDGF that was supra-maximal for elevation of [Ca\(^{2+}\)], (1 \(\mu\)g.ml\(^{-1}\), 40 nM). Even at this elevated concentration there was no accumulation of \(^{3}\text{H}\)-InsP\(_x\) (Fig 4.iv.B).
Fig. 4.iv. A) Time course of the accumulation of $[^3]H$-InsP$_x$ in adherent, serum-starved, Li$^+$-blocked SH-SY5Y cells in response to 1 mM methacholine ($\triangle$), 200 ng.ml$^{-1}$ PDGF ($\triangledown$), or vehicle ($\blacksquare$). B) $[^3]H$-InsP$_x$ accumulation under the same conditions in response to 1 mM methacholine ($\bullet$), 1 $\mu$g.ml$^{-1}$ PDGF ($\triangle$), or vehicle ($\blacksquare$). Data are mean ± S.E.M. ($n \geq 3$).
The potential effect of the Ins(1,4,5)P₃ receptor inhibitor, heparin, on PDGF-mediated ⁴⁵Ca²⁺ release in permeabilised SH-SY5Y cells.

Initially the temporal release of ⁴⁵Ca²⁺ from permeabilised SH-SY5Y cells (see section 2.iii.viii.) was investigated to ascertain an optimal time point for PDGF-mediated Ca²⁺ release from intracellular stores in a permeabilised cell system.

Determinations of ⁴⁵Ca²⁺ release were taken over a 20 min time course following agonist addition. At times up to and including 15 min, Ins(1,4,5)P₃ (25 μM) and muscarinic receptor activation (methacholine, 1 mM) consistently released approximately 70% and 50%, respectively, of the ionomycin-releasable intracellular Ca²⁺ store. In contrast, PDGF (200 ng.ml⁻¹) stimulation exhibited a temporally related increase in ⁴⁵Ca²⁺ release, from 0% at 2 min, to a maximum of 16 ± 2% (n = 3) at 15 min. Interestingly, at 20 min there was a marked reduction, compared to 15 min, of the ability of Ins(1,4,5)P₃ (59 ± 1%, n = 3; compared to 77 ± 1% at 15 min, n = 3), methacholine (22 ± 4%, n = 3; compared to 55 ± 2% at 15 min, n = 3) and PDGF (1 ± 1%, n = 3; compared to 16 ± 2% at 15 min, n = 3) to release ⁴⁵Ca²⁺ from the pre-loaded intracellular Ca²⁺ store (Fig. 4.v.A).

This study demonstrates that maximum ⁴⁵Ca²⁺ release in the presence of PDGF occurred at 15 min. Our findings are also in agreement with the previously reported optimal time for measuring muscarinic receptor-mediated release at 2 min in permeabilised SH-SY5Y cells [Willars et al., 1996]. Therefore, the effect of heparin (IC₅₀ 5-15 μg.ml⁻¹ [Michelangeli et al., 1995]), a membrane-impermeant, competitive Ins(1,4,5)P₃ receptor inhibitor, on agonist-mediated ⁴⁵Ca²⁺ release was investigated at two different inhibitor concentrations (50 μg.ml⁻¹ and 100 μg.ml⁻¹) at the optimal times for each agonist.

Heparin (50 μg.ml⁻¹) significantly inhibited both methacholine (32 ± 4%, n = 5; compared to 60 ± 4% in control cells at 2 min, n = 10; 47% inhibition; p<0.05) and PDGF-mediated (7 ± 1%, n = 4; compared to 14 ± 3% in control cells at 15 min, n = 9; 50% inhibition; p<0.05) ⁴⁵Ca²⁺ release from the ionomycin-sensitive intracellular Ca²⁺ store (Fig. 4.v.B).
At an increased heparin concentration (100 μg.ml\(^{-1}\)) there was no significant effect of heparin on the ability of PDGF to release \(^{45}\)Ca\(^{2+}\) from the intracellular store (20 ± 9%, n = 4; compared to 14 ± 3% in control cells at 15 min, n = 9). Even more surprisingly, this concentration of heparin was unable to completely inhibit muscarinic receptor-mediated \(^{45}\)Ca\(^{2+}\) release. Although significant inhibition still occurred compared to control (27 ± 3%, n = 5; compared to 60 ± 4% in control cells at 15 min, n = 10; 55% inhibition; p<0.05), there was no significant difference in the inhibition between heparin at either 50 μg.ml\(^{-1}\) or 100 μg.ml\(^{-1}\) (32 ± 4%, n = 5; compared to 27 ± 3%, n = 5, respectively) (Fig. 4.v.B).
**Fig. 4.v.** A) The temporal profile of Ins(1,4,5)P₃ (25 μM), methacholine (1 mM) and PDGF-mediated (200 ng.ml⁻¹) ⁴⁵Ca²⁺ release in permeabilised SH-SY5Y cells. B) Agonist-mediated ⁴⁵Ca²⁺ release at optimal, agonist-specific time points in the absence (control) or presence of heparin (+ 50 μg.ml⁻¹; ++ 100 μg.ml⁻¹). All data are mean ± S.E.M. (n ≥ 3) (*p<0.05).
Caffeine-mediated inhibition of the Ins(1,4,5)P₃ receptor.

Although a known activator of ryanodine-sensitive Ca²⁺ channels, caffeine at high concentration (40 mM) has been suggested to inhibit the Ins(1,4,5)P₃ receptor, non-competitively, by a mechanism that alters channel opening rather than ligand binding [Michelangeli et al., 1995 and references therein].

Using adherent populations of SH-SY5Y neuroblastoma cells, this study investigated caffeine-mediated Ins(1,4,5)P₃ receptor blockade, following PDGF and muscarinic receptor stimulation. Experiments were performed in both the presence of 1.3 mM [Ca²⁺]ₑ (Fig. 4.vi.A) and also in the absence of an extracellular Ca²⁺ gradient (~100nM, EGTA-buffered) (Fig. 4.vi.B).

Caffeine (40 mM) completely inhibited muscarinic receptor-mediated elevations of [Ca²⁺], evoked by a sub-maximal concentration of methacholine (1 μM: 0 nM, n = 3; compared to 296 ± 34 nM, n = 3, in the absence of caffeine (40 mM); 100% inhibition) and resulted in a significant reduction of the response to a maximal concentration of methacholine (1 mM: 257 ± 28 nM, n = 3; compared to 472 ± 33 nM, n = 3, in the absence of caffeine (40 mM); 46% inhibition; p<0.01). Contrastingly, at both maximal (200 ng.ml⁻¹) and sub-maximal (20 ng.ml⁻¹) PDGF concentrations, despite an apparent reduction (39% and 42%, respectively) of the PDGF-mediated elevation of [Ca²⁺], statistical analysis (unpaired, two-tailed, Student’s t-test assuming unequal variances) determined no significant reduction in the presence of caffeine at either concentration (Fig. 4.vi.A).

In the absence of extracellular Ca²⁺, caffeine (40 mM) significantly inhibited intracellular Ca²⁺ store release when cells were stimulated with a concentration of bradykinin (1 μM) that is maximal for elevation of [Ca²⁺], (175 ± 15 nM, n = 3; compared to 269 ± 11 nM, n = 3, in the absence of caffeine (40 mM); 35% inhibition; p<0.01). This GPCR agonist is reported to generate only a small amount of Ins(1,4,5)P₃ in the SH-SY5Y neuroblastoma cell [Willars and Nahorski, 1995a; Martin et al., 1999]. Under these conditions, caffeine had no significant effect on the Ca²⁺ release following methacholine (1 mM) stimulation (267 ± 33 nM, n = 4; compared to 205 ± 22 nM, n = 4, in the absence of caffeine (40 mM)), but
significantly inhibited intracellular Ca\textsuperscript{2+} release in response to a maximal (200 ng.ml\textsuperscript{-1}) concentration of PDGF (63 ± 7 nM, n = 4; compared to 106 ± 14 nM, n = 4, in the absence of caffeine (40 mM); 41% inhibition; p<0.05) (Fig. 4.vi.B).
Fig. 4 vi. Elevations of $[Ca^{2+}]_i$ in the absence (-) or presence (+) of caffeine (40 mM) following challenge of SH-SY5Y cells with A) methacholine (1 μM and 1 mM) or PDGF (20 ng.ml$^{-1}$ and 200 ng.ml$^{-1}$) in the presence of 1.3 mM $[Ca^{2+}]_o$. B) Similar responses to bradykinin (1 μM), methacholine (1 mM) and PDGF (200 ng.ml$^{-1}$) in the absence of extracellular $Ca^{2+}$. Data are mean ± S.E.M., elevations above basal $Ca^{2+}$ ($n$ ≥ 3) (*$p$<0.05, **$p$<0.01).
**PDGF-mediated Ca^{2+} signalling is unaffected by down-regulation of the Ins(1,4,5)P_3 receptor.**

SH-SY5Y cells were treated with 1 mM methacholine for the final 7 hours of their serum starvation period, including the period of fura-2 loading, to facilitate Ins(1,4,5)P_3 receptor down-regulation (see section 2.iii.ix.) [Wojcikiewicz et al., 1994a].

It was predicted that stimulation of muscarinic receptors for 7 hours would cause a marked homologous desensitisation of subsequent muscarinic receptor-mediated responses. Indeed, [Ca^{2+}], elevations in response to both maximal (1 mM) and sub-maximal (1 μM) concentrations of methacholine were substantially reduced by 7 hours pre-treatment with 1 mM methacholine (1 mM: 96 ± 8 nM, n = 6; compared to 495 ± 14 nM in control cells, n = 3; p<0.01 and 1 μM: 77 ± 20 nM, n = 4; compared to 404 ± 80 nM in control cells, n = 3, p<0.01).

The extended methacholine pre-treatment also abolished elevation of [Ca^{2+}], in response to 10 μM bradykinin (0 nM, n = 3 compared to 123 ± 17 nM, n = 6, in control cells). Under these conditions the elevation of [Ca^{2+}], following a maximal (for elevation of [Ca^{2+}]), concentration of PDGF (200 ng.ml⁻¹) was unaffected (74 ± 8 nM, n = 4; compared to 63 ± 8 nM, n = 5, in methacholine pre-treated and control cells, respectively) (Fig 4.vii.).
Fig. 4.vii. Following a preliminary incubation (7 hours) in the absence (solid bars) or presence (hatched bars) of 100 μM methacholine to down-regulate the Ins(1,4,5)P3 receptor, SH-SYSY cells were challenged with either methacholine (1 mM or 1 μM), PDGF (200 ng.ml⁻¹) or bradykinin (10 μM). Data are mean ± S.E.M., elevations above basal Ca²⁺ (n ≥ 3) (**p<0.001, *p<0.01).
**Ins(1,4,5)P₃ receptor down-regulation does not result in down-regulation of key signalling molecules.**

Extended exposure to muscarinic receptor agonist (100 μM methacholine for 7 hours) facilitated down-regulation of the Ins(1,4,5)P₃ receptor. The integrity of other key signal transduction components was examined semi-quantitatively to ensure this pre-treatment had not compromised the signalling pathway elsewhere by protein down-regulation.

Immunoblotting SH-SY5Y whole cell extract (Fig. 4.viii.) and subsequent densitometric analysis revealed that, with the exception of the Ins(1,4,5)P₃ receptor, the protein levels of other key GPCR and RTK signalling molecules such as: the G₉1₁ G-protein; PLC-β₁ and -β₃; and the PDGF receptor β-chain and PLC-γ₁, respectively; were all unchanged (see Table II).

<table>
<thead>
<tr>
<th>Protein</th>
<th>KHB</th>
<th>Methacholine (100 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G₉₁₁ G protein</td>
<td>0.651 ± 0.074</td>
<td>0.669 ± 0.080</td>
</tr>
<tr>
<td>Ins(1,4,5)P₃ receptor</td>
<td>0.648 ± 0.033</td>
<td>*0.056 ± 0.013</td>
</tr>
<tr>
<td>PDGF-β receptor</td>
<td>0.374 ± 0.096</td>
<td>0.366 ± 0.061</td>
</tr>
<tr>
<td>PLC-β₁</td>
<td>0.500 ± 0.059</td>
<td>0.574 ± 0.079</td>
</tr>
<tr>
<td>PLC-β₃</td>
<td>1.116 ± 0.087</td>
<td>1.166 ± 0.013</td>
</tr>
<tr>
<td>PLC-γ₁</td>
<td>1.435 ± 0.137</td>
<td>1.466 ± 0.198</td>
</tr>
</tbody>
</table>

**Table II:** Semi-quantitative analysis of protein by densitometer following either vehicle (KHB) or methacholine (100 μM) pre-treatment. Data are mean ± S.E.M., densitometric units (n ≥ 3) (*p ≤ 0.01).
Fig. 4.viii. Immunoblotting SH-SYSY whole cell extract demonstrated that following a 7 hour pre-treatment with either vehicle (-) or 100 μM methacholine (+), only the protein level of the Ins(1,4,5)P$_3$ receptor was markedly reduced within the SH-SYSY cell. Data shown are representative immunoblots from at least 3 independent experiments.
**PDGF-mediated Ca^{2+} signalling is unaffected by sensitisation of the Ins(1,4,5)P receptor by thimerosal.**

These experiments were performed, initially, in the presence of 1.3 mM [Ca^{2+}]	ext{e} and then repeated in the absence of extracellular Ca^{2+} (EGTA-buffered, ~100 nM). The presence of extracellular Ca^{2+} may complicate the results (see Discussion), therefore removing it allows the direct examination of agonist-mediated release from intracellular Ca^{2+} stores.

In the presence of extracellular Ca^{2+}, thimerosal (5 μM) significantly increased the elevation of [Ca^{2+}]\text{i} in response to submaximal concentrations of methacholine (1 μM: 541 ± 44 nM, n = 3; compared to 198 ± 24 nM, n = 3, in the absence of thimerosal, p<0.001) and PDGF (20 ng.ml\textsuperscript{-1}: 267 ± 24 nM, n = 3; compared to 100 ± 4 nM, n = 3, in the absence of thimerosal, p<0.01). In contrast, there was no significant increase in the elevation of [Ca^{2+}]\text{i} following stimulation with a submaximal concentration of the GPCR agonist, LPA (50 nM: 270 ± 34 nM, n = 3; compared to 198 ± 12 nM, n = 3, in the absence of thimerosal.) (Fig. 4.ix.A)

The thimerosal-mediated enhancement of PDGF-evoked elevations of [Ca^{2+}]\text{i} in the presence of 1.3 mM [Ca^{2+}]	ext{e} was concentration-dependent. At a sub-maximal PDGF concentration of 20 ng.ml\textsuperscript{-1}, increasing concentrations of thimerosal (0-5 μM) resulted in a graduated elevation of [Ca^{2+}]\text{i} following agonist stimulation (Fig. 4.ix.B).

However, in the absence of extracellular Ca^{2+}, thimerosal had no effect on the PDGF (20 ng.ml\textsuperscript{-1})-evoked elevation of [Ca^{2+}]\text{i} over basal levels (50 ± 7 nM, n = 3; compared to 48 ± 3 nM, n = 3, in the absence of thimerosal). There was still a significant increase in the elevation of [Ca^{2+}]\text{i} above basal levels in response to a submaximal (1 μM) concentration of methacholine (399 ± 26 nM, n = 3; compared to 213 ± 23 nM, n = 3, in the absence of thimerosal, p<0.01) (Fig. 4.x.).
Fig. 4.ix. A) Peak elevations of $[Ca^{2+}]_i$ following challenge with sub-maximal concentrations of methacholine (1 μM), LPA (50 nM) and PDGF (20 ng.ml$^{-1}$) in the absence (solid bars) or presence (hatched bars) of thimerosal (5 μM) and the presence of 1.3 mM $[Ca^{2+}]_c$ (**)p<0.001, *p<0.01). B) Thimerosal concentration-dependence of PDGF-mediated elevation of $[Ca^{2+}]_i$ (for temporal profile of $Ca^{2+}$ elevation, see inset) (**)p<0.01, *p<0.05). Data are mean ± S.E.M., elevations above basal $Ca^{2+}$, n ≥ 3. Inset data are representative of at least 3 independent experiments.
Fig. 4.x. In the absence of extracellular Ca$^{2+}$, the elevation of $[Ca^{2+}]_{i}$ following challenge of SH-SY5Y cells with sub-maximal concentrations of methacholine (1 μM) and PDGF (20 ng.ml$^{-1}$) in the absence (solid bars) or presence (hatched bars) of thimerosal (5 μM). Data are mean ± S.E.M., elevations above basal Ca$^{2+}$, n ≥ 3 (*p<0.01).
**PDGF does not mobilize intracellular Ca^{2+} through the activation of sphingosine kinase.**

Adherent SH-SY5Y cells were challenged with agonist and the peak elevation of [Ca^{2+}]_{i} over basal determined following a preincubation with the sphingosine kinase inhibitor, N,N-dimethylsphingosine (DMS) (30 μM, 10 min) and a non-inhibitory analogue, N-acetylsphingosine (NAS) [Edsall et al., 1998], in the absence of extracellular Ca^{2+}.

No reduction in the PDGF-mediated elevation of [Ca^{2+}]_{i} occurred in DMS treated cells challenged with a maximal concentration (200 ng.ml^{-1}) of PDGF. Surprisingly, the PDGF-mediated elevation of [Ca^{2+}]_{i} was significantly increased in the presence of the sphingosine kinase inhibitor (81 ± 12 nM, n = 5; compared to 54 ± 5 nM, n = 10, in the absence of DMS, p<0.05).

The elevation of [Ca^{2+}]_{i} following challenge with a maximal concentration (1 μM for elevation of [Ca^{2+}]_{i}) of LPA was significantly reduced, almost abolished (7 ± 4 nM, n = 9; compared to 84 ± 16 nM, n = 9, in the absence of DMS; p<0.01). Interestingly, DMS also significantly reduced the muscarinic receptor-mediated elevation of [Ca^{2+}]_{i} (226 ± 18 nM, n = 3; compared to 448 ± 30 nM, n = 3, in the absence of DMS, p<0.01) when cells were challenged with a maximal concentration of methacholine (1 mM) (Fig. 4.xi.).

At a similar concentration to DMS, NAS had no effect on the elevation of [Ca^{2+}]_{i} following either methacholine, LPA or PDGF (Fig. 4.xi.).
Fig. 4.xi. Elevation of \([\text{Ca}^{2+}]_i\), following challenge of SH-SYSY cells with agonist (solid bars): methacholine (1 mM); LPA (1 \(\mu\)M); PDGF (200 ng.ml\(^{-1}\)); or agonist in the presence of DMS (30 \(\mu\)M), to inhibit sphingosine kinase (shaded bars), or the non-inhibitory DMS analogue, NAS (30 \(\mu\)M) (hatched bars). Data are mean ± S.E.M., elevations above basal Ca\(^{2+}\), \(n \geq 4\) (*p<0.01).
**PDGF does attenuate LPA receptor-mediated Ca\(^{2+}\) signalling.**

In conditions of a low \([\text{Ca}^{2+}]_e\) (not EGTA-buffered), PDGF (200 ng.ml\(^{-1}\)) stimulation resulted in only a small elevation of \([\text{Ca}^{2+}]_i\) (102 ± 4 nM, \(n = 3\), see section 3.ii.). In contrast, LPA (1 μM) evoked a rapid, transient elevation of \([\text{Ca}^{2+}]_i\) (371 ± 39 nM, \(n = 4\)). A prestimulation with PDGF resulted in a significant reduction in the subsequent response to LPA given 5 minutes after PDGF (64 ± 8 nM, \(n = 3\); compared to 371 ± 39 nM, \(n = 4\); \(p<0.001\)) (Fig 4.xii.).

**Fig. 4.xii.** Following PDGF (200 ng.ml\(^{-1}\)) prestimulation in low \([\text{Ca}^{2+}]_e\), the subsequent LPA-mediated (1 μM) elevation of \([\text{Ca}^{2+}]_i\), was significantly reduced (\(p<0.001\)). Solid lines represent measurements of LPA release following PDGF and broken lines indicate the application of LPA alone. Data shown are representative of at least three separate experiments.
4.iv. DISCUSSION

Within this chapter, this study demonstrated that the mechanism for PDGF-mediated release of intracellular Ca\(^{2+}\) was not an Ins(1,4,5)P\(_3\)-mediated event.

In summary, challenge of the SH-SY5Y cells with methacholine evoked both an elevation of global Ins(1,4,5)P\(_3\) and an accumulation of \[^{3}H\]-InsP\(_x\) against a Li\(^+\) block of inositol metabolism. In contrast, the addition of either PDGF or vehicle were similar and resulted in no increased levels of Ins(1,4,5)P\(_3\) or \[^{3}H\]-InsP\(_x\). The inhibition of either PKC or sphingosine kinase resulted in a decrease in the Ins(1,4,5)P\(_3\) generated by muscarinic receptor activation and had no effect on the lack of Ins(1,4,5)P\(_3\) measured in response to PDGF.

Having measured no Ins(1,4,5)P\(_3\) or \[^{3}H\]-InsP\(_x\) in response to PDGF challenge, could not rule out an Ins(1,4,5)P\(_3\)-mediated mechanism completely. Therefore, a variety of protocols that targeted the Ins(1,4,5)P\(_3\) receptor were employed to define the possible role of Ins(1,4,5)P\(_3\) in PDGF-mediated intracellular Ca\(^{2+}\) store gating. Despite contrasting evidence of inhibition of the PDGF-mediated elevation of [Ca\(^{2+}\)], by caffeine and heparin, the weight of evidence presented herein suggested a novel, and Ins(1,4,5)P\(_3\)-independent Ca\(^{2+}\) release mechanism.

The thapsigargin-sensitive, intracellular Ca\(^{2+}\) store demonstrated previously (see Chapter 3) to be shared by activated PDGF and muscarinic receptors is also reported to be Ins(1,4,5)P\(_3\)-sensitive following muscarinic receptor activation in these cells [Willars and Nahorski, 1995a; Willars and Nahorski, 1995b]. Both RTKs and GPCRs are known to activate phosphoinositide-specific isoforms of PLC [reviewed in Cockcroft and Thomas, 1992; Lee and Rhee, 1995]. Thus, the generally accepted mechanism for intracellular Ca\(^{2+}\) store release by these receptors, is the activation of PLC and subsequent generation of Ins(1,4,5)P\(_3\), which then releases Ca\(^{2+}\) from the intracellular Ca\(^{2+}\) store through interaction with the Ins(1,4,5)P\(_3\) receptor [Berridge, 1987; Berridge, 1993; Putney and Bird, 1993]. This chapter aimed to determine the mechanism for the PDGF-mediated elevation of [Ca\(^{2+}\)], that was originally presumed to be Ins(1,4,5)P\(_3\)-dependent.

The generation of Ins(1,4,5)P\(_3\) was measured in agonist-stimulated cells as an index of PLC activation. A major disadvantage of using this method however, is that it reflects the net effect of Ins(1,4,5)P\(_3\) generation and metabolism. The steady
state \text{Ins}(1,4,5)\text{P}_3 \text{ levels measured in this way are only accurate if the rapid metabolism of Ins}(1,4,5)\text{P}_3 \text{ is assumed to be constant [Wojcikiewicz et al., 1994b]. It is also possible that generation and metabolism result in a new 'pseudo-steady state', with an increase in turnover, but not the global level of Ins}(1,4,5)\text{P}_3.}

The \text{Ins}(1,4,5)\text{P}_3 \text{ response to a maximal concentration of methacholine contrasted in both peak height and temporal profile to previously published observations from this laboratory [Willars and Nahorski, 1995a; Willars and Nahorski, 1995b]. Cautious of the fact that not only had the method been slightly modified (addition of agonist to cells in buffer compared to complete removal of buffer and addition of agonist), but the cells had also been serum starved prior to the experiment, it was important to establish which of these factors was responsible for the dissimilar profiles. Following an investigation into the possible causes of the reduced peak and plateau levels and the novel second rising phase, it was found that these could be attributed to the 20 hour serum starvation prior to agonist stimulation.

Given the reduced magnitude of the \text{Ins}(1,4,5)\text{P}_3 \text{ response following serum starvation, a plausible explanation for this observation was that following serum starvation there was a reduction in the number of plasma membrane-localised muscarinic receptors. Although there are undoubtedly other candidate mechanisms that could be the cause, or contribute to, this serum deprivation effect, this 'reduction in number of muscarinic receptor' hypothesis was not without substantiation. A previous observation has shown that differentiation of the SH-SY5Y cell using retinoic acid (in low serum conditions) resulted in an approximate doubling of muscarinic M₃ receptors on the cell surface (Martin et al., 1999), suggesting that these cells are able to regulate the number of receptors at the membrane in response to external stimuli. However, the results of this investigation determined no significant change in either the receptor number at the cell surface, or the Kᵦ of [³H]-NMS following serum starvation.

Robust \text{Ins}(1,4,5)\text{P}_3 \text{ production has been reported following PDGF receptor activation in several cell types (see section 4.i.). But then Zhang et al., 1996 also report robust \text{Ins}(1,4,5)\text{P}_3 \text{ production following LPA stimulation in the N1E-115 neuroblastoma, contrasting with the known mechanism for the LPA-mediated elevation of [Ca^{2+}], in the SH-SY5Y neuroblastoma cell [Young et al., 1999; Young
et al., 2000a]. Some evidence in the literature has suggested that PKC might play a negative feedback, modulatory role on the PDGF receptor [Hasegawa-Sasaki, 1988; Miyakawa et al., 1998]. In the presence of DMS, the PDGF-mediated elevation of \([\text{Ca}^{2+}]\), was elevated above control levels (results within this chapter). Although the mechanism for this effect is unknown, one possibility was sphingosine kinase-mediated negative feedback on PDGF-mediated Ins(1,4,5)P3 generation. However, during the course of this investigation there was no evidence of a PDGF-mediated elevation of Ins(1,4,5)P3 either alone, or in the presence of PKC and sphingosine kinase inhibitors.

In contrast to PDGF, Ins(1,4,5)P3 generation following muscarinic receptor stimulation was moderately inhibited by both PKC and sphingosine kinase inhibitors. The PKC inhibitor Ro31-8220 however, has been shown to affect \([\text{H}]-\text{NMS}\) binding in the SH-SY5Y cell, causing a reduction of ~20%, despite no apparent effect on Ins(1,4,5)P3 generation or Ca\(^{2+}\) release to a maximal concentration of the muscarinic agonist carbachol [Willars et al., 1996]. This antagonism of muscarinic receptors may explain the reduced peak of Ins(1,4,5)P3 production observed in the presence of this PKC inhibitor. Alternatively, the inhibition of PKC-mediated phosphorylation events may directly influence phosphoinositide signalling. Both the muscarinic receptor and PLC-\(\beta\) are among the known targets for this prolific kinase [Herrero et al., 1994; Cunningham et al., 1999; Filtz et al., 1999]. Similarly, DMS has been reported to inhibit \([\text{H}]-\text{NMS}\) binding and phosphoinositide turnover and may therefore affect Ins(1,4,5)P3 generation as a consequence of decreased agonist binding [Young et al., 2000b].

An alternative method of measuring PLC activation is the generation of \([\text{H}]-\text{InsP}_x\) against a Li\(^+\) block of phosphoinositide metabolism. This method reflects the generation of inositol phosphates only and may not be the profile of second messenger production. A more accurate understanding is gained when both Ins(1,4,5)P3 mass and \([\text{H}]-\text{InsP}_x\) are measured and compared. The metabolism of other inositol phosphates may contribute to the measurement of \([\text{H}]-\text{InsP}_x\), but only the generation of Ins(1,4,5)P3 from PtdIns(4,5)P2 hydrolysis will result in second messenger production [Willars et al., 1996].

The magnitude of Ins(1,4,5)P3 levels following agonist stimulation within cells is a dynamic process that is dependent upon both its generation and subsequent
metabolism. However, measurement of the accumulation of $[^{3}\text{H}]$-InsP$_x$ against a Li$^+$-block of inositol monophosphatase activity provides an index of total PLC activation that is independent of inositol phosphate and polyphosphate metabolism [Wojcikiewicz et al., 1993].

In agreement with other reports, muscarinic receptor activation resulted in a robust and sustained stimulation of phosphoinositide metabolism as evidenced by the accumulation of InsP$_x$ against a Li$^+$ block [Willars and Nahorski, 1995a; Willars and Nahorski, 1995b; Martin et al., 1999]. PDGF did not cause an accumulation of $[^{3}\text{H}]$-InsP$_x$ suggesting that the failure to measure an increase in global Ins(1,4,5)P$_3$ levels was not a consequence of enhanced inositol phosphate metabolism. It is worth noting that the Li$^+$-block is only truly effective under conditions that maximally stimulate the inositol phosphate regenerative cycle [Nahorski et al., 1991]. Therefore, it may not be completely effective under conditions where a small transient elevation in Ins(1,4,5)P$_3$ may occur and is difficult to observe in the accumulation of $[^{3}\text{H}]$-inositol phosphates [cf. bradykinin response -Willars and Nahorski, 1995a; Martin et al., 1999].

Due to the amplification mechanism of PLC-linked signalling pathways [Putney and Bird, 1993; Lee and Rhee, 1995; Mikoshiba, 1997], it is generally assumed that the concentration-response curve related to PLC activation will lie to the right of the concentration-response curve for downstream signalling events such as Ca$^{2+}$ mobilisation. In short, at the maximum concentration of PDGF, the elevation of [Ca$^{2+}$], may be effected by such a small quantity of Ins(1,4,5)P$_3$ that it is impossible to measure either in isolation, or under a Li$^+$-block. Unable to measure an accumulation of $[^{3}\text{H}]$-InsP$_x$ at a maximal PDGF concentration for Ca$^{2+}$ release, the concentration of PDGF was increased to 1 µg.ml$^{-1}$ (5 times the concentration required for maximal elevations in [Ca$^{2+}$]). At this concentration, the measurement is made to the right of the Ca$^{2+}$ concentration-response curve. Again there was no observed accumulation of $[^{3}\text{H}]$-InsP$_x$.

The inability to measure Ins(1,4,5)P$_3$ or $[^{3}\text{H}]$-InsP$_x$ as an index of PLC activity cannot exclude a role for this second messenger. Agonist potency and amplification mechanisms within the cell may be responsible for the deficiency of measurable second messenger [Willars and Nahorski, 1995a]. A small and localised production of Ins(1,4,5)P$_3$, possibly in close proximity to the Ins(1,4,5)P$_3$ receptor
[Tu et al., 1998], may also be responsible for the PDGF-mediated elevation of 
$[Ca^{2+}]$. To further investigate the possible role of Ins$(1,4,5)P_3$, strategies were 
utilised that influenced the target for this second messenger, the Ins$(1,4,5)P_3$ 
receptor. Thus, we investigated PDGF-mediated $Ca^{2+}$ mobilization in the presence 
of inhibitors of the Ins$(1,4,5)P_3$ receptor and either Ins$(1,4,5)P_3$ receptor down-
regulation or sensitisation.

Agonist-induced release of $^{45}Ca^{2+}$ pre-loaded into the intracellular $Ca^{2+}$ store in a permeabilised cell, allows the use of low molecular weight heparin [Ghosh et al., 
1988] (which is membrane-impermeant) to block Ins$(1,4,5)P_3$ action. Heparin has 
been demonstrated to inhibit Ins$(1,4,5)P_3$-mediated $Ca^{2+}$ release competitively via an 
interaction with the Ins$(1,4,5)P_3$ receptor [Michelangeli et al., 1995 and references 
therein]. In this way it was hoped to implicate the Ins$(1,4,5)P_3$ receptor in the 
PDGF-mediated elevation of $[Ca^{2+}]$, in the SH-SY5Y cells.

In permeabilised SH-SY5Y cells, maximal $^{45}Ca^{2+}$ release was measured at 2 
min and 15 min following muscarinic and PDGF receptor activation, respectively. In 
agreement with previous reports a significant heparin-mediated inhibition (at both 50 
and 100 $\mu$g.ml$^{-1}$) of muscarinic receptor $^{45}Ca^{2+}$ release was reliably measured 
[Kobayashi et al., 1989; Short and Taylor, 2000]. However, there was some 
inconsistency in the inhibition of PDGF-mediated $^{45}Ca^{2+}$ release by heparin. Heparin 
failed to inhibit PDGF-mediated $^{45}Ca^{2+}$ release at 100 $\mu$g.ml$^{-1}$, yet inhibited the 
release at the lower concentration of 50 $\mu$g. ml$^{-1}$. Although micro-injected heparin 
can block PDGF-mediated elevation of $[Ca^{2+}]$, in CHO cells containing transfected 
PDGF receptors [Mathias et al., 1998], endogenous receptors in vascular smooth 
muscle cells are heparin-insensitive for associated elevation of $[Ca^{2+}]$, [Huang et al., 
1991].

A major disadvantage to using permeabilised cells for this investigation was 
the probable disruption of cytosolic signalling due to the diffusion of molecular 
signalling components from the cell under these conditions. The peak of $^{45}Ca^{2+}$ 
release in a permeabilised system has been reported to differ considerably from 
agonist-mediated elevations of $[Ca^{2+}]$, in whole cells. Muscarinic receptor activation 
results in a rapid elevation of Ins$(1,4,5)P_3$ and $[Ca^{2+}]$, in whole cells (<10 seconds)
which contrasts sharply with the reported peak of Ins(1,4,5)P₃ and ⁴⁵Ca²⁺ release (2 min) in permeabilised SH-SY5Y cells [Willars et al., 1996]. Following PDGF stimulation in intact cells, there is a 20-60 second lag phase prior to agonist-mediated elevation of [Ca²⁺], and an amplification of this delay could be expected in a permeabilised cell system. This was indeed the case, as the optimal PDGF ⁴⁵Ca²⁺ release was determined to be 15 min. Data derived from studies on ⁴⁵Ca²⁺ release under these conditions may not be reliable if important signalling components have this length of time to diffuse from the cell. This may explain why the lower concentration of heparin (50 μg.ml⁻¹) was able to block PDGF-mediated ⁴⁵Ca²⁺ release, yet the higher concentration (100 μg.ml⁻¹) could not.

Furthermore, the use of heparin as an Ins(1,4,5)P₃ receptor antagonist has limitations due to several other non-specific effects, such as the activation of L-type Ca²⁺ channels, the uncoupling of G-proteins from their receptors, stimulation of ryanodine receptors and inhibition of InsP₄ binding [reviewed in Michelangeli et al., 1995]. So, despite the mean data that suggested an Ins(1,4,5)P₃-mediated Ca²⁺ release mechanism for PDGF, in general the ability of heparin to block PDGF action was somewhat inconsistent and therefore inconclusive.

Caffeine is membrane permeable and inhibits non-competitively at the Ins(1,4,5)P₃ receptor [Parker and Ivorra, 1991; Brown et al., 1992] at high concentration (40 mM). This provided another means of inhibiting a putative PDGF-activated Ins(1,4,5)P₃-mediated response. Further studies in intact SH-SY5Y cells and the presence of 1.3 mM [Ca²⁺], demonstrated that caffeine significantly inhibited an elevation of [Ca²⁺], in response to 1 mM methacholine and completely abolished the response to 1 μM methacholine. In contrast, caffeine had no significant effect on a PDGF-mediated elevation of [Ca²⁺]. However, in the absence of extracellular Ca²⁺, which therefore examined only agonist-mediated intracellular Ca²⁺ store release, caffeine was, surprisingly, unable to inhibit muscarinic receptor-mediated elevations of [Ca²⁺], to a maximal concentration of methacholine. Caffeine did inhibit both the bradykinin and PDGF receptor-mediated elevations of [Ca²⁺], under these conditions.
Again, it must be stressed that caution should be exercised when interpreting data derived from caffeine inhibition as it, like heparin, has many other suggested sites of action, in particular, the activation of ryanodine receptors [McPherson and Campbell, 1993]. Caffeine is also suggested to inhibit Ca\(^{2+}\) and ATP-mediated positive feedback on the Ins(1,4,5)P\(_3\) receptor [reviewed in Michelangeli et al., 1995].

The interpretation of these data would suggest that caffeine can inhibit PDGF-mediated Ca\(^{2+}\) store release, but in the presence of extracellular Ca\(^{2+}\), where no significant inhibition was measured, was there compensatory influx? However, why could caffeine not abolish PDGF signalling at either maximal or sub-maximal concentrations when no Ins(1,4,5)P\(_3\) generation could be measured following 200 ng.ml\(^{-1}\) PDGF stimulation? Even if PDGF was generating either small or localised amounts of Ins(1,4,5)P\(_3\), the sub-maximal concentration would be expected to be producing little or none. This similar effect on both maximal and sub-maximal concentrations of PDGF suggested a non-specific effect of caffeine on PDGF Ca\(^{2+}\) signalling and was not interpreted as caffeine-mediated inhibition of the Ins(1,4,5)P\(_3\) receptor per se. In support of this, there was an apparent (but not significant) inhibition of PDGF-mediated elevation of [Ca\(^{2+}\)], by caffeine in the presence of [Ca\(^{2+}\)]\(_e\), that was similar to the significant reduction of the PDGF Ca\(^{2+}\) response in the absence of [Ca\(^{2+}\)]\(_e\): 39% and 42% compared to 41%, respectively.

In support of this ’non-specific effect’ theory, an elevation of [Ca\(^{2+}\)], following methacholine (1 mM) was not inhibited in the absence of [Ca\(^{2+}\)]\(_e\), which implied caffeine was not inhibiting Ins(1,4,5)P\(_3\) receptor-mediated Ca\(^{2+}\) store gating. However, the muscarinic receptor-mediated elevation of [Ca\(^{2+}\)], was significantly reduced in the presence of 1.3 mM [Ca\(^{2+}\)]\(_e\). This implied some caffeine-mediated inhibition of Ca\(^{2+}\) influx under these conditions or, as has been previously suggested, inhibition of Ca\(^{2+}\) release by affecting the Ca\(^{2+}\)-mediated positive feedback on the Ins(1,4,5)P\(_3\) receptor presumed to be mediated by extracellular Ca\(^{2+}\) [Ihrose et al., 1993]. Similarly, the increased [Ca\(^{2+}\)], following bradykinin stimulation, which generates only a small peak of Ins(1,4,5)P\(_3\) in SH-SY5Y cells [Willars and Nahorski, 1995a; Martin et al., 1999], was significantly inhibited but not abolished. If caffeine was truly inhibiting the Ins (1,4,5)P\(_3\) receptor, why could it abolish the Ca\(^{2+}\) response
to 1 μM methacholine yet only inhibit the response to 1 μM bradykinin which generates significantly less Ins(1,4,5)P₃?

So, despite having measured no Ins(1,4,5)P₃ or [³H]-InsPₓ in response to challenge with PDGF, both heparin and caffeine appeared to partially inhibit PDGF-mediated elevations in [Ca²⁺]. In contrast, muscarinic receptor activation resulted in a robust and sustained Ins(1,4,5)P₃ and [³H]-InsPₓ response and rather confusingly, a component of the Ca²⁺ release mechanism was resistant to Ins(1,4,5)P₃ receptor inhibition by either heparin or caffeine. Bradykinin, which according to previous reports [Willars and Nahorski, 1995a; Martin et al., 1999] produces significantly less Ins(1,4,5)P₃ than methacholine, was also only partially inhibited by caffeine. In light of the complexity of interpreting data derived from both heparin and caffeine, which have multiple effects on phosphoinositide-mediated Ca²⁺ signalling, other methods were also employed to gather further evidence for the mechanism of PDGF-mediated elevation of [Ca²⁺].

Down regulation of the type 1 Ins(1,4,5)P₃ receptor suggested a method that could conclusively implicate Ins(1,4,5)P₃ in PDGF-mediated Ca²⁺ signalling. Under such conditions, we could expect agonists that elevate [Ca²⁺], via an Ins(1,4,5)P₃-mediated mechanism to have either abolished or significantly reduced responses. This may be due to either removal of ~90% of the Ins(1,4,5)P₃ type I receptor, or the greater diffusion time required to locate a receptor which leads to an increase in the probability that Ins(1,4,5)P₃ is metabolically removed. Following this protocol, PDGF-mediated signalling could be investigated in the reduced population of Ins(1,4,5)P₃ receptor.

Agonist-mediated elevations of [Ca²⁺], were examined in response to PDGF, bradykinin, and methacholine. Bradykinin couples through a Go₄-coupled bradykinin B₂ receptor to elevate [Ca²⁺], in these cells with a maximal effect on cytosolic Ca²⁺ at 100 nM. However, in contrast to the robust elevation of Ins(1,4,5)P₃ by activated muscarinic receptors, the bradykinin receptor generates significantly less Ins(1,4,5)P₃ [Willars and Nahorski, 1995a, Martin et al., 1999]. The complete block of [Ca²⁺], elevation to the known Ins(1,4,5)P₃-generating agonist bradykinin, and concomitant inability to reduce the PDGF-mediated elevation of [Ca²⁺], under these conditions, suggested that the localised generation of
Ins(1,4,5)P₃ in response to PDGF was not responsible for PDGF-mediated Ca²⁺ release. One caveat to this interpretation was that muscarinic receptor-mediated down-regulation of Ins(1,4,5)P₃ receptors may preferentially cause receptors to be lost in compartments accessible to Ins(1,4,5)P₃ generated in response to activated GPCRs but not activated PDGF receptors.

Despite chronic stimulation of the muscarinic receptors, a methacholine-mediated elevation of [Ca²⁺]ᵢ was still apparent although significantly reduced. A plausible explanation for this was that receptor internalisation and ligand degradation had depleted the methacholine used to down regulate the muscarinic receptors and the response was, therefore, recovering. Alternatively, in contrast to bradykinin receptors, muscarinic receptors might utilise either a sub-population of down regulation-resistant Ins(1,4,5)P₃ receptors or be capable of gating another Ins(1,4,5)P₃ receptor subtype. However, as demonstrated, the Ins(1,4,5)P₃ receptor was still significantly reduced (<10% of control) after 7 hours and the inability to completely abolish muscarinic receptor-mediated Ca²⁺ signalling was not a major concern with respect to defining the PDGF-mediated Ca²⁺ release mechanism.

SH-SY5Y cells have been shown to express predominantly (~99% of the Ins(1,4,5)P₃ receptor population) type I Ins(1,4,5)P₃ receptors [Wojcikiewicz, 1995]. The remaining 1% of Ins(1,4,5)P₃ receptors can be presumed to be type II, which is often found at very low levels in neuronal tissues [Wojcikiewicz, 1995]. In these cells, chronic (i.e. 7 hour) pre-treatment with methacholine down-regulates the type I Ins(1,4,5)P₃ receptor [Wojcikiewicz et al., 1994a]. This loss of Ins(1,4,5)P₃ receptor immunoreactivity is functionally relevant as the ability of exogenous Ins(1,4,5)P₃ to release Ca²⁺ from the stores of permeabilised cells is also reduced [Wojcikiewicz and Nahorski, 1991].

Chronic muscarinic receptor activation has been shown to modulate several other key signal transduction components via internalisation and/or degradation. Reports to date include the muscarinic receptor [Mullaney et al., 1993], the G-protein [Sorensen et al., 1997] and PLC-β₁ [Sorensen et al., 1998]. However, this study clearly demonstrated that the Ins(1,4,5)P₃ receptor down regulation protocol had no effect, at the level of protein, on these and other key signal transduction components of Ca²⁺ signalling pathways activated following ligand-dependent activation of muscarinic, bradykinin or the putative, PLC-linked for Ca²⁺, PDGF
receptors. Those examined by no means constitute a comprehensive list of the signalling intermediaries involved following activation of these receptors. Furthermore, this examination is considered cursory rather than thorough with regard to function and cannot rule out regulatory or desensitisation mechanisms that do not involve protein degradation, such as phosphorylation.

These data are in agreement with the hypothesis that the PDGF-mediated elevation of \([Ca^{2+}]\) was \(Ins(1,4,5)P_3\)-independent. The relative potency of this protocol in abolishing the \(Ca^{2+}\) response to \(G\alpha_q\)-coupled GPCRs can be presumed to be directly related to the \(Ins(1,4,5)P_3\) generation following either muscarinic or bradykinin receptor activation.

A second alternative strategy utilised thimerosal, which has been reported to sensitise the \(Ins(1,4,5)P_3\) receptor, in effect, lowering the concentration of \(Ins(1,4,5)P_3\) required to activate the receptor and allow \(Ca^{2+}\) release from the intracellular stores [Missiaen et al., 1991; Bootman et al., 1992; Kaplin et al., 1994; Thrower et al., 1996; Young et al., 1998]. In the presence of thimerosal, the efficacy of \(Ins(1,4,5)P_3\) for calcium release is enhanced, resulting in a leftward shift in concentration-response curves. This implied that at concentrations of \(Ins(1,4,5)P_3\) that do not saturate the \(Ins(1,4,5)P_3\) receptor, more \(Ca^{2+}\) should be released from the intracellular \(Ca^{2+}\) stores in the presence of thimerosal.

Therefore, the potential involvement of \(Ins(1,4,5)P_3\) receptors in PDGF-mediated \(Ca^{2+}\)-signalling was investigated using thimerosal. In the presence of 1.3 mM [\(Ca^{2+}\)], sensitisation of the \(Ins(1,4,5)P_3\) receptor using thimerosal resulted in significant increases in the ability of submaximal concentrations of both methacholine (1 \(\mu\)M) and PDGF (20 ng.m\(^{-1}\)) to elevate [\(Ca^{2+}\)]. The increased elevation of [\(Ca^{2+}\)], following PDGF was clearly demonstrated to be thimerosal concentration dependent. The G-protein coupled, sphingosine-1-phosphate dependent, LPA receptor was unaffected by this protocol and suggested a specificity for PLC-coupled receptors. In the absence of extracellular \(Ca^{2+}\) however, PDGF-mediated \(Ca^{2+}\) store release was unaffected by \(Ins(1,4,5)P_3\) receptor sensitisation whilst methacholine-activated muscarinic receptors still exhibited a significant increase in \(Ca^{2+}\) release in the presence of thimerosal.
Initially these data supported an Ins(1,4,5)P3-dependent PDGF Ca\(^{2+}\) release mechanism as the presence of thimerosal resulted in a significant increase in the ability of PDGF to elevate [Ca\(^{2+}\)]. Disconcertingly, or perhaps reassuringly, in the absence of extracellular Ca\(^{2+}\), PDGF-mediated Ca\(^{2+}\) store gating was thimerosal-insensitive. Was thimerosal affecting Ca\(^{2+}\) influx following PDGF-mediated Ca\(^{2+}\) store release? Although the effect of thimerosal on capacitative Ca\(^{2+}\) entry in SH-SY5Y cells is unknown, there is some evidence in the literature that suggests thimerosal may be able to affect the endoplasmic reticulum Ca\(^{2+}\) pump, affecting Ca\(^{2+}\) re-uptake and thus providing an indirect means of regulating cytosolic Ca\(^{2+}\) [Sayers et al., 1993; Hilly et al., 1993]. This could account for the 'apparent' sensitisation of PDGF-mediated elevation of [Ca\(^{2+}\)], only in the presence of extracellular Ca\(^{2+}\).

These data therefore suggested that the PDGF-mediated elevation of [Ca\(^{2+}\)], was indeed Ins(1,4,5)P3-independent and are in agreement with the known Ca\(^{2+}\) release mechanisms for muscarinic and LPA receptors in the SH-SY5Y cells.

The existence of the S-1-P signalling pathway in the SH-SY5Y cells, as evidenced by the LPA receptor [Young et al., 1999], prompted an investigation of PDGF-mediated sphingolipid signalling. Sphingosine kinase inhibition in the absence of [Ca\(^{2+}\)], abolished LPA-mediated Ca\(^{2+}\) release and significantly increased the peak elevation of [Ca\(^{2+}\)], in response to PDGF. Partial inhibition of the muscarinic receptor-mediated Ca\(^{2+}\) store release again suggested that under conditions of serum starvation, muscarinic receptors might access more than one mechanism for intracellular Ca\(^{2+}\) store gating. These experiments were performed in the absence of an extracellular Ca\(^{2+}\) gradient as DMS inhibits I\(_{CRAC}\) by 95% within 5 minutes of exposure [Mathes et al., 1998].

PDGF has been reported to activate sphingosine kinase resulting in the production of sphingosine-1-phosphate [Olivera and Spiegel, 1993]. This lipid-derived second messenger is thought to play a variety of trophic roles within the cell and has been demonstrated to release Ca\(^{2+}\) from intracellular stores independently of Ins(1,4,5)P3 [Ghosh et al., 1990; Zhang et al., 1991; Olivera et al., 1999; Young et al., 1999; Young et al., 2000a], but still requires the recruitment of PLC\(\gamma\) to the activated PDGF receptor [Olivera et al., 1999]. In the presence of DMS, PDGF-mediated intracellular Ca\(^{2+}\) store release was, surprisingly, elevated rather than
inhibited. A plausible theory was sphingosine kinase-mediated negative feedback on PDGF-mediated Ca\(^{2+}\) store gating. This mechanism could ultimately be PKC-mediated, although via a circuitous activation following S-1-P-dependent PLD activation and the subsequent conversion of PA to DAG [Ha and Exton, 1993; Exton, 1997b]. This indirect activation may differ from more direct PDGF-mediated PKC activation in either potency or activation of different PKC isoforms. However, within the context of this study, the mechanism remains unknown.

Sphingosine kinase may be fully responsible for the lysophosphatidic acid-induced [Ca\(^{2+}\)]\(_j\) response, via the Edg-4 GPCR, in SH-SY5Y cells. The significant reduction of the LPA-mediated elevation of [Ca\(^{2+}\)]\(_j\) in the presence of DMS (30 \(\mu\)M) is consistent with this previously reported role [Young et al., 1999; Young et al., 2000a].

In these cells, the mechanism for elevations of [Ca\(^{2+}\)]\(_j\) by activated muscarinic receptors is well documented to be Ins(1,4,5)P\(_3\)-dependent [Baird et al., 1989; Lambert and Nahorski, 1990; Wojcikiewicz et al., 1994b; Willars and Nahorski, 1995a; Willars and Nahorski, 1995b; Willars et al., 1998]. Data presented within this study (DMS-mediated inhibition of muscarinic receptor-induced Ins(1,4,5)P\(_3\) generation and elevation of [Ca\(^{2+}\)]\(_j\)) suggested a Ca\(^{2+}\) release mechanism for muscarinic receptors that is partially dependent on sphingolipid-mediated signalling. This was supported by an earlier report in HEK-293 cells, where the Ca\(^{2+}\) responses of stably transfected M\(_2\) and M\(_4\) muscarinic receptors were markedly inhibited by the sphingosine kinase inhibitors DMS and DL-threeo-dihydrosphingosine (DHS) [Meyer zu Heringdorf et al., 1998]. Interpretation of these experiments is, however, complicated by the recent observation that DMS inhibits binding of the non-selective muscarinic receptor antagonist, \(^3\)H-NMS to SH-SY5Y cells [Young et al., 2000b] despite no alteration of InsP\(_x\) accumulation or affecting the ability of exogenous Ins(1,4,5)P\(_3\) to release Ca\(^{2+}\) directly [Meyer zu Heringdorf et al., 1998].

Finally we demonstrated that PDGF-mediated heterologous desensitisation of GPCR-mediated elevations of [Ca\(^{2+}\)]\(_j\), may extend to receptors that are not PLC-coupled. Prestimulation with PDGF resulted in a significant inhibition of a subsequent muscarinic receptor-mediated elevation of [Ca\(^{2+}\)]\(_j\), which is likely not due to Ca\(^{2+}\) store depletion (see chapter 3). This study also demonstrated that PDGF
prestimulation could significantly reduce a subsequent LPA receptor-mediated elevation of \([\text{Ca}^{2+}]\).

Experimental definition of the mechanism(s) for the PDGF-mediated desensitisation of the different muscarinic and LPA receptor Ca\(^{2+}\) release mechanisms is beyond the scope of this study. However, as LPA-mediated Ca\(^{2+}\) signalling is PLC-independent in the SH-SY5Y cells and a proposed mechanism for the heterologous desensitisation of muscarinic receptor signalling may involve PLC (see section 5.iii.), these are most likely different mechanisms. Although numerous plausible mechanisms exist, such as a direct consequence of PA that is known to change the activity of many proteins \textit{in vivo}, two others of note are: the generation of LPA by the action of cPLA\(_2\) on PA following PLD activation [Moolenaar, 1995; Exton, 1997b] and; prestimulation of the PDGF receptor could prevent transactivation of the PDGF receptor by the G\(_i\)-coupled LPA receptor [Herrlich \textit{et al}., 1998; Goppelt-Struebe \textit{et al}., 2000]. Both of these mechanisms suggest that LPA-mediated signalling would be attenuated following PDGF receptor activation and there is increasing evidence for pertussis-toxin-sensitive, G\(_i\)-mediated RTK transactivation, making this a most likely mechanism.

A number of other mechanisms have been suggested to play a role in PDGF-mediated elevations of \([\text{Ca}^{2+}]\). In several cell types PI-3K has been shown to be partially responsible for PDGF-mediated Ca\(^{2+}\) release [Bonser \textit{et al}., 1991; Bae \textit{et al}., 1998; Rameh \textit{et al}., 1998]. However, where this mechanism is reported, concurrent \(\text{Ins}(1,4,5)\text{P}_3\) production is often also measured and it is unclear, therefore, whether this represents a truly \(\text{Ins}(1,4,5)\text{P}_3\)-independent release mechanism. Also, the pleckstrin homology domain of PLC-\(\gamma\) binds to the product of PI-3K action, PtdIns(3,4,5)P\(_3\) [Falasca \textit{et al}., 1998], and subsequent Ca\(^{2+}\) signalling may be partially dependent upon PtdIns(3,4,5)P\(_3\)-dependent membrane recruitment of PLC-\(\gamma\). A number of other potential mechanisms exist for the agonist-mediated mobilization of intracellular Ca\(^{2+}\), particularly through cyclic ADP-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP) [Peterson and Cancela, 1999]. cADPR releases Ca\(^{2+}\) from intracellular stores by activation of ryanodine receptors. However, caffeine does not release Ca\(^{2+}\) in SH-SY5Y cells [Martin \textit{et al}., 1999; Young \textit{et al}., 2000a] demonstrating a lack of functional ryanodine receptors in
these cells, which would suggest a mechanism independent of cADPR. Whether SH-SY5Y cells have the appropriate machinery for NAADP-mediated Ca$^{2+}$ release remains to be established.

The primary aim of this component of the current study was to define the mechanism responsible for PDGF-mediated elevations of [Ca$^{2+}$], in the SH-SY5Y human neuroblastoma cell line. The weight of evidence suggested an Ins(1,4,5)P$_3$-independent mechanism, despite some contradictory data following heparin and caffeine-mediated inhibition of the Ins(1,4,5)P$_3$ receptor. Therefore, the mechanism, although novel and Ins(1,4,5)P$_3$-independent, remains to be established.
5. AGONIST-MEDIATED REGULATION OF PLC AND PKC ISOFORMS IN THE SH-SY5Y CELL.

5.i. INTRODUCTION

Production of the second messengers DAG and Ins(1,4,5)P3 following agonist-driven hydrolysis of the minor membrane phospholipid PtdIns(4,5)P2 is attributed to the activation of phosphoinositide-specific PLC. DAG mediates the activation of several lipid-sensitive PKC isoforms, and the generation of Ins(1,4,5)P3 is a primary mechanism in most cell types for the receptor-mediated elevation of [Ca2+]. This ubiquitous signalling mechanism is one of the key signalling events for a multitude of known neurotransmitters, growth factors and extracellular hormones that signal through membrane-bound receptors [Cockcroft and Thomas, 1992; Rhee and Choi, 1992; Berridge, 1993]. Many isoforms of PLC and PKC have been identified and, despite the huge amount of information available in the literature regarding these proteins and their action, what remains to be clarified, in almost all cases, is exactly which of the various PLC and PKC isoforms are activated following agonist-mediated activation of a particular receptor.

Activation of PLC-β isoforms (130-155 kDa) is presumed to underlie PLC-dependent cellular responses following stimulation of G-protein coupled receptors (GPCRs) that signal through the Gq,11 family of G-proteins. Whilst Gα sub-units preferentially activate PLC-β1 > PLC-β3 > PLC-β4 >> PLC-β2, Gβγ subunits preferentially activate PLC-β3 > PLC-β2 > PLC-β1 [Smrcka et al., 1991; Taylor et al., 1991; Camps et al., 1992; reviewed in Lee and Rhee, 1995] (see section 1.iii.i.).

PLC-γ1 and/or -γ2 (146 kDa) are often recruited by agonist occupancy of RTKs, and rely on both tyrosine phosphorylation and recruitment to the membrane for efficient catalytic activation [Lopez-Rivas et al., 1987; Kim et al., 1991; Lee and Rhee, 1995]. Recent evidence suggesting agonist-dependent PLC-γ1 recruitment by several GPCRs has been previously discussed (see section 1.ii.iii.). This implies that agonist-driven PLC recruitment may be receptor-specific rather than receptor family-specific as PLC-γ is no longer the sole province of RTKs [Marrero et al., 1994; Dhar and Shukla, 1994; Gusovsky et al., 1993; Rao et al., 1995; Puceat and Vassort, 1996].
The most enigmatic PLC isoform remains PLC-δ (85 kDa), whose precise activation and regulatory mechanisms remain unclear. It is reported to be the most Ca²⁺-sensitive PLC isoform and a possible activation mechanism may be driven by Ca²⁺ subsequent to stimulation by any agonist that results in an elevation of [Ca²⁺], [Allen et al., 1997; Kim et al., 1999] (for a fuller discussion see Introduction section 1.ii.iv.).

Within the current study the aims were to: 1) determine exactly which PLC and PKC isoforms were present in the SH-SY5Y cell, and 2) determine which of these were activated in response to the muscarinic M₃ GPCR and platelet-derived growth factor receptor (our paradigm GPCR and RTK respectively). We asked: did the temporal profile of potential indicators of PLC activation, such as translocation and phosphorylation, coincide with temporal profiles of agonist-mediated downstream signalling events such as Ins(1,4,5)P₃ generation or elevation of [Ca²⁺]? 

PKC is a prolific serine/threonine kinase, and a ubiquitous regulatory mechanism, that can mediate phosphorylation of receptor and/or downstream signalling components, which exerts both positive and negative influences on signal transduction in both a cell and receptor-specific manner (see section 1.iv.). However, given the number of PKC isoforms, their differing activation mechanisms and the multitude of roles assigned to the different isoforms, this work sought not only to identify which PKC isoforms are present in the SH-SY5Y cell, but also to define the particular PKC isoforms involved in the regulation of a muscarinic or PDGF receptor-mediated cell response.

Following either RTK or GPCR stimulation, some PLC isoforms are recruited from the cytosol to the membrane [Kim et al., 1990; Atkinson and Yang, 1996; Banno et al., 1996; Kawakami et al., 1996; Puceat and Vassort, 1996; Matsuda et al., 2001; reviewed in Rebecchi and Pentyala, 2000], which contains the phosphatidylinositol substrate, and this provided the basis for an investigation of PLC isoform translocation following agonist-mediated receptor stimulation. The spatial separation of enzyme and substrate has always raised the possibility of localization, mediated by pleckstrin homology and C2 domains, being a regulatory mechanism for PLC, although there is little evidence in the literature to support this. So whilst translocation does not guarantee activation they are clearly linked [Rhee
and Choi, 1989; Cockcroft and Thomas, 1992; Lee and Rhee, 1995; Rhee and Bae, 1997]. Although there are other methods of measuring total PLC activation, translocation is frequently used due to its ability to differentiate the PLC isoforms [Kawakami et al., 1996; Banno et al., 1996; Matsuda et al., 2001; reviewed in Rebecchi and Pentyala, 2000 and references therein], but rarely are all the PLC isoforms within a given cell investigated. Similarly, PKC recruitment to the membrane relies on the lateral organisation of signalling complexes containing the PKC phosphorylation target at the membrane and not in the cytosol.

It was possible that if different isoforms of PLC and PKC were responsible for the different phases of downstream signalling events in response to agonist-mediated receptor activation, an agonists' ability to activate particular PLC/PKC isoforms could be the determinant of the downstream signal. This study therefore aimed to determine exactly which PLC and PKC isoforms were activated following muscarinic and PDGF receptor activation. Measurements of enzyme activity may have been indiscriminate and unable to differentiate the isotypes and thus down-regulation, translocation and phosphorylation of PLC and translocation of PKC isoforms were all investigated as an index of activation.
5.ii. RESULTS

Identification of PLC isoforms in SH-SY5Y cells.

To determine the role of PLC isoforms in downstream signalling events it was necessary to identify the PLC isoforms present in SH-SY5Y cells. Immunoblotting whole cell extracts for PLC isoform protein demonstrated that PLC-β₁, PLC-β₂, PLC-β₃, PLC-γ₁ and PLC-δ₂ were present (Fig. 5.i. A).

Antibodies to PLC-β₄, PLC-γ₂ and PLC-δ₁ (data not shown for PLC-δ₁) failed to locate any immuno-reactive proteins at the correct molecular weight when used in immunoblots of whole cell lysate. The immunoreactivity of all three antibodies was verified using rat brain lysate as a positive control. At a similar protein concentration to the SH-SY5Y cell extract (50 μg protein.lane⁻¹), antibodies to PLC-β₄ (130 kDa), -γ₂ (146 kDa) and -δ₁ (85 kDa) detected bands in the rat brain lysate at 130 kDa, 146 kDa and 85 kDa respectively, and confirmed the presence of these proteins in the rat brain lysate (Fig 5.i. B). These data confirm the immunoreactivity of these PLC antibodies.

The PLC-γ₂ antibody located a distinct band at 65-68 kDa (Fig. 5.i. A) and there is evidence in the literature that suggested this band is commonly observed, was originally classified as a PLC-α isoform, but was most likely a cross-reacting dithiol-oxidoreductase [Rhee and Choi, 1989; Cockcroft and Thomas, 1992].
A) SH-SY5Y whole cell lysate (50 μg protein/lane) probed with a range of antibodies demonstrated the presence of PLC-β, -β2, -β3, -γ1 and -δ isoforms. B) Immunoblots of rat brain lysate (lane A: 30 μg protein and lane B: 50 μg protein) confirmed the immunoreactivity of antibodies for PLC-β4, -γ2 and -δ1. Immunoblots are representative of 5 and 2 independent experiments, respectively.
Localisation of PLC isoforms in SH-SY5Y cells.

Having determined that SH-SY5Y cells expressed several PLC isoforms including members of all three families (β, γ and δ), subsequent analysis aimed to determine any possible PLC redistribution as an effect of serum starvation that may have contributed to increased agonist-mediated elevations of [Ca^{2+}] (see chapter 3, Fig 3.vii.). Following membrane/cytosol fractionation of whole cell extracts (see section 2.ii.), Western blotting with PLC isoform-specific antibodies was performed and analysed by densitometer.

There was no significant effect of serum starvation on the membrane-localisation of any PLC isoforms, except PLC-γ1. Following serum starvation, the percentage of PLC-γ1 isolated in the membrane fraction increased significantly (35 ± 3%, n = 3; compared to 15 ± 2%, n = 3, when serum maintained; p<0.05). In contrast, there was no significant difference in the membrane localisation of the other PLC isoforms within the cell: PLC-β1, 47 ± 3%, n = 3; compared to 51 ± 2%, n = 3, when serum maintained; PLC-β2, 16 ± 2%, n = 3; compared to 17 ± 4%, n = 3, when serum maintained; PLC-β3, 52 ± 5%, n = 3; compared to 53 ± 10%, n = 3, when serum maintained; PLC-δ2, 100 ± 0%, n = 3; compared to 100 ± 0%, n = 3, when serum maintained (see Fig 5.ii.).
Fig. 5.ii. Membrane distribution of PLC isoforms in the SH-SYSY cell either maintained in serum (+) or serum starved (-) for 20 hours prior to fractionation. Data are expressed PLC isoform isolated from the membrane fraction as the percentage of that PLC isoform isolated from both fractions, mean ± S.E.M. (n = 3) (*p<0.05).
**Down-regulation of PLC by muscarinic but not PDGF receptor agonist.**

Chronic stimulation of SH-SY5Y cells with the muscarinic agonist methacholine (1 mM) for 24 hours, resulted in significant reductions of most PLC isoforms (PLC-β1, 23 ± 6%; PLC-β2, 42 ± 31%; PLC-β3, 33 ± 9% and PLC-δ2, 53 ± 18%, compared to control cells (100%) that were exposed to vehicle alone, n = 4; p<0.05). However, PLC-γ1 isoform protein levels remained comparable to control cells (93 ± 29%, n = 4) under these conditions (Fig 5.iii.).

In contrast, SH-SY5Y cells chronically stimulated with PDGF-BB (200 ng.ml⁻¹) exhibited significant increases in several PLC isoforms (PLC-β3, 340 ± 101%; PLC-γ1, 209 ± 21% and PLC-δ2, 224 ± 52%, compared to control (100%), n = 4; p<0.05). There was no significant change in the levels of PLC-β1 or PLC-β2 compared to control cells (107 ± 7% and 139 ± 33%, respectively, n = 4) (Fig 5.iii.).
Fig. 5.iii. A) Immunoblots illustrating typical levels of PLC isoform protein following a 24 hour prestimulation with either vehicle (V), 200 ng.ml⁻¹ PDGF-BB (P) or 1 mM methacholine (M). Vehicle was KHB and data are representative of 4 independent experiments. B) Subsequent densitometric analysis determined levels of PLC isoform protein following chronic PDGF and muscarinic receptor stimulation compared to control cells. Data are mean ± S.E.M. (n = 4) (*p<0.05; **p<0.01).
**Agonist-mediated translocation of PLC**

In SH-SY5Y cells several of the PLC isoforms appeared to be preferentially localised at the membrane under basal (non-stimulated) conditions (see Fig 5.ii. and Fig 5.iv.). The temporal profile of the translocation of PLC isoforms was investigated following maximal concentrations of either methacholine (1 mM) or PDGF (200 ng.ml⁻¹). These concentrations were maximal for elevation of [Ca²⁺]. Translocation was examined by cell fractionation following agonist stimulation over a 15 min (30 min for PLC-γ translocation to PDGF) time course and the data expressed as the PLC isoform identified in the membrane fraction as a percentage of the total of that isoform identified in both membrane and cytosolic fractions (see section 2.ii.).

This investigation into agonist-mediated translocation of PLC determined two PLC isoforms that translocate with differing temporal profiles in response to methacholine (1 mM). Both PLC-β₁ and PLC-β₂ exhibited significant cytosol to membrane redistribution over the 15 min (p<0.05, single factor ANOVA) following muscarinic receptor activation. However, the translocation of PLC-β₁ was slow (maximal at 10 min) but sustained over the 15 min time course contrasting with the more rapid (maximal at 5 min) but transient translocation of PLC-β₂ (PLC-β₁: from 55 ± 22% membrane-associated protein, to 90 ± 1% after 10 min; PLC-β₂: from 32 ± 10% membrane-associated protein, to 84 ± 4% after 5 min, n > 4) (Fig 5.iv.).

Despite an apparent transient translocation of PLC-γ₁ (3 of 4 independent experiments exhibited a marked translocation), there was no significant change in the distribution of PLC-β₃, -γ₁ or -δ₂ isoforms over the 15 min time course (determined by single factor ANOVA), the latter isoform being located solely in the membrane fraction (Fig 5.iv. and Fig 5.v.). Representative immunoblots of the PLC isoforms in the membrane fraction of SH-SY5Y cells following methacholine (1 mM) stimulation reflect the mean data (Fig 5.v.).

Activation of RTKs has recruited PLC γ₁ to the membrane in many cell types [Claesson-Welsh, 1994; Malarkey et al., 1995; Rhee and Bae, 1997; Rebecchi and Pentyala, 2000; Matsuda et al., 2001] and so the translocation of all the PLC
isoforms was investigated following agonist-mediated activation of the PDGF receptor. PDGF-mediated PLC translocation was more specific, with only a single isoform, PLC γ1, translocating to the membrane over the 30 min time course (p<0.05, single factor ANOVA). No PLC-γ1 translocation was observed prior to 10 min and membrane localisation was still increasing at 30 min (25 ± 2% membrane-associated protein, to 49 ± 4% after 30 min, n = 4) (Fig 5.vi. A).

PDGF stimulation did not cause the re-localisation of any PLC-β isoforms to the membrane; neither did it affect the membrane-only localisation of PLC-δ2. Representative immunoblots can be seen in Fig 5.vi. B.
Fig 5.iv. Panels A) to E) illustrate the temporal profile of PLC isoform membrane localisation over the 15 minutes following muscarinic receptor activation with methacholine (1 mM). Data are mean ± S.E.M. (n ≥ 4).
**Fig 5.v.** Immunoblots of SH-SY5Y cell membrane fraction confirm the temporal profile of PLC isoform membrane localisation over the 15 minutes following muscarinic receptor activation with methacholine (1 mM). Data are representative immunoblots of at least 4 independent experiments.
Fig 5.vi. A) The temporal profile of PLC-γ1 membrane localisation (and representative immunoblot of the membrane fraction) over the 30 minutes following PDGF receptor activation with PDGF (200 ng.ml⁻¹). Data are mean ± S.E.M. (n = 3). B) Representative immunoblots of PLC-β1, -β2, β3 and δ2 which show no redistribution following PDGF stimulation. Immunoblots are representative of at least 3 independent experiments.
**PLC translocation is agonist-driven rather than an effect of Ca\(^{2+}\) or PKC activation.**

Although PLC-δ isoforms are acknowledged to be the most Ca\(^{2+}\) sensitive isoforms, all PLC isoforms require Ca\(^{2+}\) for activation. It was possible that translocation was merely a consequence of elevating [Ca\(^{2+}\)], and/or PKC activation subsequent to agonist-mediated receptor activation. These possibilities were investigated by examining the membrane localisation of PLC in conditions of elevated [Ca\(^{2+}\)], and PKC activation independent of receptor agonist.

The Ca\(^{2+}\) ionophore, ionomycin (10 μM), promoted an elevation of [Ca\(^{2+}\)], independent of receptor activation (see chapter 3, Fig 3.vi.) and resulted in no change in the membrane localisation of PLC-β\(_1\), -β\(_3\) and -γ\(_1\). There was a complete membrane to cytosol re-localisation of PLC-δ\(_2\) following ionomycin treatment, which had reached 15 ± 15% membrane-associated protein by 5 min and was completed by 15 min (Fig 5.vii. A).

Phorbol ester-mediated (PDBu, 3 μM) activation of PKC caused no significant redistribution of any PLC isoform investigated, either to or from the membrane (Fig 5.vii. B).

When cells were treated simultaneously with both ionomycin (10 μM) and PDBu (3 μM), again there was no change in the localisation of PLC-β\(_1\), -β\(_3\) and -γ\(_1\). Interestingly, PLC-δ\(_2\) translocated from the membrane fraction immediately following the addition of both compounds with a maximal decrease in membrane association occurring by 5 min (28 ± 25% membrane-associated protein) (Fig 5.vii. C). In sharp contrast to the effect of ionomycin alone, under conditions of simultaneous ionomycin and PDBu treatment, PLC-δ\(_2\) membrane-association was still measurable at 15 min (45 ± 1% membrane-associated protein).
Fig 5.vii. Agonist-independent translocation of PLC isoforms over a 15 min time course when SH-SY5Y cells are treated with A) ionomycin (10 μM), B) PDBu (3 μM) and C) ionomycin (10 μM) and PDBu (3 μM) simultaneously. Data are mean ± S.E.M. (n = 3).
**Agonist-mediated phosphorylation of PLC isoforms.**

The temporal profile of agonist-mediated phosphorylation of PLC-β1, -β3 and -γ1 isoforms was subsequently investigated in order to answer the questions: do both GPCR and RTK agonists phosphorylate different PLC isoforms? And, what was the time course of any phosphorylation?Phosphorylation of PLC isoforms was examined over the initial 15 min following methacholine (1 mM) and PDGF-BB (200 ng.ml⁻¹) stimulation.

There was a marked increase in the phosphorylation state of PLC-β1 following stimulation with methacholine. Phosphorylation was increased at 5 min and even greater at the limit of the time course, 15 min (Fig 5.viii. A). Pre-incubation of the cells with atropine (2 μM) for 5 min was sufficient to maintain phosphorylation at basal levels when subsequently stimulated with methacholine for 5 min. At a maximal concentration for Ca²⁺ release, PDGF stimulation had no effect on the phosphorylation of PLC-β1 (Fig 5.viii. A).

The most dramatic agonist-mediated phosphorylation was exhibited by PLC-β3. In response to methacholine there was no measurable increase in phosphorylation by 1 min compared to control (time 0). A marked peak elevation at 5 min, that remained high even after 15 min, could be reduced to near basal levels by pre-incubation with atropine (2 μM). The phosphorylation of PLC-β3 was rapid (elevated above control (time 0) at 1 min) and steadily increased when SH-SY5Y cells were similarly challenged with PDGF. Within these experimental paradigms, maximal phosphorylation to PDGF occurred at the last (15 min) time point (Fig 5.viii. B).

Both methacholine and PDGF stimulation resulted in the phosphorylation of PLC-γ1. Muscarinic receptor activation caused a rapid (visible at 30 sec) increase in PLC-γ1 phosphorylation that peaked over the 1-5 min time period and was reduced to near basal levels by 15 min. Atropine treatment (2 μM) prior to methacholine was unable to prevent the phosphorylation of this isoform. In contrast, PDGF-mediated phosphorylation of PLC-γ1 was slower (visible at 1 min) but peaked at 5 min and was still elevated at 15 min (Fig 5.viii. C).
Fig 5.viii. Agonist-mediated PLC isoform phosphorylation over a 15 min time course in SH-SY5Y cells. Phosphorylation of A) PLC-β1, B) PLC-β3 and C) PLC-γ1 were investigated in response to methacholine (1 mM) or PDGF-BB (200 ng.mL⁻¹). Following a 5 min pre-incubation with 2 μM atropine (A), methacholine stimulation for 5 min examined a requirement for receptor occupancy. Immunoblots are representative of at least three independent experiments.
**PDGF-AA stimulation results in phosphorylation but not translocation of PLC-$\gamma_1$.**

Challenging SH-SY5Y cells with PDGF-AA (200 ng.ml$^{-1}$) was unable to evoke an elevation of $[\text{Ca}^{2+}]_i$ (see Chapter 3). As these cells are reported to express PDGF-$\alpha$ receptors [Pahlman et al., 1992], was the PDGF-BB-mediated elevation of $[\text{Ca}^{2+}]_i$, due to preferential recruitment and/or phosphorylation of PLC-$\gamma_1$?

The translocation of PLC-$\gamma_1$ in response to PDGF-AA (200 ng.ml$^{-1}$) was investigated over a 30 min time course and resulted in no significant recruitment to the membrane fraction (as determined by single factor ANOVA, see Fig 5.ix. A). Like PDGF-BB however, PDGF-AA stimulation resulted in an elevated level of PLC-$\gamma_1$ phosphorylation that was maximal by 5 min and remained maximal even after 15 min (Fig 5.ix. B).
Fig 5.ix. A) Percentage of the total PLC-γ1 localised in the membrane fraction (and representative immunoblot of the membrane fraction) over the 30 minutes following PDGF receptor activation with PDGF-AA (200 ng.ml⁻¹). Data are mean ± S.E.M. (n = 3). B) Phosphorylation of PLC-γ1 over a 15 min time course in SH-SY5Y cells. Both immunoblots are representative of at least three independent experiments.
Identification of the PKC isoforms in the SH-SY5Y cell.

To implicate any PKC isoforms in downstream signalling events following muscarinic or PDGF receptor activation, it was first necessary to identify the PKC isoforms present in the SH-SY5Y neuroblastoma cell. Immunoblotting whole cell extract for PKC isoform protein demonstrated that six (of a possible 10 to which antibodies were available) PKC isoforms, representing all three PKC families were present.

Protein bands were identified at molecular weights close to the known molecular weights of conventional PKCα and γ (82 and 80 kDa, respectively); atypical PKCτ, λ and ζ (74, 74 and 72 kDa, respectively); and novel PKCε (90 kDa) (Fig. 5.x.).

Fig. 5.x. SH-SY5Y whole cell lysate (50 μg protein.lane\(^{-1}\)) probed with a range of antibodies demonstrated the presence of PKCα, γ, τ, λ, ζ and ε isoforms. Immunoblots are representative of at least three independent experiments.
Agonist-mediated translocation of PKC

As an index of activation the translocation of PKC isoforms was examined over 5 min following stimulation with either methacholine (1 mM), PDGF (200 ng.ml⁻¹) or bradykinin (1 μM), at maximal concentrations for agonist-mediated elevation of [Ca²⁺]. In serum starved SH-SY5Y cells PKCε was evenly distributed between the membrane and cytosol, whilst PKCα, γ, τ, λ and ζ were primarily cytosolic (time 0, left and right-hand panels, Fig 5.xi.). Phorbol ester (PDBu, 3 μM) stimulation for 5 min provided a suitable control for lipid-sensitive PKC isoforms and routinely resulted in translocation of PKCα, γ and ε (p<0.05) from the cytosol to the membrane fraction (right-hand panels, Fig 5.xi.).

This investigation into agonist-mediated translocation of PKC demonstrated one PKC isoform that translocated following muscarinic receptor stimulation with methacholine. An apparent biphasic translocation of PKCε to the membrane was rapid, visible at 1 min, (51 ± 6% membrane-associated protein, increasing to 75 ± 10% after 1 min and 85 ± 1% after 5 min, n = 3; p<0.01) and there was no significant redistribution of other PKC isoforms (as determined by single factor ANOVA) (Fig 5.xi. A).

In contrast, PDGF stimulation resulted in the recruitment of both PKCε and PKCζ to the membrane. PKCε demonstrated a more rapid translocation (45 ± 3% membrane-associated protein, to 84 ± 2% after 5 min, n = 3; p<0.01), whereas the translocation of PKCζ was apparent only after 1 min (44 ± 6% membrane-associated protein, to 79 ± 7% after 5 min, n = 3; p<0.05) (Fig 5.xi. B).

Stimulation with another GPCR agonist, bradykinin, resulted in the translocation of PKCε (51 ± 4% membrane-associated protein, to 70 ± 3% after 5 min, n = 3; p<0.05) and no other PKC isoform (Fig 5.xi. C) (all degrees of significance determined by single factor ANOVA).
Fig 5.xi. Agonist-dependent translocation of PKC isoforms over a 5 min time course when SH-SY5Y cells are treated with A) methacholine (1 mM), B) PDGF (200 ng.ml⁻¹) and C) bradykinin (1 μM). Right-hand panels show PDBu (3 μM) controls for each agonist. PKC isoforms are represented as follows: PKCα (■, dotted lines); PKCγ (▲, broken lines); PKCe (▼, solid lines); PKCι (●, dot-dash lines); PKCλ (○, solid lines); and PLCζ (□, dot-dot-dash lines). Data are mean ± S.E.M. (n = 3) (*p<0.05).
5.iii. DISCUSSION

This study identified a number of PLC isoforms (PLC-β₁, -β₂, -β₃, -γ₁ and -δ₂) and PKC isoforms (PKCα, γ, ε, τ, λ and ζ) within the human SH-SY5Y neuroblastoma cell. Down-regulation of all three PLC-β isoforms and PLC-δ₂ following chronic exposure to methacholine suggested a muscarinic receptor-mediated regulation of these isoforms and acute receptor activation resulted in the agonist-mediated translocation of just two of these isoforms, PLC-β₁ and -β₂. In contrast, PLC-β₁, -β₁ and -γ₁ were all phosphorylated within 5 min of methacholine challenge. Chronic PDGF receptor activation with PDGF-BB, resulted in a significant elevation of PLC-β₃, -γ₁ and -δ₂ protein and acute receptor activation resulted in translocation of only PLC-γ₁, which along with PLC-β₃ exhibited rapid agonist-dependent phosphorylation.

Translocation of a single PKC isoform, the 'novel' PKCε was observed following both muscarinic and bradykinin GPCR activation. PDGF receptor activation recruited the 'atypical' PKCζ isoform to the membrane as well as PKCε. PDGF-AA, which did not elevate [Ca²⁺], in these cells (see Chapter 3) did stimulate PLC-γ₁ phosphorylation but resulted in no increased membrane localisation.

One of the aims of this thesis was to determine if multiple PLC isoforms contribute to the downstream phosphoinositide and Ca²⁺-mediated signalling of GPCR and RTKs. If this were occurring, would chronic activation of these receptors lead to a down-regulation of the PLC isoforms involved and thus provide clues as to which isoforms were activated by a given receptor?

One of the known regulatory mechanisms of the phosphoinositide-signalling pathway involves the down-regulation of key intermediary signalling molecules, by proteolysis, when under chronic agonist stimulation. This has been successfully used to implicate or identify proteins that may play a role in signal transduction or regulatory mechanisms following the activation of a particular receptor. Chronic stimulation of muscarinic receptors has been reported to decrease the levels of cell-surface receptor, G-protein, endoplasmic reticulum Ins(1,4,5)P₃ receptor and more recently PLC-β₁ [Mullaney et al., 1993; Wojcikiewicz et al., 1994a; Sorensen et al., 1997; Sorensen et al., 1998]. In the latter case, chronic muscarinic receptor
activation for 24 hours with the agonist oxotremorine-M reduced the level of PLC-β1, but had no effect on PLC-γ1 or -δ1 in SH-SY5Y cells [Sorenson et al., 1998]. Although our SH-SY5Y clone expressed no PLC-δ1, but instead expressed PLC-δ2, the results presented within this chapter suggest that multiple PLC isoforms may be jointly responsible for the downstream signalling of muscarinic receptors. This lends credence to the hypothesis that the recruitment of multiple PLCs may be a determinant of the phosphoinositide and Ca2+ signalling profile.

Chronic muscarinic receptor stimulation with methacholine demonstrated the down-regulation of all the PLC isoforms present except for PLC-γ1. This implies a role for PLC-β1, -β2, -β3 and -δ2 in mediating the intracellular response to this agonist, or alternatively, that a common intracellular signal, such as Ca2+ or PKC etc. can regulate PLC in a relatively non-specific manner. Muscarinic receptor-mediated elevations of Ins(1,4,5)P3 or [Ca2+], were biphasic (see chapters 3 and 4) and this study suggested no involvement of PLC-γ1 in acute muscarinic receptor responses. The trophic effects of PDGF complicate interpretation of the PDGF-mediated ‘down-regulation’ data. In SH-SY5Y cells PDGF has a stimulation index of 2.5 (250% increase in cell number over a 24 hour period), based on [3H]-thymidine incorporation experiments [Hynds et al., 1995]. This correlates well with the altered levels of PLC-β3, -γ1 and -δ2. However, this also implies that PDGF was able to regulate PLC-β1 and -β2 protein levels following exposure of the cells to chronic agonist stimulation as, despite an increase in cell number, there was no increase of these isoforms compared to unstimulated cells.

As PLC-β1 is also reported as a primary nuclear PLC, involved in the nuclear phosphoinositide cycle and activation of nuclear PKC [Sun et al., 1997; Neri et al., 1998], it seems logical that its regulation would also be coupled to trophic factors. Indeed, in Swiss 3T3 cells IGF-I has been shown to activate nuclear PLC-β1 independently of PLC present in the plasma membrane fraction. Blocking PLC-β1 expression with anti-sense RNA inhibits DNA synthesis following such IGF-I treatment [Manzoli et al., 1997]. PLC-β2, -β3, -γ1, and -γ2 have also been identified within the nucleus of HL-60 cells [Bertagnolo et al., 1997] and, in general, nuclear levels of PLC differ in an isoform and splice variant manner in different cell types,
suggesting a complex, and as yet unknown, regulation [reviewed in Rebecchi and Pentyala, 2000 and references therein].

Although PLC-γ1 couples growth factors to trophic signalling pathways, it does not appear to be down-regulated at the level of protein by either methacholine or PDGF treatment. Indeed, during transformation, differentiation and proliferation, PLC-γ translocation to the nucleus has been observed, remaining at a high nuclear level in terminally differentiated cells [Diakonova et al., 1997; Bertagnolo et al., 1997]. This isoform is known to exhibit its mitogenic properties even in the absence of any catalytic activity [Smith et al., 1994] and the absence of regulation at the level of protein cannot preclude a role for PLC-γ1 in the downstream second messenger-mediated signalling of PDGF. Its involvement in more classical RTK-coupled signalling pathways, such as the MAP kinase cascade and mitogenic events, would imply a tight regulation of this isoform were it also involved in other signalling cascades. Perhaps PLC isoforms exhibiting resistance to down-regulation by chronic receptor activation do so due to some commonality between them, which is independent of receptor family, such as nuclear translocation for example.

Although activation and translocation are not synonymous, as PLC-γ1 can translocate in the absence of tyrosine phosphorylation following GPCR activation [Di Salvo and Nelson, 1997], they are usually coincident following growth factor stimulation [Cockcroft and Thomas, 1992; Berridge, 1993; Noh et al., 1995]. Under resting conditions, equilibrium exists between the cytosolic and membrane distribution of most PLC isoforms, although in many cell types PLC-β isoforms are reported as strongly associated with the particulate fraction [Vaziri and Downes, 1992; Rebecchi and Pentyala, 2000]. Results within this chapter support these prior claims with both PLC-β1 and -β3 evenly distributed between the membrane and cytosolic fractions of SH-SY5Y cells. In agreement with general consensus, results presented here identify PLC-γ as a primarily cytosolic enzyme under resting conditions. However, under conditions of serum starvation, there is a small, yet significant, increase in the amount of membrane-associated PLC-γ1. Even then, this enzyme is still preferentially located in the cytosolic fraction of SH-SY5Y cells. This may be a truer representation of the ‘resting’ distribution of this isoform as PLC-γ1.
may be desensitised when cells are maintained in the presence of serum and therefore, growth factors.

Which PLC isoforms are recruited in an agonist-dependent manner? Are there differences in the temporal profiles that can be correlated with downstream signalling events? Does a single agonist recruit multiple isoforms? Determination of the answers to these questions would hopefully provide some insight into the mechanism of intracellular Ca\textsuperscript{2+} store release following either muscarinic or PDGF receptor stimulation.

Only PLC-\(\beta_1\) and -\(\beta_2\) exhibit any significant membrane redistribution following muscarinic receptor activation, but the translocation is slow compared to agonist-mediated elevation of \([\text{Ca}^{2+}]_i\). Both PLC isoforms are also down-regulated in response to 1 mM methacholine and this suggests that both isoforms may play a role in the downstream signalling of this agonist. Although there was no significant translocation of PLC-\(\beta_3\), this isoform has an unusually high membrane distribution in these cells under resting conditions and it may be that sufficient PLC-\(\beta_3\) is already present to mediate any role of this isoform in the downstream signal. These data suggest a complex profile of PLC isoform activation, which may involve PLC-\(\beta_3\) and/or PLC-\(\beta_1\) for the rapid peak of \(\text{Ins}(1,4,5)P_3\) and Ca\textsuperscript{2+} signalling following methacholine, and one or more from PLC-\(\beta_1\), -\(\beta_2\), -\(\beta_3\) and -\(\delta_2\) for the plateau component. Despite statistical analysis refuting any claim that PLC-\(\gamma_1\) is recruited following muscarinic receptor stimulation, both the graph and immunoblot would lend credence to speculation that this potential, transient translocation does in fact occur (and marked translocation was observed in 3 of 4 independent experiments), but more experiments are required to answer this question. Other methods of examining translocation were considered, such as: microinjection or electroporation of PLC antibodies; over-expression of PLC isoforms; and the use of immunocytochemical methods; but all were rejected as one of the strengths of this study was the investigation of endogenous receptors at endogenous levels in a neuronal cell type. PLC-\(\gamma\) visualisation using immunocytochemistry was attempted and although antibody specificity was determined, presumably the endogenous level of PLC-\(\gamma\) and the relatively subtle changes involved resulted in no viable data.
In sharp contrast, PDGF receptor activation recruits only PLC-γ1 to the membrane and no other isoform. This translocation is measurable at 10 min and beyond, not correlating well with the downstream Ca^{2+} signalling. However, given that no Ins(1,4,5)P_{3} is measured in response to PDGF, perhaps PLC-γ1 recruitment is not a requirement for PDGF-mediated elevation of [Ca^{2+}], in the SH-SY5Y cell.

This study therefore demonstrates a lack of correlation between differential agonist-driven translocation of PLC and acute downstream signalling events, such as Ins(1,4,5)P_{3} generation and elevation of [Ca^{2+}].

One important question remained however: was an elevation of [Ca^{2+}] and PKC activation sufficient to drive PLC activation? If this were the case, it would hint at a more general mechanism of PLC activation that was independent of receptor type. Ionomycin and PDBu were used to elevate intracellular [Ca^{2+}] and activate PKC, respectively, independent of ligand-induced receptor-driven events. An agonist-independent elevation of [Ca^{2+}], resulted in no PLC translocation except for PLC-δ2, which migrated from the membrane to the cytosol and was completely cytosolic by 15 min. This could be attributed to some membrane disruption by ionomycin; however, there was no significant effect on the localisation of PLC-β and γ isoforms. Furthermore, this effect was specific for PLC-δ2 that is wholly located in the membrane fraction under normal conditions. This may suggest that agonists that elevate [Ca^{2+}], also regulate the localisation of PLC-δ2. This is further supported by the co-application of ionomycin and PDBu. Under these conditions, PLC-δ2 still exhibited a significant translocation to the cytosolic fraction, but the presence of PDBu, and consequently PKC activation, attenuated this Ca^{2+}-mediated effect. Thus, was Ca^{2+} regulating, in this case desensitising, PLC-δ2 by removing this PLC from the vicinity of its’ substrate? And secondly, was PKC responsible for the attenuation of this Ca^{2+}-induced phenomenon?

These data implied that an elevation of [Ca^{2+}], possibly from a particular source, i.e. extracellular via VOCC or ROCC, may in fact inactivate PLC-δ2 despite its’ Ca^{2+} sensitivity for activation [Allen et al., 1997; Kim et al., 1999]. Both methacholine and PDGF are demonstrated to elevate [Ca^{2+}], within this study. Yet PLC-δ2 is also demonstrated to remain entirely at the membrane under conditions of agonist-mediated receptor activation. This implies that both agonists, that are also
demonstrated to recruit PKC following receptor activation, may be capable of regulating PLC-δ₂ localisation. Alternatively, Ca²⁺ influx may activate PLC-δ, which in turn hydrolyses PtdIns(4,5)P₂. As the PH domain of PLC-δ has an affinity for Ins(1,4,5)P₃ comparable to that for PtdIns(4,5)P₂ [Rebecchi et al., 1992; Cifuentes et al., 1993], the subsequent production of Ins(1,4,5)P₃ may compete PLC-δ off the membrane and into the cytosol. Indeed, this has been recently observed using an overexpressed green fluorescent protein (GFP)-tagged PLC-δ PH domain in cells [Hirose et al., 1999; Nahorski, S.R. – personal communication]. Previous reports using overexpressed GFP-tagged PLC-δ PH domains had reported transient translocation from the membrane following agonist stimulation [Stauffer et al., 1998; Varnai and Balla, 1998] and similar results have been demonstrated with GFP-tagged intact PLC-δ following PtdIns(4,5)P₂ depletion during osmotic stress [Fujii et al., 1999]. However, if sustained agonist-mediated Ins(1,4,5)P₃ production may be PLC-δ-dependent [Kim et al., 1999], a mechanism to maintain membrane localisation of this isoform would seem likely and it should also be considered that the specificity of PLC-δ membrane interactions will provide a limited number of ‘correct’ attachment sites. Indeed, a very recent observation has demonstrated that PLC-δ₁ (PH domain and intact protein) did not distribute uniformly at the membrane of NIH 3T3 cells, but rather is discretely localised to actin-rich membrane regions [Tall et al., 2000], consistent with the notion that PtdIns(4,5)P₂ clusters at focal adhesions [Lee and Rhee, 1995; Rebbechi and Pentyala, 2000]. The presence of the Ca²⁺-dependent phospholipid-binding C2 domain may provide a second membrane anchorage point for PLC-δ [Rizo and Südhof, 1998], which functions only during elevated [Ca²⁺], when an agonist-driven elevation of Ins(1,4,5)P₃ may be saturating the PH domain. Furthermore, the presence of multiple EF-hand domains in PLC-δ isoforms, that do bind Ca²⁺ and are important for activity but do not contribute to the Ca²⁺-sensitivity of this isoform [Drayer et al., 1995], may provide a hydrophobic surface following Ca²⁺ binding capable of mediating protein-membrane attachment. Although agonist-specific activation of PKC may be entirely responsible for this effect, it is more likely that the mechanism is more complex and requires additional regulatory components.
As catalytic activation of PLC-γ₁ is usually coincident with tyrosine phosphorylation, GPCR and RTK-mediated phosphorylation of this isoform was initially investigated in an attempt to implicate PLC-γ₁ in the PDGF-mediated Ca²⁺ release mechanism. The ubiquitous nature of phosphorylation as a regulatory mechanism led to an investigation of the temporal phosphorylation state of the other (PLC-β and δ) isoforms. One important aspect of this study is the inability, within these experimental paradigms, to distinguish tyrosine and serine/threonine phosphorylation.

Only muscarinic receptor activation resulted in an increase in the phosphorylation of PLC-β₁. In contrast, both methacholine and PDGF induced phosphorylation of the PLC-β₃ isoform. Both muscarinic receptor-mediated phosphorylations were attenuated by the addition of the muscarinic receptor antagonist, atropine. The surprising phosphorylation of PLC-β₃ by PDGF was also very rapid and easily observed at 1 min. The function of this 'heterologous' phosphorylation is speculated upon later in this discussion. Together, these data might suggest a role for both PLC-β₁ and –β₃ in the phosphoinositide and Ca²⁺ signalling following muscarinic receptor activation. The slower phosphorylation of PLC-β₁ may suggest an involvement in the later phases of the Ins(1,4,5)P₃ and Ca²⁺ response (plateau), whilst the more rapid and maximal phosphorylation of PLC-β₃ would suggest a role in the peak, early phases. Interestingly, Xu et al., 2001 have recently demonstrated that ERK can phosphorylate nuclear PLC-β₁ in Swiss 3T3 cells resulting in activation of this isoform. Whether this is a general regulatory mechanism or a consequence of localisation of PLC-β₁ within the nucleus is unknown but the data presented here would suggest that this is a complex regulatory mechanism as PDGF receptor activation, which activates ERK in these cells (data presented later, see Chapter 6), does not result in PLC-β₁ phosphorylation.

Although the phosphorylation of PLC-γ₁ is well documented in a variety of cell types, the receptor-mediated phosphorylation of PLC-β and δ isoforms is infrequently examined. However, evidence does support a PLC-β₁ phosphorylation by PKC (α or ε) [Ozawa et al., 1993], whilst PLC-β₂ is a known target for PKA [Liu and Simon, 1996]. More recently, PLC-β₃ has been revealed as a target for not only PKC but also PKA. Dual phosphorylation by PKC and PKA has been proposed
as the molecular basis for differential inhibition of PLC-β₃, which can be activated independently, and simultaneously, by Gαq and Gβγ subunits [Smrcka and Sternweis, 1993; Ali et al., 1997]. This is further supported by the PKC-mediated PLC-β₁ and PKA-mediated PLC-β₂ phosphorylations, as these isoforms are principally Gα and Gβγ activated respectively. This suggested that PKC target sites on PLC specifically inhibit Gα binding, whilst PKA-mediated phosphorylation is specifically inhibiting Gβγ activation [Ali et al., 1997].

Most studies have revealed that elevated cAMP levels suppress PLC activation whilst PKC-mediated phosphorylation had no effect on PLC-β₁ activity in vivo [Rebecchi and Pentyala, 2000 and references therein]. This implies that Gβγ-regulated PLC desensitisation could be responsible for the agonist-mediated desensitisation of downstream phosphoinositide and Ca²⁺ responses. In turn, this suggests a role for PLC-β₃ and -β₂ in these cellular responses, but a primarily nuclear role for PLC-β₁, which is only desensitised by PKC in vitro [Litosch, 1997]. There are no reports of a PKC-dependent phosphorylation of PLC-β₂, the agonist-dependent phosphorylation of which is, unfortunately, missing from this study.

Following PDGF stimulation the phosphorylation of PLC-γ₁ occurred fairly rapidly, within 1 min. The slight delay in phosphorylation is consistent with the lag phase of PDGF-mediated elevation of [Ca²⁺], in response to this agonist. This implies that unless the phosphorylation is not associated with the catalytic function of PLC-γ₁, but with some other function (e.g. as an adaptor protein [Pei et al., 1997]), then PDGF-mediated elevation of [Ca²⁺], would presumably be dependent upon the hydrolysis of PtdIns(4,5)P₂ and the production of localised Ins(1,4,5)P₃. However, following the conclusions drawn in Chapter 4, such as the inability of Ins(1,4,5)P₃ receptor down-regulation or thimerosal sensitisation to affect PDGF-mediated elevation of [Ca²⁺], this also seems unlikely. As will be discussed, the subsequent translocation of PKC isoforms could be dependent upon DAG derived from sources other than the hydrolysis of PtdIns(4,5)P₂.

The phosphorylation of PLC-γ₁ in response to muscarinic receptor activation is transient and, given the rapid elevation of [Ca²⁺], following methacholine stimulation, unlikely to play a major role in downstream phosphoinositide and Ca²⁺ signalling. This is in agreement with previous reports of angiotensin II-mediated
PLC-γ1 phosphorylation that is reported as rapid (by 15 sec) and transient (no visible phosphorylation at 10 min) [Ushio-Fukai et al., 1998]. Although any phosphorylation of PLC-γ1 by trophic factors is most likely on tyrosine residues, there is a serine target, Ser1248. Both PMA and elevated cAMP levels, which activate PKC and PKA respectively, are known to result in serine phosphorylation of this isoform leading to a reduction in tyrosine phosphorylation and activity [Ali et al., 1997]. More likely, PLC-γ1 provides a link between this classical GPCR and pathways normally the province of RTK activation, such as Ras, the MAP kinase cascade and mitogenosis through its adaptor function [Pei et al., 1997]. Consistent with this proposal, muscarinic receptor activation in the SH-SY5Y cell has been demonstrated to significantly increase cell proliferation to the same degree as foetal calf serum (260% and 240%, respectively) [Pam White – personal communication], as demonstrated by [3H]-thymidine incorporation.

PKC is involved or implicated in the regulation of a large number of proteins, enzymes and cellular processes. It is a prolific serine/threonine kinase and thus usually plays a regulatory role by phosphorylation of its target proteins resulting in a modified activity. To complicate the issue however, there are at least 11 known PKC isoforms, which themselves are regulated by multiple mechanisms [Nishizuka, 1995; Newton, 1997; discussed in greater detail in section 1.iv].

Of particular relevance to this study, a PKC isoform is known to phosphorylate PLC-γ1 on Ser1248 as a component of the regulatory mechanism of this signalling molecule, with a subsequent decrease in the tyrosine phosphorylation and activity of this isoform [Rhee and Choi, 1992; Ali et al., 1997]. PKC has also been implicated in the phosphorylation of PLC-β1, which may or may not result in a concomitant decrease in catalytic activity [Ozawa et al., 1993; Filtz et al., 1999; Rebecchi and Pentyala, 2000]. Down-regulation of PKC is reported to significantly increase Ins(1,4,5)P3 production following PDGF stimulation in Swiss 3T3 cells [Miyakawa et al., 1998] and cause an enhancement of PDGF receptor-PLC-γ1 coupling and inositol phosphate formation in WFB cells [Hasegawa-Sasaki et al., 1988], implying a negative modulatory role for PKC. It was possible that our inability to measure Ins(1,4,5)P3 production following PDGF stimulation in the SH-
SY5Y cells (see Chapter 4) was the result of a small, localised production regulated by such a feedback mechanism. Recruitment of certain PKC isoforms to activated PDGF receptors may have indicated DAG production was occurring and thereby implicated PLC in the Ca$^{2+}$ response to this agonist. However, PtdIns(4,5)P$_2$ hydrolysis is not the only cellular source of DAG, as activation of phosphatidylcholine-specific PLC, or breakdown of PA, could also generate DAG without a concomitant increase of cellular Ins(1,4,5)P$_3$ levels.

This prompted an investigation of the agonist-dependent translocation of PKC isoforms following muscarinic, bradykinin and PDGF receptor activation. Again, it must be stressed that translocation is not interpreted as activation, but is used as an indication of the possibility activation has occurred.

Interestingly, all three agonists resulted in the translocation of the 'novel' PKC$\varepsilon$ isoform. This isoform is implicated not only in increased growth rates, but also in the positive regulation of plasma membrane L-type Ca$^{2+}$ channels [Xuan et al., 1994; Mathias et al., 1997]. Evidence in the literature suggested that desensitisation of GPCR signalling by a PKC-mediated phosphorylation of PLC-$\beta_3$ involved either PKC$\alpha$ or PKC$\varepsilon$ [Ozawa et al., 1993; Strassheim et al., 1998]. In contrast to previous reports of carbachol-dependent muscarinic receptor-mediated translocation of PKC$\alpha$ in the SH-SY5Y cell [Willars et al., 1996], neither methacholine, bradykinin nor PDGF initiated the translocation of this isoform in these cells, despite a reproducible translocation by phorbol ester. Although, the reason for this discrepancy is unknown, it is possibly an effect of serum starvation as this study has demonstrated a serum starvation effect on characteristic Ins(1,4,5)P$_3$ generation by methacholine (see Chapter 4). This would imply that PKC$\varepsilon$ may be responsible for some or all of the phosphorylation of PLC-$\beta_3$ reported herein. To further support this conclusion, PDGF also recruits PKC$\varepsilon$ with a temporal profile not dissimilar to that of the PDGF-mediated phosphorylation of PLC-$\beta_3$ reported herein.

PDGF stimulation also resulted in the translocation of the 'atypical' PKC$\zeta$ isoform that has known roles in mitogenesis and activation of MAP kinase signalling [Diaz-Meco et al., 1994; Sanz et al., 1994; Berra et al., 1995]. This is not entirely unexpected as PDGF is a known mitogen in the SH-SY5Y cell.
So can any of the questions regarding an agonist's ability to sequentially activate PLC isoforms and the role of PKC isoforms as determinants of the downstream signalling be answered? In the context of all the results gathered during the course of this study, the most plausible hypothesis is as follows: muscarinic receptor activation by methacholine resulted in a peak and plateau profile of phosphoinositide and Ca\(^{2+}\) responses within the SH-SY5Y cell (see Chapters 3 & 4). This agonist also recruited PLC-\(\beta_1\) and -\(\beta_2\) to the membrane fraction and phosphorylated PLC-\(\beta_1\), -\(\beta_3\) and -\(\gamma_1\). The phosphorylation of PLC-\(\beta_1\) had a similar temporal profile to the translocation. The rapid peak phase of Ins(1,4,5)P\(_3\) and Ca\(^{2+}\) production may be a consequence of PLC-\(\beta_3\) activation, which did not translocate to the membrane but over 50% of which was already present in the membrane fraction. This may or may not be assisted by PLC-\(\beta_1\), of which over 50% of the total cellular protein was also present in the membrane. The proposed PKCe-mediated phosphorylation of PLC-\(\beta_3\) would be one possible explanation or contributory factor for the partial desensitisation of second messenger-coupled signalling which then declines to a sustained plateau level. In these cells, phorbol ester-mediated activation of PKC has been demonstrated to attenuate muscarinic receptor-mediated generation of Ins(1,4,5)P\(_3\) [Willars et al., 1996], whilst within this study, inhibition of PKC by Ro31-8220 resulted in decreased Ins(1,4,5)P\(_3\) production (see chapter 4, Fig 4.iii.). Agonist-independent Ca\(^{2+}\) release was also demonstrated to facilitate this inhibitory effect of PKC [Willars et al., 1996] and together these data may suggest that more than one PLC or PKC isoform is involved in this complex regulation. Established models for desensitisation of Ins(1,4,5)P\(_3\) and Ca\(^{2+}\)-mediated signalling include: receptor phosphorylation and/or internalisation [Tobin and Nahorski, 1993; reviewed in Tobin, 1997; Pitcher et al., 1998]; desensitisation of PtdIns-specific PLC [Rebecchi and Pentyala, 2000 and references therein]; and Ca\(^{2+}\) pool depletion [Fatatis et al., 1994; Willars and Nahorski, 1995b]; but are not thought to include substrate supply [Willars et al., 1998 and references therein].

The sustained signalling component could be a consequence of either PLC-\(\beta_2\) recruitment or PLC-\(\delta_2\) activation secondary to Ca\(^{2+}\) release within the cell. Results here suggested that the membrane association of PLC-\(\delta_2\) was being actively
regulated by the agonist-activated muscarinic receptors. It was always possible that PLC-β₁ was also involved in this phase of the muscarinic receptor-mediated response; however, the translocation of this isoform may be a nuclear translocation in preparation for cell division although the PH domain of PLC-β₁ has been recently demonstrated to bind PtdIns(3)P [Razzini et al., 2000]. PLC-γ₁ translocation (although not significant) may indeed occur and thus, provide a rapid transient link to mitogenic pathways and the transcription of the early genes required for cell division. The phosphorylation of PLC-γ₁ by agonist-activated muscarinic receptors was likely mediated by PKC and designed to prevent catalytic activity, thus allowing the putative transient recruitment, which mirrors phosphorylation, to engage only the adaptor functions of PLC-γ₁ and not its catalytic activity.

In sharp contrast, PDGF recruited only PLC-γ₁ to the membrane and notably phosphorylation of this isoform occurred some 10 min prior to any visible translocation. The phosphorylation may occur on activating tyrosine residues or on inhibitory serine residues; the nature of this phosphorylation is currently unknown. PDGF-mediated elevations of [Ca²⁺], were unlikely to be Ins(1,4,5)P₃-mediated (see Chapter 4) and therefore the catalytic function of PLC-γ₁ was not required by this agonist in the SH-SY5Y cell line. However, the integral role that PLC-γ₁ plays in mitogenesis has an absolute requirement for its presence at the membrane where it clusters at focal contacts, binding to cytoskeletal elements. PtdIns(4,5)P₂ is an integral lipid in the micro-domain of focal contacts and essential for their normal assembly, yet no Ins(1,4,5)P₃ generation was measured in response to PDGF (see Chapter 4). Presumably PDGF utilised PLC-γ₁ only for its trophic and adaptor functions within this cell line. The leading argument both for and against this, is the phosphorylation of PLC-γ₁ by PDGF-AA that did not recruit PLC-γ₁ to the membrane and was unable to elevate [Ca²⁺], within the SH-SY5Y cell (see Chapter 3). This suggested that PLC-γ recruitment following receptor activation might be the deciding factor in the ability of PDGF to elevate intracellular Ca²⁺. However, in response to PDGF-BB stimulation, results within this study demonstrated that PLC-γ₁ was not recruited to the membrane until 5-10 minutes after receptor occupation, by which time the agonist-mediated elevation of [Ca²⁺], had returned to basal levels. In vascular smooth muscle cells, both PDGF-AA and -BB stimulate tyrosine
phosphorylation of PLC-γ1, yet unlike PDGF-BB, PDGF-AA was unable to promote DNA synthesis [Inui et al., 1994]. Interestingly, the PDGFα receptor-PLC-γ complex has been demonstrated to be more stable than the corresponding PDGFβ receptor-PLC-γ complex, but PDGFα receptor activation resulted in less tyrosine phosphorylation of PLC-γ and less Ins(1,4,5)P₃ generation [Eriksson et al., 1995]. This supports the hypothesis that PLC-γ was not recruited solely for its catalytic function that does require phosphorylation-dependent activation of the enzyme.

Why then did PDGF phosphorylate PLC-β₃? Persistent PDGF receptor activation has been demonstrated herein to significantly reduce a subsequent muscarinic receptor-mediated elevation of [Ca²⁺]. This was not an effect of intracellular store depletion by PDGF (see Chapter 3) and may be attributable to this RTK-mediated phosphorylation of PLC-β₃. PDGF recruited two PKC isoforms (ε and ζ) that may be responsible for this phosphorylation and, given that PKCζ has known roles only in mitogenic signalling, it is possible that PDGF utilised PKCε in a manner similar to muscarinic receptors to desensitise this PLC isoform. This further supports the hypothesis that PLC-β₃ was responsible for the early phase of muscarinic receptor-mediated Ins(1,4,5)P₃ and Ca²⁺ signalling, despite its lack of recruitment to the membrane.
6: FUNCTIONAL SIGNIFICANCE OF PDGF-MEDIATED
ELEVATION OF [Ca^{2+}]_{i}

6.i. INTRODUCTION

Much of the present study has concentrated on aspects of phosphoinositide
signalling and the mechanism for a PDGF-mediated elevation of [Ca^{2+}]_{i}. However,
these studies indicated that elevation of cytosolic Ca^{2+} is not a general feature of
RTK activation in the SH-SY5Y neuroblastoma cell (see Chapter 3, Fig 3.i.). Thus,
one of the aims of this study was to try and address the functional role of PDGF-BB-
mediated elevation of [Ca^{2+}]_{i}.

Although elevation of [Ca^{2+}]_{i} clearly has many roles, it must play a specific
role following PDGF receptor activation and that specific function is not required by
other mitogenic RTKs such as the EGF receptor. In SH-SY5Y cells, PDGF evoked
an elevation of [Ca^{2+}], whilst EGF caused no elevation above basal (see Chapter 3,
Fig 3.i.) and both are generally acknowledged to be potent, MAP kinase-activating
mitogenic factors in most cell types. Furthermore, although PDGF-AA-mediated
mitogenicity is unknown in the SH-SY5Y cells, it can be as equally mitogenic as
PDGF-BB in some cell types [Eriksson et al., 1992; Bazenet and Kazlauskas, 1994],
but is often considered a weak mitogen [Inui et al., 1994] and does not elevate
[Ca^{2+}]_{i} in the SH-SY5Y cell (see Chapter 3, Fig 3.ii.). Does Ca^{2+} therefore play a
role in mitogenesis and activation of MAP kinase in response to PDGF-BB?
Activation of the muscarinic receptor population in SH-SY5Y cells also resulted in
an elevation of [Ca^{2+}], (see Chapter 3, Fig 3.ii.) and is a reported mitogen in this cell
(see section 5.iii.) and this prompted the question: how dependent upon Ca^{2+} was
mitogenic signalling following agonist-mediated receptor activation?

However, the integration and/or convergence of RTK and GPCR signalling
may occur independently, antagonistically or synergistically at more than just one
protein downstream of the receptor and there will undoubtedly be receptor-specific
interactions. The GPCR transactivation of PLC-γ and the PDGF and muscarinic
receptor-mediated activation of small GTP-binding proteins of the Ras superfamily
provide two examples that have already been identified and are discussed elsewhere
(see sections 1.i. and 1.ii.iii. and 5.iii.). Furthermore, a growing body of evidence
has indicated that transactivation and cross-talk between RTKs and pertussis toxin-
sensitive, G i-coupled GPCRs may be a widespread aspect of MAPK activation via
these receptor families [Linseman et al., 1995; Rao et al., 1995; Daub et al., 1996;
Daub et al., 1997; Gutkind, 1998; Conway et al., 1999] (see section 1.ii.iii.). In
short, the complexity of RTK and GPCR heterologous regulation is only just
beginning to be appreciated after many years of assuming they were distinct receptor
families employing distinct signalling mechanisms.

Activation of both RTK and GPCR MAP kinase-coupled receptors (see Fig
6.i.) triggers a series of phosphorylation events within the cell involving the
sequential activation of Ras, Raf, MAPK kinase and finally MAP kinase that utilises
a unique dual serine/threonine and tyrosine phosphorylation mechanism. MAP
kinases phosphorylate other regulatory proteins, including transcription factors and
other protein kinases, resulting in the regulation of DNA synthesis and many other
effector proteins. There are several parallel MAP kinase pathways that follow this
premise within most cells and the central elements of the three distinct and well-
characterised MAP kinase pathways are the terminal pathway-specific kinases: ERK
(1 and 2, p44 and p42 respectively), SAPK1/JNK and p38 (see Fig 6.i.). Thus, Ras-
mediated ERK and Rac/Cdc42-mediated JNK and p38 cascades are all independent,
architecturally homologous, MAP kinase pathways that may be activated following
mitogenic or cellular stress stimuli. Often, the SAPK1/JNK and p38 cascades are
activated in response to cellular stress and inflammatory cytokines rather than
mitogens [Cobb and Goldsmith, 1995; Waskiewicz and Cooper, 1995; Kyriakis and
Avruch, 1996; Cuenda et al., 1997; Gutkind, 1998].

In addition to the differences between PDGF-AA and -BB signalling
mentioned earlier, a second line of evidence also strongly suggested a role for
PDGF-mediated elevation of [Ca 2+], in the efficient activation of MAP kinase. The
modulation of ERK activation by PI-3K in response to both RTK and GPCR
activation is well evidenced [Hawes et al., 1996; Kranenburg et al., 1997; Lopez-
Ilasaca et al., 1997; Duckworth and Cantley, 1997; Conway et al., 1999]. PI-3K has
also been reported as an essential component in PDGF receptor-mediated cell
motility [Wennström et al., 1994; Kundra et al., 1994]. Although catalytic
inhibition, expression of a dominant negative p85 sub-unit or disruption of PDGF
receptor and PI-3K association has no effect on chemotactic responses [Higaki et al., 1996; Matsumoto et al., 1999], inhibition of PI-3K has been demonstrated to suppress PDGF-mediated elevations of \([\text{Ca}^{2+}]\), by about 40-50% [Bonser et al., 1991; Vossebeld et al., 1997; Falasca et al., 1998; Rameh et al., 1998], which correlates with the frequently reported attenuation of ERK activation. The proposed mechanism for this partial PI-3K-dependency of PDGF-mediated elevations of \([\text{Ca}^{2+}]\), involves the product of PI-3K activation, PtdIns(3,4,5)P_3. PtdIns(3,4,5)P_3 has been demonstrated to bind directly to the C-terminal SH-2 and the N-terminal PH domains of PLC-\(\gamma_1\) [Falasca et al., 1998; Rameh et al., 1998] and directly activate PLC-\(\gamma\)-dependent PtdIns(4,5)P_2 hydrolysis \textit{in vitro} [Bae et al., 1998]. Thus, PI-3K activation may result in an enhanced membrane association, thereby increasing substrate availability, and/or activity.

Therefore, the logical place to begin trying to understand the functional significance of PDGF-mediated elevation of \([\text{Ca}^{2+}]\), was considered to be the MAP kinase signal transduction pathway. Ideally, a direct study of agonist-driven cell proliferation in the absence of agonist-mediated elevations of \([\text{Ca}^{2+}]\), would have provided the best data for these comparisons. However, protocols to examine proliferation, such as \(^{3}\text{H}\)-thymidine incorporation and DNA absorption spectra, often involve extended incubations, which, in the presence of intracellular \(\text{Ca}^{2+}\)-chelating agents, might have complicated matters and made the results uninterpretable. Therefore, using a \(\text{Ca}^{2+}\)-chelating agent, BAPTA, to inhibit agonist-mediated elevations of cytosolic \(\text{Ca}^{2+}\), the subsequent effect on agonist-driven MAP kinase phosphorylation (as an index of activation) was investigated.
**Fig 6.i.** Divergent protein kinase cascades link both GPCRs and RTKs to MAP kinase activation. A MAP kinase pathway consists of a MAPK kinase kinase (MAPKKK) or MEKK, which activates a MAPK kinase (MAPKK) or MEK that in turn activates a MAP kinase or ERK. Currently 3 mammalian MAPK pathways are distinguished by the terminal kinase: the MAP kinase or ERK pathway; the SAPK/JNK pathway; and the p38 pathway [Gutkind, 1998].
6.ii. RESULTS

Effect of BAPTA on the agonist-mediated elevation of $[Ca^{2+}]$. 

Adherent populations of serum-starved SH-SY5Y cells were loaded for 60 min in KHB (containing fura-2-AM) with or without the addition of BAPTA-AM (30 μM) [Tsien, 1980; Masgrau et al., 2000]. Prior to agonist stimulation, PDGF-BB (200 ng.ml$^{-1}$), EGF (200 ng.ml$^{-1}$) and methacholine (1 mM), the cells were transferred to nominally Ca$^{2+}$-free KHB to ensure no elevation of cytosolic Ca$^{2+}$ occurred.

Muscarinic receptor activation by methacholine routinely evoked a rapid (<10s), transient peak elevation of $[Ca^{2+}]_i$ (598 ± 36 nM, n = 3), that returned to basal levels within 2 min (see Fig 6.ii. A). EGF receptor activation exhibited no elevation of $[Ca^{2+}]_i$ (see Fig 6.ii. B) and PDGF stimulation, following a lag phase (20-60s), resulted in a slow transient elevation of $[Ca^{2+}]_i$ (103 ± 6 nM, n = 3) that had returned to basal levels within 4 min (see Fig 6.ii. C).

In contrast, the presence of BAPTA within the cell caused complete inhibition of methacholine and PDGF-mediated elevations of $[Ca^{2+}]_i$ and had no effect on EGF, which did not elevate $[Ca^{2+}]_i$ (see Fig 6.ii. all panels).
Fig 6.ii. $[Ca^{2+}]_i$ in adherent, control (solid lines) and BAPTA-loaded (broken lines) SH-SYSY cells following stimulation with A) methacholine, B) EGF, and C) PDGF at the concentrations indicated and in nominally $Ca^{2+}$-free KHB. Data are representative of at least three independent experiments.
**Effect of BAPTA on the agonist-mediated phosphorylation of ERK.**

Adherent populations of serum starved SH-SY5Y cells plated into 6 well multidishes were stimulated with PDGF-BB (200 ng.ml⁻¹), EGF (200 ng.ml⁻¹) or methacholine (1 mM) following a 60 min preincubation in KHB with or without the addition of BAPTA-AM (30 μM). Cells pre-loaded with BAPTA were then stimulated in nominally Ca²⁺-free KHB to ensure no elevation of cytosolic Ca²⁺ occurred, whilst ‘control’ agonist-mediated ERK phosphorylation was generated in the presence of 1.3 mM [Ca²⁺]ₑ.

Muscarinic receptor activation in the absence of BAPTA caused a rapid phosphorylation of ERK (1 and 2) that was near maximal at 5 min, peaked at 30 min (3.30 ± 0.23, fold over basal, n = 4) and was sustained to the limit of the 60 min time course. In BAPTA-loaded cells, methacholine evoked a similar rapid phosphorylation of ERK that peaked at 15 min (3.44 ± 0.53, fold over basal, n = 4) and again was sustained to the limit of the time course (see Fig 6.iii. A). There was no statistically significant difference between results obtained in the absence or presence of BAPTA over the time course studied (as determined by Student’s 𝑡-test on individual time points).

Activation of the EGF receptor had a similar effect in either the presence or absence of BAPTA and resulted in rapid ERK phosphorylation that peaked at 5 min and 15 min, respectively (3.71 ± 0.54 and 3.28 ± 0.33, respectively, fold over basal, n = 4). ERK phosphorylation then slowly declined over the remainder of the 60 min time course (see Fig 6.iii. B).

In contrast to both methacholine and EGF, stimulation with PDGF evoked a slower, more transient phosphorylation of ERK in both the presence and absence of BAPTA. In both cases, the peak of ERK phosphorylation was identified at 15 min (2.78 ± 0.50 (+BAPTA) and 3.74 ± 0.27, (+Ca²⁺), fold over basal, n = 4) and there was a significant inhibition of PDGF-mediated ERK phosphorylation at this time point following BAPTA loading of the cells (26% inhibition, p<0.05, see Fig 6.iii. C).
**Fig 6.iii.** A 60 min time course of ERK phosphorylation in SH-SY5Y cells stimulated with A) methacholine (1 mM), B) EGF (200 ng.ml⁻¹), and C) PDGF-BB (200 ng.ml⁻¹). Cells were pre-incubated for 60 min in either (■) KHB, and then stimulated in presence of 1.3 mM [Ca²⁺]₀ or (▲) KHB containing 30 μM BAPTA-AM, and then stimulated in nominally Ca²⁺-free KHB. Data are mean ± S.E.M. (n = 4). The phospho-ERK antibody recognised only two bands corresponding to p42/p44, ERK-2 and ERK-1 respectively. Immunoblots are representative of four independent experiments. (*p<0.05, Student’s t-test)
6.iii. DISCUSSION

These data demonstrate a functional relevance of PDGF-mediated elevation of \([\text{Ca}^{2+}]\). Loading the cells with BAPTA-AM [Tsien, 1980], which is converted to BAPTA within the cell by non-specific mono-esterases, abolished agonist-mediated elevations of \([\text{Ca}^{2+}]\), in response to muscarinic and PDGF receptor activation. Importantly, BAPTA-loading has been demonstrated to have no effect on agonist-mediated Ins(1,4,5)P\(_3\) generation [Kim et al., 1999] Using the agonist-mediated phosphorylation of ERK 1 and 2 as an index of MAP kinase activation [Cobb and Goldsmith, 1995], only PDGF-mediated ERK phosphorylation was identified as \(\text{Ca}^{2+}\)-dependent. Clearly, a more extensive investigation would be required to dissect the mechanism of this \(\text{Ca}^{2+}\)-dependence, and even measurements of ERK activity would have been useful in correlating ERK phosphorylation with activation. Unfortunately, due to time considerations, these questions, such as: does the source of \(\text{Ca}^{2+}\) (i.e. extracellular/intracellular) affect MAPK activation?; What is the effect, if any, of reducing the magnitude of PDGF-mediated peak ERK phosphorylation?, will have to remain unanswered.

PDGF, EGF and muscarinic receptors can all activate multiple signalling pathways within most cells (see section 1.i.) and all are known activators of MAP kinase signalling pathways. In neurons, MAP kinase activation has been implicated to play roles in synaptic plasticity, memory and learning aside from more classical trophic effects [Nathanson, 2000]. Increasingly, the mechanisms of potential cross-talk (i.e. transactivation and desensitisation) between RTK and GPCR MAP kinase-coupled receptors are becoming the subject of research, but as yet, are poorly understood (for fuller discussion see section 1.ii.iii.). Convergence of RTK and GPCR MAP kinase activation was originally suggested at Raf and this hypothesis was supported by potentiation of phorbol ester and EGF-mediated MAP kinase activation in Cos-1 cells over-expressing Raf [Howe et al., 1992]. However, Gβγ-mediated activation of Ras, which is a small GTP-binding protein that lies directly upstream of Raf in proposed signal transduction pathways, is now widely acknowledged as the most likely point of convergence for signals emerging from GPCRs with those generated by RTKs (see Fig 6.i.) [Koch et al., 1994; Malarkey et
al., 1995; reviewed in Gutkind, 1998]. The potential for Ca\textsuperscript{2+}-induced regulation of agonist-mediated MAP kinase activation is therefore, huge.

Accumulating evidence in the literature supports the widely held view that the important factors regarding MAP kinase signal transduction are not just MAP kinase activation, but more importantly the strength and duration of the signal. The findings presented within this chapter are in line with this hypothesis, as methacholine, EGF and PDGF all stimulated ERK phosphorylation with differing temporal profiles. What is interesting about these findings is the implication that the distinct Ca\textsuperscript{2+}-dependent and independent pathways for MAP kinase activation are not the reserve of specific receptor families. Both EGF and PDGF are classical RTKs and whilst efficient PDGF-mediated ERK phosphorylation had a partial requirement for Ca\textsuperscript{2+}, EGF-mediated ERK phosphorylation did not.

PDGF, EGF and muscarinic receptor activation are widely acknowledged to stimulate PKC-dependent and PKC-independent, Ras-dependent or independent ERK and SAPK1/JNK pathways in various cell types [reviewed in Avruch et al., 1994; Cano and Mahadevan, 1995; Malarkey et al., 1995; Heldin and Westermark, 1999 and references therein]. Calcium has also been demonstrated to play a key role in Ras-dependent ERK activation in neuronal cells [Finkbeiner and Greenberg, 1996; Ebinu et al., 1998] and Ras has been demonstrated to directly activate Ca\textsuperscript{2+} channels in neuroblastoma x glioma hybrid cells [Hescheler et al., 1991]. This suggests that in the SH-SY5Y neuroblastoma cell line, PDGF-mediated ERK activation was Ras-dependent whilst EGF and muscarinic M\textsubscript{3} receptors may activate a Ras-independent mechanism. As PLC-coupled muscarinic M\textsubscript{1} receptors can transactivate the EGF receptor [Huang et al., 1993; Tsai et al., 1997], it was entirely possible that EGF and muscarinic receptors were utilising a similar mechanism. However, very recent evidence would suggest otherwise. Thus, both c-src and Pyk2 kinases are essential for GPCR-induced tyrosine phosphorylation of the EGF receptor, but neither the EGF receptor, c-src or Pyk2 are required for MAP kinase activation [Andreev et al., 2001].

PDGF-mediated activation of p38, an ERK homologue, has been identified in porcine aortic endothelial (PAE) cells and TIG103 fibroblasts, resulting in cell motility responses such as cell migration and actin reorganisation, but was not required for DNA synthesis. This activation of p38 was independent of PLC-\(\gamma\),
SHP-2, c-src, RasGAP, Crk and PI-3K, all proteins implicated in ERK activation, but did require Ras [Matsumoto et al., 1999] rather than Rho-family GTPases, Rac-1 or Cdc42 which are well established as the regulators of RTK-induced motility responses [Ridley et al., 1992; Nobes and Hall, 1995; Kyriakis and Avruch, 1996; Hooshmand-Rad et al., 1997; Gutkind, 1998]. In PAE cells, PDGF did not activate JNK, although JNK activation has been reported in response to PDGF receptor α-chain, but not β-chain, stimulation in NIH 3T3 fibroblasts and may therefore be a consequence of receptor isoform-specific events [Yu et al., 2000]. Interestingly, others have failed to show PDGF-mediated JNK activation in NIH 3T3 cells [Coso et al., 1995]. The reasons for this discrepancy are unclear but could be a consequence of clonal variation in receptor isoform expression.

There is a wealth of contradictory evidence available with regard to growth factor signalling, for example: often PDGF-AA and –BB are considered equally potent mitogens [Eriksson et al., 1992], yet PDGF-AA is generally considered a weak mitogen in vascular smooth muscle due to an inability to phosphorylate RasGAP [Inui et al., 1994]. In those cell types for which PDGF-AA is mitogenic a Ras-independent activation of ERK is the favoured hypothesis. It is clear that events tend to be cell-type specific (even varying between clones of the same cell) and therefore the most crucial evidence is generated from the same cell line.

The results of the present investigation are consistent with previous reports where EGF and muscarinic receptor activation in SH-SY5Y cells resulted in increased tyrosine phosphorylation and activity of p42 ERK. In the case of muscarinic receptors, this was a PKC-dependent event that could be reproduced by phorbol ester challenge and attenuated by PKC inhibition, whilst EGF-mediated MAP kinase activation was PKC-independent [Offermanns et al., 1993]. Also, mitogenicity of PDGF and EGF has been demonstrated in SH-SY5Y cells and resulted in a 300% and 250% increase in cell number over 24 hours, respectively (assessed by [3H]-thymidine incorporation) [Pam White – personal communication]. Although the muscarinic M3 receptor is identified as the predominant PLC-coupled muscarinic receptor in SH-SY5Y cells, and is reported to be Gq/G11 as well as Gq-coupled when transfected into CHO cells and over-expressed [Burford et al., 1995], there is an SH-SY5Y population of Gi-coupled muscarinic M2 receptors [Lambert et
al., 1989] that do not activate PLC but can stimulate MAP kinase activation [Winitz et al., 1993; Wylie et al., 1999].

Thus, perhaps elevation of [Ca\textsuperscript{2+}] and MAP kinase activation are receptor subtype-specific events, although the muscarinic M\textsubscript{3} receptor has been demonstrated to efficiently activate both ERK and JNK when transfected into CHO cells whilst muscarinic M\textsubscript{2} receptors activated only ERK. In these CHO cells, ERK activation following muscarinic M\textsubscript{2} and M\textsubscript{3} receptor activation was demonstrated as a Ca\textsuperscript{2+}-independent and PKC-dependent event [Wylie et al., 1999], which is in agreement with the results of this study and previous reports [Cobb and Goldsmith, 1995; Marais et al., 1998]. This ERK activation was a muscarinic M\textsubscript{3} receptor phosphorylation-dependent event, which had no endocytotic requirement [Budd et al., 1999; Budd et al., 2001], contrasting with similar studies on the \(\beta_2\)-adrenergic receptor [Daaka et al., 1998]. In contrast, JNK activation by muscarinic M\textsubscript{3} receptors was demonstrated as a Ca\textsuperscript{2+}-dependent event whilst muscarinic M\textsubscript{2} receptors did not significantly activate JNK in CHO cells [Wylie et al., 1999]. It may therefore, be of interest to ascertain whether PDGF can activate JNK in the SH-SY5Y cells and whether Ca\textsuperscript{2+} has any effect on this MAP kinase pathway. Furthermore, JNK activation is generally reported following activation of receptors coupled to PtdIns(4,5)P\textsubscript{2} hydrolysis [Bogoyevitch et al., 1995; Zohn et al., 1995; Shapiro et al., 1996; Ramirez et al., 1997], which does not occur following PDGF receptor activation in SH-SY5Y cells (see section 4.ii.).

To conclude, the numerous identified mechanisms for both GPCR and RTK activation of MAP kinase signal transduction complicate any irrefutable conclusions based on the results presented within this Chapter and will most likely be cell-type-specific events. However, what is clear from these findings is that PDGF-mediated activation of ERK is partially dependent on elevation of [Ca\textsuperscript{2+}], whilst EGF and muscarinic receptors in the SH-SY5Y neuroblastoma cell recruit a Ca\textsuperscript{2+}-independent mechanism for ERK activation.
Chapter 7: SUMMARY AND CONCLUDING DISCUSSION

The initial aims of this study encompassed the investigation and comparison of neuronal phosphoinositide-specific PLC-coupled signal transduction mechanisms presumed to function following the activation of GPCRs and RTKs. Although there is now widespread evidence for the recruitment of PLC-γ by GPCRs, either directly or by transactivation of RTKs, the previous assumption was that GPCRs activate PLC-β, whilst RTKs activate PLC-γ. Signalling via these two receptor families was previously considered as separate and distinct events and only now is it appreciated that the heterologous interactions and regulation of different receptor families may be far more frequent and complex events.

Both GPCRs and RTKs can activate PLC-mediated, Ins(1,4,5)P₃-dependent, intracellular Ca²⁺ store release mechanisms in a cell type-dependent manner. Agonist-driven generation of Ins(1,4,5)P₃, and subsequent elevation of [Ca²⁺]ᵢ, can display complex temporal profiles and this suggested the possible involvement of more than one PLC isoform. Furthermore, growth factors are often identified with trophic cell effects, with scant information available regarding acute RTK-mediated signalling. Therefore, initial protocols were designed to answer the questions: do growth factor-mediated elevations of [Ca²⁺]ᵢ occur in a neuronal context, and if they do, what is the mechanism and function of this response? And secondly, are the different phases of complex GPCR and RTK-mediated phosphoinositide signalling a consequence of multiple PLC isoform recruitment by agonist occupied receptors?

This study utilised the SH-SY5Y human neuroblastoma cell line that endogenously express several GPCRs (including muscarinic, bradykinin and LPA receptors) and RTKs (including EGF and PDGF), which would therefore couple to endogenous signalling mechanisms. An insight into the signalling mechanisms in these cells could provide a basis for future studies in primary neurons, where the regulation of signal transduction mechanisms at synapses is undoubtedly more complex.
As stated previously, activation of GPCRs is presumed to activate PLC-β isoforms and accumulating evidence in the literature would also suggest that PLC-γ recruitment is a widespread phenomenon amongst receptors of this family. RTKs have been assumed to activate only PLC-γ and there is currently no evidence for an RTK-mediated regulation or recruitment of other PLC isoforms. Furthermore, the possibility exists that any receptor or membrane depolarisation-mediated elevation of \([\text{Ca}^{2+}]\), could activate the most \(\text{Ca}^{2+}\)-sensitive, PLC-δ isoform. Thus, one of the aims of this investigation, which was to try and define which PLC isoforms were involved in mediating phosphoinositide-linked signal transduction mechanisms, is complicated by the existence of some 10 PLC isoforms discovered to date, several of which are known to activate multiple signalling pathways.

Very recently, a novel PLC isoform with RasGEF (GTPase exchange factor activity for Ras) activity has been identified and has been described as the founding member of a new, 4th family of PLC isoforms, PLC-ε (~210 kDa), based on predicted structure [Lopez et al., 2001; Song et al., 2001]. This PLC isoform is closest in homology to PLC-β isoforms, is activated specifically by \(\text{G}_\alpha_{12}\) and may provide the missing link for receptors such as the thrombin and LPA receptors that activate \(\text{G}_\alpha_{12}\) Ras-dependent MAP kinase pathways through an unidentified mechanism [Wadsworth et al., 1997]. Interestingly, very few effector molecules are known for the \(\text{G}_{12/13}\) family of G-proteins, which have identified roles in cellular and cytoskeletal changes [Gutkind, 1998]. Recently another GEF, p115RhoGEF has been identified as a specific \(\text{G}_\alpha_{13}\)-coupled effector [Kozasa et al., 1998]. Both PLC-ε and p115RhoGEF exhibit GEF activity, but for two distinct families of small G-proteins, Ras and Rho. Importantly, over-expressed PLC-ε in Cos-7 cells is recruited to the membrane following EGF receptor stimulation in a Ras-dependent manner and may provide another phosphoinositide-coupled effector for activated RTKs [Song et al., 2001].

In the current study, the emphasis was on agonist-mediated elevations of \(\text{Ins}(1,4,5)\text{P}_3\), and subsequent elevation of \([\text{Ca}^{2+}]_i\), and the determination of which PLC and PKC isoforms were recruited following GPCR and RTK receptor occupancy. The PLC-linked muscarinic \(\text{M}_3\) receptor has already been identified in
SH-SY5Y cells and subsequent phosphoinositide signalling has been well characterised. However, a comparative study required the identification of a paradigm PLC-linked RTK. Following a screen of the SH-SY5Y cells with several growth factors, a PDGF-BB-mediated elevation of $[\text{Ca}^{2+}]_i$ was identified. Although originally presumed to be phosphoinositide-linked for $\text{Ca}^{2+}$, the PDGF receptor-mediated intracellular $\text{Ca}^{2+}$ store release mechanism was investigated and determined to be an apparently novel and Ins(1,4,5)P$_3$-independent mechanism.

However, what was clear from the results of this present investigation was that, despite PLC-γ recruitment by ligand-activated PDGF receptors, which is in accord with the generally held view of RTK signalling, an elevation of $[\text{Ca}^{2+}]_i$ was not a general feature of RTKs in the SH-SY5Y cells. Only, PDGF-BB, not PDGF-AA, EGF, bFGF or NGF resulted in an elevation of $[\text{Ca}^{2+}]_i$. PDGF-AA, which did not elevate $[\text{Ca}^{2+}]_i$ in these cells did stimulate PLC-γ1 phosphorylation but resulted in no increased membrane localisation. This might suggest that phosphoinositide hydrolysis is also not a general feature of RTK signalling in neuronal cell types, despite Zhang et al., 1996 reporting PDGF-mediated inositol phosphate production in N1E-115 neuroblastoma cells.

Indeed this does appear to be the case for PDGF-mediated signalling in SH-SY5Y cells, as PDGF gated intracellular, thapsigargin-sensitive $\text{Ca}^{2+}$ stores, yet no generation of Ins(1,4,5)P$_3$ was measured and no accumulation of $[^3\text{H}]$-InsP$_x$ occurred against a Li$^+$ block. Furthermore, down-regulation of the Ins(1,4,5)P$_3$ receptor, inhibition by heparin or sensitisation by thimerosal had no effect on PDGF-mediated $\text{Ca}^{2+}$ store release. Thus, this study proposes that PDGF-mediated elevations of $[\text{Ca}^{2+}]_i$ in the SH-SY5Y cells is a novel and as yet unidentified Ins(1,4,5)P$_3$-independent mechanism. Several other plausible mechanisms have been identified, such as: S-1-P; cADPR; and NAADP for example; and although this study demonstrated no S-1-P-dependent PDGF-mediated $\text{Ca}^{2+}$ release mechanism, due to time considerations every possibility could not be investigated.

The muscarinic receptor agonist methacholine exhibited biphasic elevations of $[\text{Ca}^{2+}]_i$ and global Ins(1,4,5)P$_3$ and an accumulation of $[^3\text{H}]$-InsP$_x$ against a Li$^+$ block of inositol monophosphate metabolism. Both PDGF-BB and methacholine-mediated $\text{Ca}^{2+}$ responses were composed of intracellular $\text{Ca}^{2+}$ store release and $\text{Ca}^{2+}$ influx.
across the plasma membrane. Protocols resulting in depletion of the intracellular Ca\(^{2+}\) store, such as thapsigargin and methacholine prestimulation completely inhibited the elevation of [Ca\(^{2+}\)]\(_i\) to a subsequent challenge with PDGF-BB. Antagonism of muscarinic receptor signalling with atropine allowed the PDGF-mediated elevation of [Ca\(^{2+}\)]\(_i\) to return in a time-dependent manner. In contrast, prestimulation with PDGF was unable to ablate a subsequent Ca\(^{2+}\) response when challenged with methacholine, although a significant reduction did occur. This heterologous desensitisation was even more apparent in a nominally Ca\(^{2+}\)-free buffer and did not occur as a consequence of intracellular Ca\(^{2+}\) store depletion. Challenging SH-SY5Y cells with the Ca\(^{2+}\) ionophore, ionomycin, demonstrated that whilst methacholine was efficiently depleting the intracellular Ca\(^{2+}\) store, PDGF was unable to significantly reduce the content of the store and thus, must rely more heavily on extracellular Ca\(^{2+}\) to mediate intracellular effects.

This study also demonstrated a similar PDGF-mediated heterologous regulation of LPA receptors, which in the SH-SY5Y cells do not rely on PLC but S1P for elevation of [Ca\(^{2+}\)]\(_i\). The identification of these regulatory mechanisms is beyond the scope of this thesis but has been discussed elsewhere (see section 3.iii. and 5.iii.).

This study identified a number of PLC isoforms (PLC-\(\beta_1\), -\(\beta_2\), -\(\beta_3\), -\(\gamma_1\) and -\(\delta_2\)) and PKC isoforms (PKC\(\alpha\), \(\gamma\), \(\epsilon\), \(\iota\), \(\lambda\) and \(\zeta\)) within the human SH-SY5Y neuroblastoma cell. Down-regulation of all three PLC-\(\beta\) isoforms and PLC-\(\delta_2\) following chronic exposure to methacholine suggested a muscarinic receptor-mediated regulation of these isoforms and acute receptor activation resulted in the agonist-mediated translocation of just two of these isoforms, PLC-\(\beta_1\) and -\(\beta_2\). In contrast, PLC-\(\beta_1\), -\(\beta_3\) and -\(\gamma_1\) were all phosphorylated within 5 min of methacholine challenge. Chronic PDGF receptor activation with PDGF-BB, resulted in a significant elevation of PLC-\(\beta_3\), -\(\gamma_1\) and -\(\delta_2\) protein and acute receptor activation resulted in translocation of only PLC-\(\gamma_1\), which along with PLC-\(\beta_3\) exhibited rapid agonist-dependent phosphorylation.
Translocation of a single PKC isoform, the 'novel' PKCε was observed following both muscarinic and bradykinin GPCR activation. PDGF receptor activation recruited the 'atypical' PKCζ isoform to the membrane as well as PKCε.

Dissection of a specific PLC isoform activity is difficult as most PLC activity measurements account for all PLC isoforms within a cell. Therefore other methods, such as recruitment and/or phosphorylation, are useful indices of agonist-mediated PLC activation. PDGF-BB stimulation did result in translocation of PLC-γ from the cytosol to the membrane after 10 min, but this did not correlate well with agonist-driven elevations of [Ca²⁺]. Phosphorylation of PLC-γ also occurred in response to this agonist, at a much earlier time of 1 min, which was more in accord with elevation of [Ca²⁺], than translocation, but without determining the tyrosine and serine/threonine components of this phosphorylation, no definite conclusions regarding the activation of PLC-γ can be drawn from these data. The rather more complex translocation and phosphorylation profiles in response to muscarinic receptor activation suggest that the hypothesis of multiple PLC isoform recruitment following receptor activation is a valid one and is discussed in more detail previously (see section 5.iii.).

The current study has demonstrated a functional relevance of PDGF-mediated elevation of [Ca²⁺]. Although this is unlikely to be the sole effect of PDGF-mediated elevations of [Ca²⁺], indeed a Ca²⁺-dependent depression of PDGF-mediated NMDA receptor currents has been identified in hippocampal pyramidal neurons [Lei et al., 1999], MAP kinase activation is a signalling pathway common to RTK activation and thus lends itself well to this comparison rather than a cell type or receptor-specific event. Using the agonist-mediated phosphorylation of ERK 1 and 2 as an index of MAP kinase activation [Cobb and Goldsmith, 1995], only PDGF-mediated ERK phosphorylation was identified as Ca²⁺-dependent.

In summary, this Thesis investigated GPCR and RTK phosphoinositide-coupled signal transduction in a neuronal cell type. The PDGF receptor was identified as distinct among several endogenous RTKs due to an ability to elevate [Ca²⁺], suggesting acute signalling at this level might play a specific role(s) following
activation of this RTK. One such role was identified as a $\text{Ca}^{2+}$-dependent component of MAP kinase activation in response to PDGF stimulation in SH-SY5Y cells.

But, how relevant is this to neuronal signalling? Terminally differentiated neurons are unlikely to require mitogenic stimuli, and although PDGF has been demonstrated to play anti-apoptotic roles in some cell types, other survival factors, such as NGF and the family of brain-derived neurotrophic factors are predominant in neuronal survival. The fact that PDGF has been demonstrated to have acute effects in neurons such as: modulation of type A $\gamma$-aminobutyrate receptors, sodium channels and NMDA receptors; and inhibition of the excitatory transmission between CA1 hippocampal neurons, through a Src and $\text{Ca}^{2+}$-dependent mechanism [Valenzuela et al., 1995; Valenzuela et al., 1996; Hilborn et al., 1998; Lei et al., 1999]; emphasises the importance of future studies in defining the PDGF-mediated mechanism of elevation of $[\text{Ca}^{2+}]$, in neuronal cell types.
APPENDIX A

Alterations in PLC and PKC Isoform expression following RA-induced SH-SY5Y cell differentiation.

A preliminary investigation into the possible mechanism for the reduced elevation of \([\text{Ca}^{2+}]\), in response to PDGF and methacholine in the SH-SY5Y_Ra6 cells (see Chapter 3, Fig 3.viii.) examined the protein levels of the PLC and PKC isoforms (see Figs A.i. and A.ii., respectively). A reduction in the expression levels of the PDGF receptor has been previously demonstrated in 3T3-L1 cells following differentiation [Vaziri and Faller, 1996]. Although this may occur in SH-SY5Y cells and be entirely responsible for the reduction in agonist-mediated elevation of \([\text{Ca}^{2+}]\), alterations in PLC and PKC expression levels is often reported following cell differentiation (see later).

Both PLC and PKC isoforms are involved in multiple signalling pathways in differentiated and undifferentiated neuroblastoma cells and neurons. Alterations in the expression levels of both PKC and PLC have defined, cell-type-specific roles during and after differentiation, but what has only recently been appreciated, is the significance of the nuclear phosphoinositide cycle in this process.

Following RA-induced differentiation, the level of all PLC isoforms decreased, the most dramatic changes exhibited by PLC-\(\beta_2\) and \(\delta_2\), which were reduced to undetectable levels (see Fig A.i.).

In contrast, these preliminary observations suggested some PKC isoforms were up-regulated (\(\iota\) and \(\zeta\)), whilst others were down-regulated (\(\alpha\)) or remained unaltered (\(\epsilon, \gamma\) and \(\lambda\)) (see Fig A.ii.).
Fig A.i. The expression levels of PLC isoforms in SH-SY5Y<sub>R6</sub> cells compared to undifferentiated (control, 100%) SH-SY5Y cells. Data are mean (n = 2).

Fig A.ii. The expression levels of PKC isoforms in SH-SY5Y<sub>R6</sub> cells compared to undifferentiated (control, 100%) SH-SY5Y cells. Data are mean (n = 2).
One of the major difficulties with obtaining and interpreting these data was the problem of uneven cell growth. Differentiating SH-SY5Y_{R_{A6}} cells grow very much slower than their undifferentiated counterparts and cell lysate protein was equalized prior to gel loading. Of course, this makes the assumption that both SH-SY5Y and SH-SY5Y_{R_{A6}} cells express equivalent amounts of total protein.

Although, PLC-β₁ is considered to be the major nuclear PLC isoform, PLC-β₂, -β₃, -γ₁ and -γ₂ have all been identified in the nucleus of various cells [Bertagnolo et al., 1997; Zini et al., 1997; Neri et al., 1998]. It is generally agreed that the nuclear phosphoinositide cycle (PLC-mediated and recruiting PKC) is vital for cell differentiation processes, but alterations in expression levels following differentiation may be cell type specific. For example, PLC-γ₁ is increased following differentiation of cardiac myocytes [Lee et al., 1995a], yet decreased following U937 cell differentiation [Lee et al., 1995b]. Interestingly, elevated levels of PLC-γ₁ have been implicated in apoptosis following cell differentiation [Min et al., 1999]. Similarly, PLC-β₃ and -γ₁ expression was decreased following RA-induced differentiation of NT2-N cells [Novak et al., 2000] yet PLC-β₃ remains unchanged in the osteoblast-like MC3T3-E1 cells [Dohjima et al., 1997]. Data from the present study show the loss of two isoforms, PLC-β₂, which has been suggested to play a key role in differentiation [Bertagnolo et al., 1997] and PLC-δ₂. It is possible that PLC-β₂ is not required following differentiation and most likely that the expression of another PLC-δ isoform is induced following the loss of PLC-δ₂. Further work is needed to confirm these speculations.

Interestingly, although ERK-mediated phosphorylation of nuclear PLC-β₁ has been implicated in activation and mitogenesis of IGF-I stimulated Swiss 3T3 cells [Xu et al., 2001], nuclear and not cytoplasmic PLC-β₁ has also been implicated in inhibition of differentiation in Friend erythroleukemia cells [Matteucci et al., 1998].

Alterations in PKC isoform expression levels following cell differentiation is an even more complex subject, but major roles for some PKC isoforms commonly occur in the differentiated phenotype in a cell type-independent manner. PKC₀ has been implicated in the process of differentiation in rat and human skeletal muscle.
[Chalfant et al., 2000; Boczan et al., 2000], PC-12 cells [Sparatore et al., 2000b] and LAN-5 human neuroblastoma cells [Sparatore et al., 2000a]. The same reports also indicate that following differentiation; this isoform is down-regulated and remains at low levels. In both neuronal-like cell types, PC-12 and LAN-5, PKC\(\theta\) was identified as primarily nuclear and peri-nuclear and translocated to the cytosol following differentiation.

PKCe and \(\beta\) isoforms are often up-regulated following neuronal differentiation, but in contrast to PKC\(\beta_{(I \text{ and } II)}\), PKCe remains high in the differentiated cell [Yoshimura et al., 1997; Oehrlein et al., 1998;]. PKCe has been implicated in neurite outgrowth in differentiated human neuroblastoma [Ziedman et al., 1999; Silei et al., 2000] and PC-12 cells [Brodie et al., 1999], probably via MAP kinase activation. Interestingly, in U937 cells, Fc\(\gamma\) receptor I activation recruits essentially Ca\(^{2+}\)-independent PKC isoforms to the membrane (\(\delta, \varepsilon\) and \(\zeta\)), yet following differentiation recruits classical Ca\(^{2+}\)-dependent isoforms (\(\alpha, \beta\) and \(\gamma\)) [Mendelez et al., 1999].

PKC\(\zeta\), which has long been implicated in mitogenesis, also translocated to the nucleus during RA-induced differentiation of HL-60 cells [Bertolaso et al., 1998], and is necessary but not sufficient for differentiation. Furthermore, over expression of the atypical PKC\(\iota\) or \(\zeta\) isoforms increases neurite outgrowth during NGF-induced PC-12 differentiation, whilst removal of these isoforms blocks differentiation [Wooten et al., 1999]. PKC\(\iota\) was also demonstrated to play a major role in survival signalling, essential for inhibition of apoptosis following serum withdrawal in these cells.

Isoform switching is generally thought to occur at the level of protein and the expression of different PLC and PKC isoforms is tightly coupled to neuronal differentiation, and even to the distinct phases of differentiation. The complex events following PLC-coupled receptor activation can drive both cytoplasmic and nuclear phosphoinositide signalling. Dissection of these spatially distinct cycles, which may activate simultaneously, is proving difficult and the available literature is suggestive of increasingly complex and possibly cell type specific mechanisms.
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