Regulation of α_1-adrenoceptor-linked phosphoinositide breakdown in cultured glia: role of protein phosphatases

by

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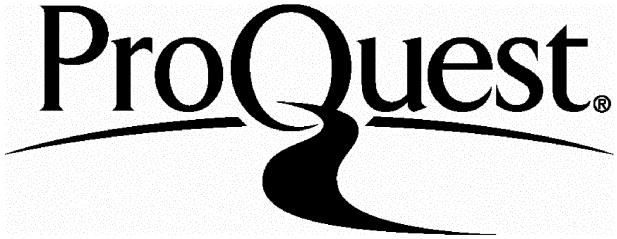

For my Parents

زدانش به اندر جهان چیز نیست

تن مرده و جان نادان یکی است

فردوسی طوسی

"There is nought in the world better than learning

Without this the lifeless body and ignorant soul are one"

Ferdowsi, Persian Poet, 940-1020 AD

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This study attempts to identify the protein phosphatases and kinases involved in regulating receptor-mediated phosphoinositide metabolism in astrocytes derived from newborn rat cerebral cortex and maintained in culture. Noradrenaline produced a dose and time dependent accumulation of [^{3}H] inositol phosphates which was mimicked by A61603 and blocked by RS17053 indicating the involvement of α_{1A}-adrenoceptors in this response. Okadaic acid inhibited noradrenaline- evoked [^{3}H] inositol phosphate accumulation in a time ($t_{1/2}$, 5 min) and concentration (IC_{50}, 0.3μM) dependent manner; an effect which was reversed by the non-selective protein kinase inhibitor staurosporine. This suggests that protein phosphatase inhibition in these cells allows a kinase-mediated down regulation of receptor activity. A myristoylated protein kinase C inhibitor, at 10μM, reversed the effect of okadaic acid by some 60% whilst the cAMP-dependent protein kinase inhibitor, at the same concentration was without an effect. The involvement of protein kinase C in this response was further confirmed with the use of a protein kinase C activator, phorbol 12-myristate 13-acetate which inhibited noradrenaline-stimulated [^{3}H] inositol phosphate accumulation (IC_{50} 3nM) but was not additive with okadaic acid. The inhibitory effect of okadaic acid on noradrenaline-evoked [^{3}H] inositol phosphate accumulation was overcome by long term phorbol ester treatment, thereby down regulating protein kinase activity in these cells. Pre-treatment of cultures with okadaic acid produced a loss of phosphatase activity and an increase in protein kinase C activity in these cultures. The effect of noradrenaline was found to be largely dependent upon extracellular Ca^{2+}. Ca^{2+} uptake into glial cells stimulated by α_1-adrenoceptor activation was blocked in cultures pre-treated with a phosphatase inhibitor (okadaic acid) or kinase activator suggesting that receptor-coupled Ca^{2+} influx is regulated by phosphorylation. Ca^{2+} release was also stimulated by α_1-adrenoceptor activation but potentiated in cultures pre-treated with okadaic acid. Results thus point towards a role for a protein kinase in the mechanism by which receptors are coupled to and regulate phosphoinositide metabolism in glial cultures. The identity of particular protein phosphatases and protein kinases involved in this aspect of glial cell function are hereby a subject for investigation.

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AA	arachidonic acid
AC	adenylate cyclase
AD	Alzheimer's disease
AKAP	A kinase anchoring protein
8-BrcAMP	8-bromo cyclic AMP
BSA	bovine serum albumin
CaMK	Ca^{2+} / calmodulin-dependent kinase
CaMKK	Ca^{2+} / calmodulin-dependent kinase kinase
cAMP	cyclic adenosine monophosphate
CNS	central nervous system
CRAC	Ca^{2+} release activated Ca^{2+} channel
DAG	diacylglycerol
dcAMP	dibutryl cAMP
DNase	deoxyribonuclease
DSP	dual specific protein phosphatase
DTT	dithiothreitol
EBSS	Earle's Balanced Salt Solution
EDTA	ethylenediamine tetraacetic acid
ER	endoplasmic reticulum

GFAP	glial fibrillary acidic protein
GPI	glycerophosphoinositol
I-1	inhibitor-1
I-2	inhibitor-2
IBMX	isobutyl methyl xanthine
iNOS	inducible nitric oxide synthase
IP	inositol phosphate
IP_1	inositol monophosphate
IP_2	inositol 2,4-bisphosphate
IP_3	inositol 1,4,5-trisphosphate
IP_3R	inositol 1,4,5-trisphosphate receptor
IP_4	inositol 1,3,4,5-tetrakisphosphate
IP_5	inositol pentakisphosphate
IP_6	inositol hexakisphosphate
LTD	long term depression
LTP	long term potentiation
MAP	microtuble associated protein
MAPK	mitogen activated protein kinase
MEK	mitogen activated protein kinase kinase

mRNA	messenger RNA
NA	noradrenaline
NFT	neurofibrillary tangles
NMDA	N-methyl-D-aspartate
OA	okadaic acid
PA	phosphatidic acid
PAF	platelet activating factor
PI	phosphatidylinositol
PIP	phosphatidylinositol - 4 -phosphate
PIP_2	phosphatidylinositol 4,5-bisphosphate
PIP_3	phosphatidylinositol 3,4,5-trisphosphate
PK	protein kinase
PKI	protein kinase A inhibitor
PKA	cyclic AMP-dependent protein kinase
PKC	protein kinase C
PHF	paired helical filaments
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethylsulphonyl fluoride

PP	protein phosphatase
PP1	protein phosphatase 1
PP2A	protein phosphatase 2A
PP2B	protein phosphatase 2B
PP2C	protein phosphatase 2C
PTK	protein tyrosine kinase
PTP	protein tyrosine phosphatase
PS	phosphatidylserine
RACKS	receptors for activated C-kinase
RICKS	receptor for inactive C-kinase
ROC	receptor operated Ca^{2+} channel
SOC	store operated Ca^{2+} channel
SBTI	soya bean trypsin inhibitor
SMOC	second messenger operated Ca^{2+} channel
STICKS	substrates that interact with C kinase
TCA	trichloroacetic acid
VOC	voltage operated Ca^{2+} channel

CHAPTER 1
INTRODUCTION

1.1 Signal transduction

During intercellular communication, extracellular signals are registered by the cell and converted into intracellular reactions. Signal transmission into the cell interior takes place by reaction chains, in which several individual reactions generally run in sequence and involve many molecules. The first step in signal transmission is always the reception of the signal by the target cell.

In a multitude of signalling pathways, conversion of the extracellular signal takes place with the help of a transmembrane receptor where the signal molecule may not penetrate the target cell, but rather it binds on the extracellular side of the transmembrane receptor. This conducts the signal into the interior of the cell and sets an intracellular signal chain in motion, which finally triggers a defined biochemical response of the target cell. Cell surface receptors can be ion-channel-, enzyme- or G-protein-linked.

A physiologically important aspect of signal transmission via transmembrane receptors is receptor regulation. The cell has various mechanisms available which control the number and activity of transmembrane receptors. The aim of regulation is to either weaken signal transmission during conditions of long lasting stimulation or to strengthen it by up-regulating receptors, thereby increasing their density. The structural elements

involved in regulation of receptor activity are generally located in the cytosolic domain. These are, above all, amino acid sequences on receptors that permit its phosphorylation by protein kinases (PKs).

1.1.2 Intracellular messengers

One of the main signal transduction mechanisms involves receptor-G-protein-second messenger coupling. G-protein-linked receptors are the largest family of cell-surface receptors. The intracellular messenger substances are formed or released by specific enzyme reactions during the process of signal transduction, these then regulate the activity of particular proteins which, in turn, control the appropriate biological response.

Two of the most widely used intracellular messengers are cyclic adenosine monophosphate (cAMP) and Ca^{2+}. Changes in their concentrations are stimulated by distinct pathways and most G-protein-linked receptors are coupled to one or the other of them. Messenger substances with hydrophobic character, such as diacylglycerol (DAG) or the phosphatidylinositol (PI) derivatives, are located in the plasma membrane. The hydrophobic messengers reach membrane-associated effector proteins by diffusing through the plasma membrane where they regulate their activity. Hydrophilic messengers with good aqueous solubility, such as cAMP and the inositol phosphates (IPs), are

localized in the cytosol and reach their protein substrates in the cytosol (Fisher et al., 1992). Intracellular messenger substances can be formed and degraded again in specific enzyme reactions. Large amounts of messenger substances can be rapidly created and inactivated via these enzymatic pathways.

cAMP is a nucleotide synthesised within the cell from ATP by the action of a membrane bound enzyme, adenylate cyclase (AC). The general role of cAMP is to activate a cAMP-dependent protein kinase (PKA). The concentration of cAMP is controlled primarily by two means, namely via new synthesis by AC and degradation by phosphodiesterases (Houslay and Milligan, 1997). In addition to AC, the activity of which is subject to diverse regulation, the cAMP phosphodiesterases are also an important point of attack for controlling cAMP levels. There are phosphodiesterases regulated by Ca^{2+}/calmodulin and by protein phosphorylation. More than 10 different isoforms of phosphodiesterase are known, which vary in their cyclic nucleotide specificity and in their regulation (Housley and Milligan, 1997). An increase in cAMP concentration over the threshold required for activation of PKA may be reached via both paths, via the stimulation of AC and/or inhibition of the phosphodiesterase. Many different drugs, hormones and neurotransmitters produce their effects by increasing or decreasing the catalytic activity of AC and thus raising or lowering the concentration of cAMP within the cell. Messengers such as Ca^{2+} may be stored in special storage organelles, from which they can be rapidly released by a signal. Ca^{2+} may be released in

a location-specific manner and also removed from the cytosol according to its site of release. It is therefore possible for the cell to create signals that are spatially and temporally limited (Abdel-Latif, 1996). The release of Ca^{2+} from internal stores is invariably associated with the receptor-linked metabolism of membrane inositol phospholipids.

1.1.3 Metabolism of inositol phospholipids

The initial discovery that hormones have effects on phosphoinositide metabolism was made by Hokin and Hokin (1953). Since then it has become apparent that a large number of different agonists can stimulate an increase in the metabolism of membrane phosphoinositides. These agonists include classical neurotransmitters such as acetylcholine, noradrenaline (NA), histamine, in addition to the more complex molecules such as peptides and growth factors.

Between 2 and 8% of the lipids of eukaryotic membranes are inositol containing lipids. The three main forms of these are PI, phosphatidylinositol - 4 -phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP_2), with PI accounting for more than 80% of the total inositol lipid content. PIP_2 is the starting point for formation of the second messengers DAG and inositol 1,4,5-trisphosphate (IP_3) (Berridge and Irvine, 1984).

These molecules are generated from PIP_2 via the action of phospholipase C (PLC). PI is sequentially phosphorylated by specific kinases such that phosphate groups are inserted onto the 4 (PIP) and 5 (PIP_2) positions of the inositol ring. PIP_2 can be further phosphorylated by a 3-kinase to yield $PI(3,4,5)P_3$ (PIP_3),which serves a role in the signalling cascade of particular signal molecules (Berridge, 1993). Both compounds are messengers that can activate further specific reaction chains. IP_3 initiates the release of Ca^{2+} from internal stores, whilst DAG stimulates the activity of protein kinase C (PKC).

DAG diffuses through the plasma membrane where it has an important messenger function in that it activates PKC to phosphorylate specific proteins (Takai et al., 1979, Kishimoto et al., 1980; Nishizuka, 1983). The activation of PKC by DAG is a complex process which, depending on the isoform of PKC, requires Ca^{2+} and phosphatidylserine (PS) as cofactors. A particular fascinating aspect of PKC is its activation by tumour promoting phorbol esters which substitute for DAG. The second messenger function of DAG is terminated by two mechanisms; DAG can be either phosphorylated to phosphatidic acid (PA) by a DAG kinase or can be hydrolysed to monoacylglycerol by a DAG lipase. DAG kinase is very active in the brain. Mammalian cells contain at least 50 structurally distinct molecular species of DAG, whose fatty acyl groups can be poly-, di- or mono- unsaturated, or saturated (Wakelam,1998). Inositol lipids which make up 5-10% of the total phospholipid content of most mammalian cells are mainly polyunsaturated (Hodgkin et al., 1998). Most DAG species activate PKCs in vitro but

there are some preference for those which are polyunsaturated. Saturated DAGs are generally poor activators (Wakelam,1998). Figure 1a shows how these intracellular second messengers are produced as a result of receptor activation.

IP_3 diffuses through the cytoplasm to specific receptor proteins located on portions of the endoplasmic reticulum (ER). By binding to these specific receptor proteins, IP_3 is able to release internal stores of Ca^{2+} (Berridge, 1987). The Ca^{2+} released by IP_3 stimulation activates many Ca^{2+} dependent processes. IP_3 is subject to rapid degradation via phosphatases to compounds without any signalling activity. In addition, there are many other IP derivatives (Berridge and Irvine, 1989). IP_3 can be further phosphorylated to form inositol 1,3,4,5-tetrakisphosphate (IP_4), inositol 1,3,4,5,6-pentakisphosphate (IP_5) and inositol hexakisphosphate (IP_6) as well as metabolised to form the lower inositols, inositol 2,4-bisphosphate (IP_2) and inositol monophosphate (IP_1) (Majerus, 1996). Of the more highly phosphorylated inositol compounds, IP_4in particular is attributed a regulatory function where it is proposed to aid Ca^{2+} entry or mobilises intracellular Ca^{2+} in conjunction with IP_3 (Irvine, 1992). Higher IPs (IP_5 and IP_6) are present at high resting concentrations in cells and there is evidence they can influence Ca^{2+} entry into neurons. IP_6 may also have a role in the regulation of exocytosis (Rana and Hokin, 1990). The IPs are linked into a metabolic cycle in which they can be degraded and regenerated. Via these pathways, the cell has the ability to replenish stores of IP derivatives according to demand. Figure 1b shows this pathway of phosphoinositide metabolism.

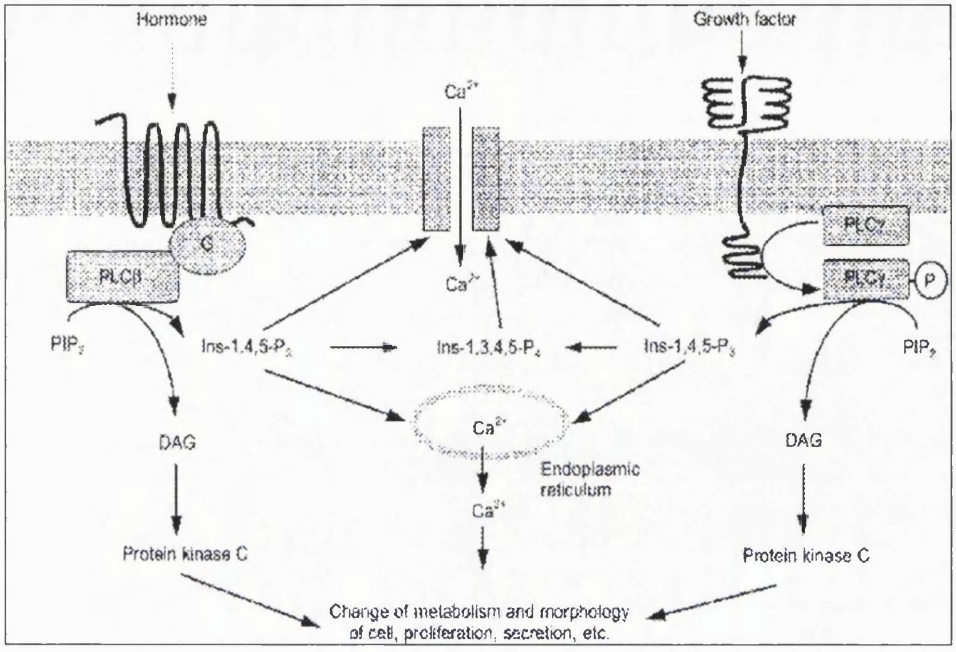

Figure 1a. Signalling linked to IP_3 and DAG formation in cells.

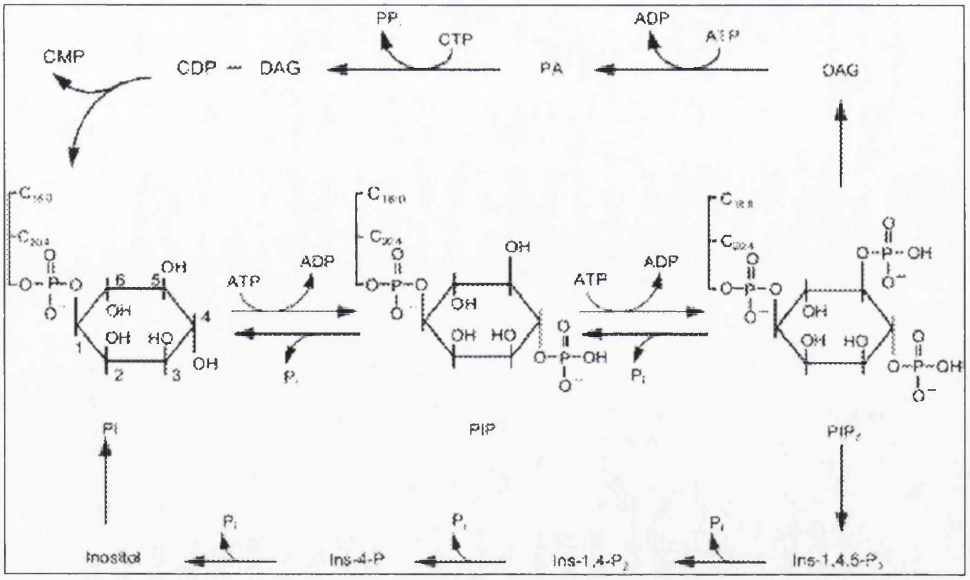

Figure 1b. The main pathway of phosphoinositide metabolism

(Both adapted from Krauss, 1999)

By disrupting the IP cycle, Li^+ results in a decrease in free inositol with a corresponding increase in the level of IP_1. This inhibitory action of Li^+ greatly amplifies the response and can thus be employed in assays to detect receptor-mediated breakdown in a wide range of different tissues including the brain (Berridge et al., 1982).

PLC, which occurs in different subtypes in the cell, is a key enzyme in the metabolism of phosphoinositides and hydrolyses PI, PIP and PIP_2. There are four classes of PLC enzymes ($\beta,\gamma,\delta,\epsilon$) of which the β,γ and δ isoforms predominate in brain (Berridge et al., 1999). Two signalling pathways regulate the PLC activity of the cell. One is the activation of PLCβ by G-proteins, in the other PLCγ is activated by transmembrane receptors with intrinsic or associated tyrosine kinase activity (Rhee et al., 1989). Majerus et al. (1986) showed that PI and PIP are also substrates for PLC, but that in these cases the enzyme is Ca^{2+} requiring. It is therefore possible that agonist induced IP accumulation in the absence of extracellular Ca^{2+} is the result of PIP_2 breakdown and that in the presence of Ca^{2+}, PI and/ or PIP may also be metabolised, possibly as a result of an influx of extracellular Ca^{2+}.

1.2 Receptor regulation

The regulation of receptor expression and activity is important in the physiology of an organism and can occur at any of the steps between gene transcription to turnover of the

receptor protein itself. Receptor proteins are synthesised by the cells that express them and the level of expression itself is controlled by receptor-mediated events. Receptor density is sensitive to changes in the cellular environment as well as to altered concentrations of the ligand (Poste, 1984). Agonist stimulation can cause receptor desensitisation, which is characterised by a rapid reduction in response to the agonist, whereas down-regulation often occurs after prolonged agonist treatment and is manifested as a decrease in receptor density. Receptors are not always degraded as removal of agonist rapidly restores receptor function (Heck and Bylund, 1998). By this type of mechanism a cell can adjust its sensitivity to the concentration of a stimulating ligand.

Receptor regulation frequently involves a rapid ligand-induced phosphorylation of receptors, which leads to the slower down-regulation of the number of receptor molecules on the target cell. The best understood example of this phenomenon is the β-adrenergic receptor, which activates AC via the stimulatory G-protein G_s. When cells are exposed to a high concentration of adrenaline, they can desensitise within minutes by two pathways which depend on β_2-adrenergic receptor phosphorylation. In one, the rise in cAMP caused by adrenaline binding activates PKA, which then phosphorylates the β_2 receptor on a serine residue, thereby interfering with the receptor's ability to activate G_s. In the other, the activated β_2 receptor becomes a substrate for β-adrenergic receptor kinase, whereby phosphorylation causes the inhibitory protein β arrestin to bind and prevents the receptor from activating G_s (Sibley and Lefkowitz, 1987).

β-adrenergic receptors are expressed throughout the body and are targets for various agents. Brodde and co-workers (1992) have shown that the human heart possesses both β_1- and β_2-adrenoceptors, though the amount of β_2-adrenoceptors is higher in the atria (about 30% of the total β-adrenoceptor population) than in the ventricular myocardium (about 20%). In chronic heart failure, cardiac β-adrenoceptor function decreases due to endogenous downregulation by the elevated catecholamines, and this decrease is related to the severity of the disease. On the other hand, bronchodilators used by asthmatics which are β-agonists such as salbutamol, activate β_2-adrenoceptors predominantly on airway smooth muscle which causes bronchodilitation and helps reverse the wheezing during an asthma attack (Johnson, 1998).

Other receptor/effector systems exhibit qualitatively similar regulatory phenomena, suggesting that phosphorylation may represent a general mechanism for regulating receptor function. Examples include the PKC-mediated phosphorylation of platelet-activating factor (PAF) receptors which is induced by activation of other chemoattractant receptors and inhibits both coupling of the PAF receptors to G proteins and mobilisation of intracellular Ca^{2+} stores (Richardson, 1996).

1.2.1 The α_1-adrenoceptors

Phospholipases of type Cβ are activated by $G_{q/11}$ proteins which are linked to various 7-

helix transmembrane receptors. These include the α_1-adrenoceptors which have been shown to be coupled to inositol phospholipid metabolism (Leeb Lundberg et al., 1987). An agonist acting at the α_1-adrenoceptor binding site, such as NA, causes $G_{q/11}$ to activate PLC dependent hydrolysis of PIP_2. Thus the α_1-adrenoceptors are phosphoinositide linked receptors.

There are three subtypes of α_1 adrenoceptor: α_{1A}, α_{1B} and α_{1D} and all are coupled to PLC and the resultant production of IP_3 and DAG (Graham et al., 1996). The affinities and selectivities of drugs for α_1-adrenoceptor subtypes have been determined primarily by competition for radioligand binding to heterologously expressed recombinant subtypes (Graham et al., 1996). Most antagonists, including prazosin, show little or no selectivity between the three known α_1-adrenoceptor subtypes. However, a variety of drugs with varying degrees of selectivity have been found such as RS17053 which is α_{1A} selective, BMY7378 which is α_{1D} selective and AH11110A which is selective for α_{1B} (Garcia-Sainz and Torres-Padilla, 1999; Burt et al., 1998). Most tissues express mixtures of the three subtypes in different densities and ratios, and in most cases responses to α_1-adrenoceptor selective agonists are probably due to activation of more than one subtype (Graham et al., 1996).

The α_1-adrenoceptor is present in many tissues including the brain, heart, blood vessels, liver, kidney, prostate and spleen (Guarino et al., 1996). In these tissues the α_1-adrenoceptors mediate a variety of physiological effects such as neurotransmission and

vasoconstriction. Long term effects of α_1-adrenoceptor stimulation include expression of c-fos and c-jun proto-oncogenes and cell growth and proliferation (Wu et al., 1992). The α_1-adrenoceptors have also been shown to be substrates of PKs. The ability of phorbol esters to inhibit the action of NA suggests that the event is likely to be mediated through receptor phosphorylation by PKC (Leeb-Lundberg et al., 1985).

It is known that agonist stimulation leads to homologous desensitisation of these receptors and current evidence indicates that such decreases in receptor activity is associated with receptor phosphorylation. Receptor phosphorylation seems to involve G protein-receptor kinases and receptor phosphorylation sites have been located in the carboxyl tail (Garcia-Sainz et al., 2000). These authors have also indicated that inhibition of protein phosphatases (PPs) increases the phosphorylation state of α_1-adrenoceptors, in particular those of the α_{1B} subtype, and that this effect seems to involve the actions of PKC (Alcantara-Hernandez et al., 2000).

1.2.2 Protein phosphorylation

One of the mechanisms involved in the modulation of receptor function is the phosphorylation and dephosphorylation of specific amino acid residues by intracellular PKs and PPs, respectively. The actual phosphorylation level is the result of a delicate balance between the two enzymes. PKs transfer a phosphate from ATP to a protein,

typically phosphorylation at a serine, threonine or tyrosine residue (Hunter, 1991). PPs, on the other hand remove the phosphate group, i.e. dephosphorylate the protein.

The reversible phosphorylation of proteins is the principal mechanism by which intracellular functions are regulated by extracellular signals. A third of all proteins in mammalian cells are thought to contain covalently bound phosphate; and PKs, PPs and their regulatory subunits are likely to account for about 5% of all human gene products. More than 2000 PKs and 1000 PPs have already been encoded in higher eukaryotes (Barford et al., 1998).

It is now acknowledged that the regulation of protein phosphorylation requires the coordinated control of both PKs and PPs and that the regulation of PPs is as complex and elegant as that of PKs. Nearly all aspects of cell life are regulated by reversible protein phosphorylation which is defined by PKs and PPs acting on specific cellular proteins. Phosphorylation on serine and threonine residues together account for over 95% of total cellular protein phosphate and are recognised as key elements in the regulation of such diverse pathways as glycogen metabolism and cell surface receptor signalling. Phosphorylation on tyrosine residues, which accounts for less than 5% of phosphate bound has been linked broadly with the regulation of the cell cycle (Sun and Tonks, 1994). The phosphorylation status of a protein is dynamic and for any phosphorylated protein at any given point in time the extent of this modification is dependent upon the

relative activities of both PKs and PPs.

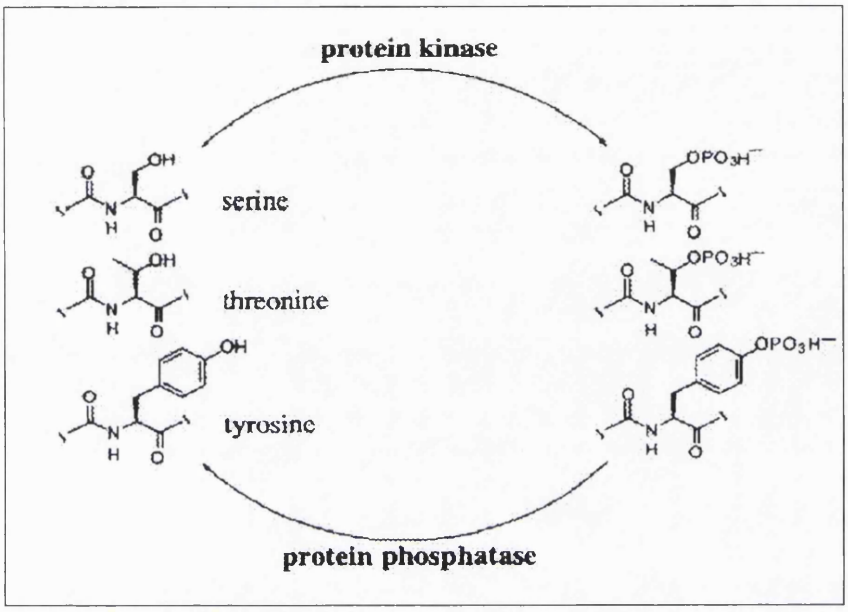

Figure 2. Phosphatase/kinase cycle (adapted from Sheppeck et al., 1997)

Concomitant control of PKs and PPs provides the cell with the capacity to rapidly switch proteins from their phosphorylated to dephosphorylated state to meet differing physiological demands. This is perhaps best illustrated during the eukaryotic cell division cycle where decisions to proceed through different stages are made by the timely phosphorylation of specific cell cycle regulators. Thus, phosphorylation/dephosphorylation events act as switches or checkpoints that ensure that a cell has fulfilled the requirements to proceed to the next cell cycle stage. Errors in checkpoint control form the most prevalent basis for aberrant cell growth seen in human

cancers. Abnormal protein phosphorylation is implicated in the aetiology of other diseases such as diabetes, inflammation and Alzheimer's disease (AD) (Wilson, 1997).

AD is a neurodegenerative disorder that is characterised by neuronal loss and the formation of neuritic plaques, neurofibrillary tangles (NFTs) and neurophil threads. The neuritic components of plaques surrounding the amyloid core, NFTs, and neurophil threads contain paired helical filaments (PHFs) that are composed of microtubule-associated protein (MAP) tau in an insoluble, abnormal highly phosphorylated state (Goedert et al.,1992). A pool of abnormally hyperphosphorylated tau (PHF -τ) not aggregated into PHFs has been found in AD (Kopke et al.,1993), suggesting that abnormal hyperphosphorylation might precede its polymerisation into PHFs. The major function of tau in the normal brain, is to promote microtubule assembly and is physiologically regulated by its phosphorylation state (Lindwall et al., 1984).

The abnormal highly phosphorylated PHF -τ has a much reduced ability to bind to microtubules and to promote their assembly (Bramblett et al., 1993). As a likely consequence, the axonal cytoskeleton and transport processes might be disturbed, and neuronal viability compromised. Metabolism of the amyloid precursor protein and its processing into β-amyloid is also regulated by protein phosphorylation (Gong et al., 1994). It might therefore, be hypothesised that an imbalance between protein phosphorylation and dephosphorylation is central to the pathological mechanism of AD.

This disturbance might be the result of either higher activities of PK, lower activities of PPs, or both. To date, phosphorylation of only serine and threonine but not of tyrosine residues have been observed in AD (Tanaka et al., 1998). Previous studies have shown that in AD abnormally hyperphosphorylated tau can be dephosphorylated by PP2A, PP2B and to a lesser extent by PP1 to a normal - like state, and that the activities of these enzymes are decreased in the AD brains (Sontag et al., 1999; Gong et al., 2000). All phosphorylation sites that have been identified in tau preparations from AD brains are therefore serine/threonine residues.

1.2.3 Classification of protein phosphatases

PPs are defined according to three structurally distinct gene families. The PPP and PPM families encode protein serine/threonine phosphatases, whereas the protein tyrosine phosphatase (PTP) family includes both tyrosine - specific and dual specific phosphatases (DSPs) (Barford et al., 1998). The PPP class shares a common phosphatase domain and includes phosphatase type 1 (PP1), phosphatase type 2A (PP2A), phosphatases type 2B (PP2B, calcineurin) and the novel phosphatases. PP1, 2A and 2B share 39 - 59% sequence homology in their catalytic domains and are inhibited by the PP inhibitor okadaic acid (OA) (Takai et al., 1992). The PPM class consists of several closely related isoforms that have very little sequence homology with the PPP family and include the phosphatase PP2C which is Mg^{2+}-dependent and insensitive to OA. Table 1 shows the different isoforms and their nomenclature.

PPP family

Catalytic subunit	**Regulatory subunits**
PP1c	G_M, G_L, M_{110} + M_{21}, NIPP-1, RIPP-1, R110, p53BP2, L5, sds22, RB gene product, inhibitor-1, DARPP-32, inhibitor-2, splicing factor, kinesin-like protein, 134.5 (Herpes simplex), R5
PP2Ac	A subunit (PR65)
	B subunit (PR55, PR72, PR61), eRF1, PTPA, SET, polyoma middle and small T antigens, SV40 small T antigen
PP2B	B-subunit, calmodulin, AKAP-79
Novel protein phosphatases of the PPP family	
PPP1:	PPY, Ppz1, Ppz2, Ppq1
PPP2A:	PP4, PP6, PPV 6A, sit4, Ppc1, Ppg1
PPP5:	PP5, RdgC

PPM family

PP2C

Arabidopsis ABI1, *Arabidopsis* KAPP -1

Pyruvate dehydrogenase phosphatase

Bacillus subtilis SpoIIE phosphatase

Protein–tyrosine phosphatases family

Tyrosine-specific phosphatases

Cytosolic, nonreceptor forms PTP1B, SHP-1, SHP -2

Receptor-like, transmembrane forms CD45, RPTPμ, RPTP

Dual-specificity phosphatases

CDC25

Kinase-associated phosphatase

MAP kinase phosphatase -1

Table 1. Nomenclature of Protein Phosphatases (adapted from Barford et al., 1998)

1.2.4 Protein serine/threonine phosphatases

Of the PP families, the relatively better studied are the serine/threonine PPs. It has been discovered that besides the control of the key enzymes of metabolic pathways, these PPs participate in the regulation of gene transcription and cell division in eukaryotes (Barford, 1995). PP1, PP2A, PP2B and PP2C are the major serine/threonine-specific PPs whose catalytic subunits are present in the cytoplasm of animal cells. These PPs can be classified according to their substrate specificity, metal ion dependence and sensitivity to inhibitors based on a classification system proposed by Cohen and Cohen (1989).

Three of these enzymes, PP1, PP2A, and PP2B, are members of the same gene family, while PP2C appears to be distinct. Of the four types, PP1 and PP2A exhibit overlapping substrate specificities in vitro, accounting for almost all measurable activity towards phosphoproteins regulating cellular processes (Sim, 1991). PP1 and PP2A do not have an absolute requirement for divalent cations, whereas PP2B and PP2C are Ca^{2+}/calmodulin and Mg^{2+}-dependent, respectively (Cohen, 1992). PP2B is active on a much more restricted range of substrates, and PP2C is structurally unrelated to the other types. Several isoforms have also been identified for each type and novel PPs which are structurally related and share similar homology have also been discovered (Barford et al., 1998; Cohen, 1997).

Each PP is present at significant concentrations in skeletal muscle, heart muscle, liver,

brain and adipose tissue, although the relative amounts differ considerably. The members of the PP1/2A/2B family share a common catalytic core domain of roughly 300 amino acids (Cohen and Cohen, 1989; Takai et al., 1992). In eukaryotes, PP1 and PP2A represent the most quantitatively significant source of serine/threonine PP activity in mammalian cells (Cohen, 1991) and are therefore the primary focus of the research described in this thesis.

Given that protein phosphorylation participates in virtually every aspect of cellular regulation, it is not surprising that dysfunctions in protein phosphorylation-dephosphorylation processes can have serious consequences. Many poisons and pathogens target this portion of the cell's command and control network. It has long been known that PPs are the targets for over 50 environmental toxins (Millward et al., 1999). Many of these are also tumour promoters, suggesting the importance of serine/threonine PP inhibition in unregulated cell growth. The role of PP1 and PP2A as negative regulators of the cell cycle is exemplified by the ability of various inhibitors, including OA, calyculin A and microcystin, to accelerate and enhance cell cycle progression (Fujiki and Suganuma, 1999). Not surprisingly, these inhibitors are classified as tumour promoters where they mediate their effects by prematurely activating cell cycle progression and by inhibiting vital mitotic processes.

1.2.4.1 Protein phosphatase 1

PP1 is one of the most highly conserved eukaryotic proteins. This high degree of conservation reflects the fact that PP1 plays a fundamental role in many cellular processes such as protein synthesis, glycogen metabolism and cell division (Faux, 1996). Four mammalian isoforms of the PP1 catalytic subunit are generated from three genes. These isoforms are highly conserved showing 90% amino acid sequence identity. With the exception of PP1γ2, which is predominantly expressed in testes, the other isoforms, PP1α, PP1β and PP1γ1 are widely expressed in mammalian tissues (Strack et al., 1999). The functional importance of the different PP1 isoforms remains unclear.

Studies in many eukaryotic systems all point to a crucial role for PP1 in cell cycle progression, and an absolute requirement for mitotic exit (Cyert et al., 1989). There is some evidence that PP1 also plays a role in regulating the progression of cells into and out of the cycle. For example, PP1 can dephosphorylate ribosomal protein S6 (Oliver et al., 1988), a protein whose phosphorylation is induced by growth factor treatment and has been correlated with increased cell growth. PP1 also has also been shown to have an important role in the regulation of glycogen degradation as its catalytic subunit is associated with a glycogen binding protein which mediates a targeted localisation of the catalytic subunit to glycogen so that a close spatial orientation of PP and its substrates, the enzymes of glycogen metabolism, is created (Hubbard and Cohen, 1993).

PP1 has a number of important functions in brain. Association with a wide variety of regulatory/targeting subunits is thought to be instrumental in directing the PP to specific subcellular locations and substrates. Brain PP1 is present in membrane fractions (Sim et al., 1994) and synaptic junctions (Shields et al., 1985) and is associated with neurofilaments (Strack et al., 1997). Immunoblotting has detected the PP1γ2 isoform in brain, which had previously been thought to be testes specific, in addition to the α, β, and γ1 isoforms (Strack et al., 1999). Subcellular fractionation studies demonstrate that PP1β and PP1γ1 are selectively associated with different cytoskeletal elements, PP1β with microtubules and PPγ1 with the actin cytoskeleton; whereas PP1β localizes to a discrete area of the soma, PP1γ1 is highly enriched in dendritic spines and presynaptic terminals of cultured neurons (Ouimet et al., 1995). These results show that PP1 isoforms are targeted to different neuronal cytoskeletal compartments with a high degree of specificity, presumably by isoform-specific association with regulatory/targeting proteins. Furthermore, the synaptic localization of PP1γ1 indicates that it is this isoform that is involved in the regulation of synaptic phosphoproteins such as neurotransmitter receptors and ion channels implicated in synaptic plasticity (Strack et al., 1999).

1.2.4.2 Protein phosphatase 2A

PP2A is by far the most abundant type 2 serine/threonine PP in mammalian cells, where it can be found largely in the cytoplasm, but also in the nucleus. The enzyme is composed

of the C catalytic subunit bound to two regulatory subunits termed A and B. The A subunits function as scaffolds that link the C subunit to any one of a number of different B subunits. Along with the A subunits, the B subunits dictate the substrate specificity and probably the subcellular localisation of the PP2A heterotrimeric complex (Shenolikar and Nairn, 1991). The A subunit is believed to provide structural support to the C subunit, and the B subunit is believed to determine the subcellular localisation and substrate specificities of the C subunit (Hunter, 1995; Wera and Hemmings, 1995). There are two known forms of the C subunit, but these are very similar functionally and are considered to be isoforms of each other. In contrast, there appear to be multiple forms of the B subunit which are expressed differentially in mammalian tissues. To date, two A subunits, two C subunits and over 20 B subunits have been identified. This suggests the existence of numerous PP2A complexes in mammalian cells (Wera and Hemmings, 1995). It also appears that the B subunits expressed in a particular cell specify the cell type-specific functions of the PP.

PP2A regulates multiple aspects of cell growth and metabolism. The ability of this widely distributed heterotrimeric enzyme to act on a diverse array of substrates is largely controlled by the nature of its regulatory B subunit. A fraction of brain PP2A associates with microtubules and may play a role in regulating phosphorylation of microtubule-associated proteins (MAPs). The efficient association of PP2A with microtubules is dependent on an anchoring activity present in a brain protein fraction containing

microtubule-associated and microtubule-interacting proteins (Price et al., 1999).

Changes in microtubule structure and dynamics are essential for the maintenance of cell shape, cell motility, cell division and vesicle transport. The phosphorylation of MAPs is an important component in the control of microtubule dynamics (Maccioni et al., 1995). These MAPs serve both to stabilise microtubules against disassembly and to mediate their interaction with other cell components. Two major classes of MAP can be isolated from brain in association with microtubules, high molecular weight proteins which include MAP1 and MAP2 and tau proteins (Skoufias and Scholey, 1993). A pool of PP2A is associated with microtubules in non neuronal cells and in brain (Sontag et al., 1995). A more general role for PP2A targeting in regulating the cellular cytoskeleton comes from studies showing that PP2A is associated with neurofilaments (Saito et al., 1995). More recently, cytostatin, an inhibitor of cell adhesion and a powerful anti-metastatic drug was also demonstrated to inhibit PP2A selectively (Kawada et al., 1999).

1.2.4.3 Protein phosphatase 2B

PP2B is a widely distributed Ca^{2+}/ calmodulin-dependent PP. It represents the major component of the class of calmodulin binding proteins in the brain, accounting for about 1% of the total protein in the brain (Depaoli-Roach et al., 1994). PP2B is a heterodimer composed of equal amounts of A (61kDa) and B (19kDa) subunits. The A subunit

contains the catalytic and calmodulin binding domains, whereas the B subunit binds to Ca^{2+} and is highly homologous to calmodulin (Klee et al., 1988). Three isoforms of PP2B subunit A have been demonstrated by cloning of mammalian genes and designated as Aα, Aβ and Aγ. The Aα and Aβ isoforms are expressed in mammalian brain and the Aγ isoform is testis-specific (Muramatsu et al., 1993). PP2B has a much narrower in vitro substrate specificity than either PP1 or PP2A which is consistent with its specialised functions in the nervous system, T lymphocytes and other cells.

PP2B plays a key role in inflammation and immunosuppression. The activity of PP2B is regulated by a series of proteins termed immunophilins (Shenolikar, 1995). These proteins were first identified as the targets of potent immunosuppresive drugs, such as cyclosporin and FK506. Both the A and B subunits are involved in the binding of immunophilins. The immunosuppresive /immunophilin-mediated reduction in PP2B activity results in loss of the ability of Ca^{2+} to activate T lymphocytes, which mediates the immunosuppresive effects of these drugs. PP2B has drawn much attention as the target of two clinically important immunosuppresive drugs, cyclosporin and FK506 (Liu et al., 1993). It has been speculated that the immunophilins may be physiological regulators of PP2B and the immunosuppresive drugs simply stabilise the formation of immunophilin/PP2B complexes to potentiate PP inhibition (Cardenas et al., 1994). There is also a strong co-localisation of PP2B and FK506-binding proteins in the brain (Dawson et al., 1994).

PP2B shows relatively low activity in the heart, spleen, liver and testes but is highly enriched in brain tissue, particularly in the striatum and hippocampus and is localised along dendritic spines (Depaoli-Roach et al., 1994). This is consistent with its proposed role in memory by modulating long term potentiation (LTP) and long term depression (LTD) of synaptic efficacy (Mitsuhashi et al., 2000). Over expression of PP2B in transgenic mice produced deficits in long-term memory and a constraint on LTP. Other neurological studies have shown PP2B to be abundantly expressed in areas of the brain, which are vulnerable to stroke, epilepsy and neurodegenerative disease (Strack et al., 1997). It has been suggested that high level activity of PP2B predisposes neuronal cells to apoptotic cell death and that PP2B inhibitors reduce such susceptibility (Asai et al., 1999). Some of the neurological and physiological abnormalities of Down's syndrome may also be attributed to PP2B inhibition. In this context, DSCR1 is a family of proteins coded by chromosome 21, that are over-expressed in the brain and striated muscles of Down's syndrome patients, and which inhibit PP2B signalling pathways (Kinsbury and Cunningham, 2000).

PP2B also appears to function in the long-term maintenance of cardiac hypertrophy or myopathic disease states. Consequently, the PP2B inhibitors, cyclosporin A and FK506 protect against heart ischemia (Lim et al., 2000). PP2B also regulates the Ca^{2+} channel activity of the ryanodine and IP_3 receptors via an immunophilin-mediated interaction. Cameron et al. (1995) showed that PP2B anchored to the IP_3 receptor via the

immunophilin FKBP12, thereby regulating the phosphorylation status of the receptor and resulting in a dynamic Ca^{2+}-sensitive regulation of IP_3 -mediated Ca^{2+} flux.

1.2.4.4 Protein phosphatase 2C

PP2C shares no homology with other PPs, even within the catalytic domain, and this is reflected in the fact that the PP inhibitors used to characterise the roles of other PPs, such as the OA class of compounds, have no effect on PP2C. The lack of pharmacological agents able to modulate PP2C activity has hampered the understanding of its role in regulating cell function. However, it does display a degree of overlapping substrate specificity particularly with PP1 and PP2A. PP2C is a monomeric protein of approximately 43kDa, and include the subtypes PP2Cα, PP2Cβ, PP2Cγ and PP2Cδ (Wang et al., 1995). These PPs require metal cations, Mg^{2+} or Mn^{2+}, for dephosphorylation activity. Beyond this dependence, which is unlikely to regulate the PP in vivo, not much is known about its regulation or functional roles. PP2Cs are widely expressed but most abundant in heart and skeletal muscle and is present in brain extracts at levels four-fold higher than skeletal muscle (Ingebritsen et al., 1983).

Functions that may require PP2C include reversal of PK cascades activated by stress and regulation of apoptosis, cell cycle progression and gene expression (Tong et al., 1998). PP2Cδ has 30% homology in its catalytic domain with other PP2Cs and is expressed

ubiquitously with the highest expression in the kidney, liver and muscle. The activity of PP2Cδ is dependent on Mn^{2+}, but unlike other PP2Cs isoforms, this activity is inhibited by Mg^{2+} (Gong et al., 2000).

1.2.5 Protein tyrosine phosphatases

PTPs are specific for tyrosine residues on target proteins and are unable to dephosphorylate serine/threonine residues. For many years it was thought that the PPs that acted on the aliphatic hydroxyl groups of serine/threonine did not act upon the aromatic hydroxyl groups of tyrosine, and vice-versa. Several exceptions to this pattern have been encountered and are referred to as dual-specific PPs (DSP) which belong to the PTP family. PTPs consist of an extracellular domain, a membrane spanning region and a cytosolic catalytic domain. PTPs and DSPs are characterised by a conserved PTP motif containing catalytically essential cysteine and arginine residues (Saito and Streuli, 1991). Over 40 PTPs have been characterised, each of which posses a 230 amino acid catalytic domain and contain a number of regulatory subunits that appear to be essential for subcellular localisation and regulation of enzymatic activity (Barford., 1996).

The PTPs can be roughly divided into two groups: receptor PTPs (also called receptor-like PTPs) and cytoplasmic PTPs. Both groups catalyse the hydrolysis of a tyrosine phosphate by a common mechanism and correspondingly, both groups have a

homologous catalytic domain (Neel and Tonks, 1997). The identification of extracellular ligands for PTPs could provide critical insights into the specific functions and regulation of these enzymes. In cells of neural tissue, for example, a surface protein, contactin, has been identified as a ligand for the extracellular domain of a PTPα. This complex may regulate aspects of neuronal development (Zeng et al., 1999).

The extent of PTP phosphorylation of signal proteins is determined both by the activity of the protein tyrosine kinase (PTK) and also the activity of PTPs. If the total activity of both enzymes in the cell is considered, it is found that there is a preponderance of PTP activity. In contrast, the activities of the serine/threonine specific PKs and PPs are approximately balanced. It is estimated that the activity of the PTPs is about 3-4 orders of magnitude higher than the activity of the PTKs. With this relationship, it is not surprising that the net level of tyrosine phosphorylation in the cell is very low and often only transient. Appropriate regulation of tyrosine phosphorylation is essential for axon growth and guidance; evidence from invertebrates indicates that receptor PTPs are required for correct axon growth during CNS development (Wang and Bixby, 1999). Several PTP isoforms are expressed in the brain, including PTP-κ which participates in homophilic cell-cell interactions in vitro and PTP-ζ which is a neurite-promoting cell adhesion molecule for CNS neurons (Shen et al., 1999).

1.2.6 Novel protein phosphatases

A number of novel PPs have been identified including PP4, PP5, PP6 and PP7. PP4 (formally known as PPX) and PP6 are structurally related to PP2A, sharing 65% and 57% amino acid homology, respectively, and show intriguing and highly restricted subcellular localisations, pointing to specialised functions in cells (Chen et al., 1994; Cohen , 1997; Huang and Honkanen, 1998). PP4, PP5 and PP6 are all present in all mammalian tissues. PP4 is 45% identical to PP1, but only in its catalytic domains (Kloeker and Wadzinski, 1999). PP5 contains a catalytic domain common to PP1, PP2A, PP2B, PP4 and the PP6 family of enzymes and an extended N-terminal domain containing four 34-amino acid repeat motifs. PP7 contains a catalytic core domain similar to the other PPs, but has unique N- and C-terminal regions and shares about 35% identity with other known PPs (Kutuzov et al., 1998). Other recent additions to these classes include relatives of PP1: PPZ1, PPZ2, PPQ; relatives of PP2A: PPV, PPG; and a new PP2B, rdgC.

PP5 is < 45% related to the PP1/2A/2B family and is located in all examined human tissues, predominantly in the nucleus (Chen et al., 1994). PPY from drosophila also has homology that is intermediate between PP1 and PP2A, although it is more closely related to PP1 than PP2A (Dombradi et al., 1989). PPZ is found in mammalian brain and has 68% and 44% homology with PP1 and PP2A, respectively (Cohen et al., 1990). PP2Bw is a Ca^{2+} and calmodulin dependent PP that is 62% homologous to PP2B. PPZ and

PP2Bw were found by da Cruz e Silva (1991) to be, in fact, yeast enzymes. PPQ is 60% identical in its c-terminal domain to PPZ. Although a number of novel PPs such a PP4 and PP5 are found in all tissues, some are tissue specific; RdgC is found only in the eye, whereas PPY is located only in the testis (Kutuzov et al., 1998).

PP4 is one of the most conserved novel PPs and has been shown to be highly concentrated at centromeres indicating that it may play a role in nucleation of microtubules (Sheppeck, 1997). By creation of a mutant drosophila, Hastie and Cohen (1998) demonstrated that PP4 is essential for the nucleation of microtubules that form the mitotic spindle. PP4 is strongly detected in centromeres and is detected throughout all stages of mitosis except telophase. An OA-sensitive PP is known to participate in initiation of microtubular growth at centromeres, and although this was originally thought to be PP2A, PP4 now seems a more likely candidate (Sheppeck, 1997). This is supported by the observation that Drosophila embryos with a defective PP2A catalytic subunit show elongated, disorganised microtubles emanating from centromeres in all directions (Snaith et al., 1996). Despite this wide range of PPs the majority of PP activity within the cell is attributable to PP1 and PP2A (Huang and Honkanen, 1998).

1.2.7 Phosphatase inhibitors

Much of the present knowledge of serine/threonine PPs has grown out of studies

involving their inhibition. A variety of compounds are capable of specifically inhibiting serine/threonine PPs. In 1976, Huang and Glinsmann isolated two endogenous PP inhibitors from rat skeletal muscle, termed inhibitor 1 (I-1) and inhibitor-2 (I-2). Until this point only non specific PP inhibitors, such as NaF or vanadate, were available. I-1 and I-2 appear to be widely distributed in various tissues, including brain. Phosphorylated I-1 and its homologue DARP-32 are inhibitors of PP1 but substrates of PP2A and PP2B. I-2 is unrelated and is thought to be a chaperone for PP1. It inhibits PP1 activity with a K_i of 3.1nM, and unlike I-1, does not need to be phosphorylated to inhibit PP1. I-2 forms a stable and inactive complex with the PP1 catalytic subunit (Makintosh and Makintosh, 1994).

A drug that has proven to be extremely useful for the study of the roles of PPs in cellular regulation is the tumour promoter OA. OA is a product of marine dinoflagellate metabolism and is the causative agent of diarrhetic shellfish poisoning. OA is thought to trigger, by phosphorylation of control proteins, Na^+ release by intestinal cells thus causing diarrhoea. OA is a complex polyether derivative of a 38 - carbon fatty acid and its binding to PPs appears to be non-competitive in nature (Bialojan and Takai, 1988). Even though there is a striking homology between the catalytic domains of PP1 and PP2A, OA exhibits a degree of PP selectivity - subnanomolar concentrations (IC_{50} 0.1 - 1nM) abolish PP2A activity, whereas 10 - 100 fold higher concentrations (IC_{50} 1 - 10nM) are required to inhibit PP1. PP2B is inhibited only at high micromolar concentrations

(IC_{50} > 5μM), and PP2C is insensitive to OA. PP2A can therefore be taken as the proportion of activity inhibited by 1nM OA, PP1 as the activity resistant to 1nM OA (but inhibited by 1μM OA) and PP2C as the Mg^{2+} dependent activity resistant to 1μM OA (Cohen et al., 1990). OA is also membrane permeable making it a particularly useful drug in studies using intact cells.

The sensitivities of both PP1 and PP2A to OA have been remarkably conserved, and are virtually identical in organisms as diverse as mammals, yeast and even higher plants (MacKintosh and Cohen, 1989). Sensitivity to OA may be related to the highly homologous catalytic domains shared by PP1, PP2A and PP2B.The fact that PP2C shares very little sequence homology, even within the catalytic domain, may explain the insensitivity of PP2C to OA (Denu et al., 1996) The differential sensitivity of PP1 and PP2A to OA has proved extremely useful in classifying unknown PP activities and can be exploited to determine the relative contributions of particular PPs in mixed PP preparations.

OA appears to exert its effects by inhibiting PP1 and PP2A leading to an increase in the phosphorylation state of many cellular proteins, an effect which is equivalent in many ways to activating PKs, such as PKC activation by phorbol esters (Cohen et al., 1990). OA is a very potent, non-phorbol ester-type tumour promoter on mouse skin (Suganuma, 1988) and has been shown to stimulate glucose transport in adipocytes (Haystead et al.,

1989). OA rapidly stimulates protein phosphorylation in intact cells, and behaves like a specific PP inhibitor in a variety of metabolic processes. It is therefore apparent that OA is an extremely valuable tool with which to test the physiological role of protein phosphorylation in intact cells.

Phosphatase	**IC_{50} (nM)**	**References**
PP1	20-100	(Bialojan and Takai, 1988)
PP2A	0.1-1.0	(Cohen et al., 1996)
PP2B	>5000	(Cohen, 1991)
PP2C	no inhibition	(Cohen, 1991)
PP3	3.0-5.0	(Honkanen et al., 1991)
PP4	0.2	(Brewis et al., 1993)
PP5	<1.0	(Chen et al., 1994)
PP6	not determined	(Bastians and Ponstingl, 1996)
PP7	no inhibition	(Huang and Honkanen, 1998)

Table 2. Inhibition of protein phosphatases by okadaic acid.

Another toxin, calyculin A, has been isolated from sea sponges. Although unrelated to the structure of OA, it inhibits PP2A with similar potency but was found to inhibit PP1 at concentrations 30 times lower than OA (Ishihara et al., 1989). Tautomycin is related in structure to OA but is produced by soil bacteria. Mycrocystins, like mycrocystin -LR, are unrelated in structure to OA and are produced by cyanobacteria. Both inhibit PP2A with

similar potency to OA but are even more potent inhibitors of PP1 than OA. Despite their diversity of structure, there is evidence that OA, tautomycin, mycrocystin -LR, and inhibitors 1 and 2 interact at the same site on PP1 (MacKintosh and Klumpp., 1990). Microcystins with their acidic groups, two potential hydrogen binding sites and a hydrophobic side chain, have been proposed as being the crucial common features for binding to PPs.

1.2.8 Protein kinases

The PKs are one of the largest protein families in the cell. PKs participate in critical processes ranging from cell growth and division to memory formation and retrieval. Frequently, PKs are themselves activated or inactivated through phosphorylation by other PKs, and intracellular signals often pass through several PKs in a linear phosphorylation cascade before reaching their destinations. Several hundred different PKs are known in mammals, most of which are serine/threonine or tyrosine specific. In addition there are some PKs that phosphorylate other amino acids. Based on the nature of the acceptor amino acids, four classes of PKs can be distinguished - serine/threonine PKs phosphorylate a protein with serine/threonine residues; tyrosine PKs phosphorylate tryrosine residues; histidine PKs phosphorylate histidine residues and can also phosphorylate lysine and arginine residues; aspartate or glutamate PKs create a mixed phosphate carboxylate anhydride (Hunter, 1991). Whilst comprehensive data are

available on structure and function of the serine/threonine and tyrosine PKs, both the other families of PKs are incompletely characterised. The serine/threonine PKs is the family focussed on this thesis.

1.2.8.1 Serine/threonine kinases

Due to the complexity and sheer number of the subfamilies now identified only a selection of the serine/threonine specific PK family in vertebrates are discussed here. PKs do not recognise any fixed amino acid sequence, rather they recognise families of related sequences. There are many other PKs that show no close relationship to these serine/threonine subfamilies. These include PKs with two-fold specificity, in that they can phosphorylate serine/threonine and also tyrosine residues. An example of a PK with two-fold specificity is the MAP kinase kinase, also known as MEK (Robinson and Cobb, 1997).

PKs can exist in active and inactive forms, which is why they are able to perform the function of a switch in signalling pathways. This transition between inactive and active forms of the PK may be controlled by different mechanisms which include binding of activator or inhibitor proteins, ligand controlled binding of regulatory subunits and activation of co-factors (Kemp et al., 1994). Of the serine/threonine PKs, PKA and PKC are the best characterised. Table 3 shows some of the more common PK subfamilies.

PKs regulated by a cyclic nucleotide	PKA
	cGMP dependent PK
Diacylglycerol regulated PKs	PKC isoforms
Ca^{2+}/calmodulin regulated PKs	γ subunit of phosphorylase kinase
	myosin light chain kinase
	Ca^{2+}/calmodulin dependent PKII
Ribosomal S6 PK	
Serpentine receptor kinase	β-adrenergic receptor kinase
Casein Kinase II	
Glycogen synthase kinase	
CDC2 kinases	
Mitogen activated kinases (MAPs)	
Mos/Raf PKs	

Table 3. Subfamilies of the serine/threonine specific protein kinases

(adapted from Krauss,1999)

1.2.8.2 Protein kinase C classification

Like most of the serine/threonine specific PK family, the PKC family shows significant heterogeneity. Based on criteria such as sequence, stimulation and regulation, at least 12 different subtypes of PKC have been discovered in mammals (Way et al., 2000).

Conventional PKCs (cPKC) (α, βI, βII and γ), are activated in vitro and in vivo by the co-factor PS in a Ca^{2+}-dependent manner. In addition, they bind to and are activated by DAG, which increases the specificity of the enzyme for PS and also increases the affinity of the enzyme for Ca^{2+}. Novel PKCs (nPKC) (δ, ϵ, η and θ), are also activated by DAG and require PS as a co-factor, but do not require Ca^{2+}. This may partly be due to the absence of a classical C2 domain which is involved in the binding of Ca^{2+}. Atypical PKCs (aPKC) (ζ and ι / λ), are Ca^{2+}-independent and do not require DAG for activation although PS regulates their activity (Liu, et al.,1998; Mellor and Parker, 1998; Newton, 1997). An additional family may be considered by the more recently discovered PKCμ which is also known as protein kinase D. PKCμ, like the aPKCs, does not respond to either DAG or Ca^{2+}, though apparently still requires PS as a cofactor. Yet another more distantly related family includes the PKC-related kinases (Ouyang et al., 1999).

The existence of the many subtypes of PKC suggests that each may perform specific functions. PKC isoforms are widely distributed in mammalian tissues and some are localized to specific tissues. PKCγ is expressed only in brain and spinal cord, whereas η, θ and λ are most abundant in the lung, skin and skeletal muscle (Mochly-Rosen and Kauver, 2000). PKCθ is present in skeletal muscle and hematopoietic cells, and PKCβ in pancreatic islet cells, monocytes, brain and retinal tissue (Kanashiro and Khalil, 1998).

1.2.8.3 Protein kinase C structure and function

PKC is a single polypeptide with an N-terminal regulatory region and a C-terminal catalytic region. Initial cloning of the cPKC group demonstrated that the polypeptide structure comprises four conserved (C1–C4) and five variable regions (V1–V5). Regions C1 and C2 present at the N-terminal (~20–70 kDa) constitute the membrane-targeting regulatory domains that are required for interaction with DAG and phorbol esters, PS and Ca^{2+}. Incorporated within the C1 site are two cysteine-rich zinc fingers that comprise tandem C1A and C1B repeats that bind DAG and phorbol esters; by contrast, the C2 site is involved in Ca^{2+}-dependent membrane binding (Zang et al., 1995).

Differences in the structure of the various PKC isoforms are associated mainly with the conserved region; the cPKC family contains each of the four conserved regions, whereas nPKC possesses a C2-like region that does not bind Ca^{2+} and might have a role in PKC translocation or protein–protein interactions (Liu et al., 1998). The aPKC family also possesses a C2-like region but only one cysteine-rich zinc finger. The C3 site, present at the C-terminal (~45 kDa) catalytic domain, is involved in binding to ATP, whereas C4, also present in the C-terminal, contains the domain required for the recognition of substrates to be phosphorylated (Gallicano et al., 1997).

The majority of non activated PKC is located in the cytosolic fraction and is translocated

to the membrane upon activation. Thus many functions of PKC in signalling pathways are closely linked with the membrane association of the enzyme. Activation of PKC, initiated by addition of phorbol esters for example, is associated with a redistribution of the enzyme from the cytosol to the membrane. An increase in cellular Ca^{2+} is also sufficient for the redistribution, whereby Ca^{2+} is directly involved in translocation of PKC (Divecha et al., 1993). An equilibrium seems to exist between cytosolic and membrane bound forms of PKC thus it can be assumed that this equilibrium can be shifted in the direction of membrane association with the help of the cofactors Ca^{2+}, PS and DAG.

PKC plays a pivotal role in proliferation, malignant transformation and differentiation of cells. It is a key constituent of a cascade of biochemical events triggered by mitogens and hormones via cell surface receptors, whereby DAG activates PKC by increasing its affinity for Ca^{2+}. A role for PKC has been identified in a variety of disease states including diabetes, heart disease and cancer (Teicher et al., 1999). Furthermore, roles for individual PKC isoforms in clinical states are emerging. For example, over-expression of PKCβII is implicated in cardiomyopathy, heart failure and diabetic cardiovascular complications. Inhibitors of PKC block the development of ischaemic preconditioning and, at the same time, block the translocation of PKCϵ, indicating that this isoform is specifically responsible for the protection afforded by ischaemic preconditioning (Teicher et al., 1999). Many substrates of PKC are membrane proteins and it is evident that membrane association of PKC is of great importance for the phosphorylation of these

proteins.

There is substantial evidence that PKs, through phosphorylation of substrate proteins, play a significant role in information processing in the brain, including processes underlying memory formation. An increasing body of evidence indicates that PKC catalysed phosphorylation plays an important role in hippocampal LTP, which is a long-lasting form of synaptic plasticity found in the mammalian brain that displays a number of properties that make it a likely synaptic mechanism underlying learning and memory (Bliss and Collingridge, 1993). PKC is highly expressed in mammalian brain and the γ isoform seems to be restricted to neuronal tissue.

Another molecular model for memory fixation, LTD in the cerebellum, depends on an increased cation influx via AMPA-type glutamate receptors and DAG/IP_3 release induced by metabotropic glutamate receptors (Mulkey et al., 1993). This leads to an activation of PKC which seems to be a prerequisite for LTD although the corresponding substrate proteins have not yet been identified (Soderling and Derkach, 2000). The stimulatory effects of PKC catalysed phosphorylation on excitatory amino acid receptors are accompanied by an inhibition of $GABA_A$ and glycine receptors i.e. the major inhibitory receptors of the brain. It should be emphasised that besides PKC other PKs have also been implemented in regulating synaptic plasticity (Raymond et al., 1993).

1.2.8.4 Protein kinase C binding proteins

Since the early 1990s it has become clear that in addition to binding to lipids, PKC can also interact with proteins via protein-protein interactions. These interactions play an important role in the localization and function of PKC isozymes. Isozyme specificity appears to be mediated in part by association of each PKC isozyme with specific anchoring proteins. These proteins may be grouped together on the basis of the conformation of the PKC required for binding, cofactor requirement, and whether or not the proteins are PKC substrates. At least four classes of PKC binding protein have now been identified. The members of the first group of PKC binding proteins were all shown to be substrates that interact with C kinase (STICKs) (Ron and Kazanietz, 1999). A number of other PKC binding proteins are not substrates and are, therefore, candidates for a targeting role.

PKC in an active conformation will bind to receptors for activated C kinase (RACKs). RACKS comprise a group of proteins that bind PKC at a site distinct from the substrate binding domain. Binding is insensitive to PS, suggesting that a direct protein-protein interaction is involved. The first RACK to be identified (RACK1) is a protein which enhances the activity of PKC several fold, thereby promoting specific substrate phosphorylation by both targeting and activation of the kinase (Mochly-Rosen et al.,1991). Since RACKS bind only the activated forms of PKC, it has been suggested that

another group of proteins bind PKC in its inactive state. It has been proposed that receptor for inactive C-kinase (RICKS) target PKC to different subcellular locations from RACKS and specific PKC mediated phosphorylation is regulated by the movement of PKC from RICK to RACK (Mochly-Rosen and Gordon,1998). Each isozyme may have several RICKS that anchor it to different subcellular sites in the inactive state. Similarly, there might be several RACKS that anchor PKC in the activated states. Klauck and co-workers (1996) showed that inactive PKCs will interact with A kinase anchoring protein (AKAPs) in particular AKAP79 and AKAP250. It is likely that the unique cellular functions of PKCs are determined by the binding of isozymes to specific anchoring molecules in close proximity to particular subsets of substrates and away from others.

1.2.8.5 Protein kinase C inhibitors and activators.

Experimental evidence for the involvement of PKC in a given biological response has generally been based on the use activators and inhibitors. Effects of inhibitors which may interact either with the ATP binding site, with the DAG binding site or with the lipid binding site of PKC also interact with other PKs and non-PKC phorbol ester binding proteins (Parker, 1999). Thus many inhibitors are not specific for PKC alone and experimental data obtained with them should be interpreted cautiously.

Inhibitors of PKC can be classified according to their site of interaction within the PKC

protein structure. Inhibitors of the regulatory domain can target the phospholipid or phorbol-ester binding site, whereas inhibitors of the catalytic domain are directed to either the substrate site or ATP binding site (Hofmann, 1997). The most promising drugs developed to date as selective PKC inhibitors or as agents on which a new drug design could be based are the inhibitors of the ATP binding site.

The microbial alkaloid staurosporine was identified over 20 years ago as a potent inhibitor of PKC activity and is an example of a drug acting at the ATP binding site. Staurosporine, an indolocabazole, was discovered in the course of screening extracts of the bacterium *Streptomyces* for constituent alkaloids with PKC-inhibitory activity, and it has become the 'lead' compound among PKC inhibitors with therapeutic potential. It turned out to be a potent, albeit non selective, inhibitor of PKC, with an IC_{50} for PKC inhibition of around 10^{-9} M. It can potently inhibit both serine/threonine and tyrosine PKs with a reported ranking order of potency: phosphorylase kinase > PKC > PKA > myosin light chain kinase > p60 src (Ruegg and Burgess, 1989).

The discovery in the late 1970s that plant-derived tumour-promoting phorbol esters, such as phorbol-12-myristate-13-acetate (PMA), substitute for DAG in the activation of PKC (Castagna, 1982) engendered the realisation that phorbol esters are regulators of cell proliferation, differentiation and malignant transformation. PMA is a potent tumour promoter and specific activator aPKC and cPKCs. Unlike DAG, PMA is not readily

metabolised, and treatment of cells results in prolonged activation of PKC (Newton, 1995).

Phorbol esters have been shown to induce or inhibit cell differentiation and proliferation, to activate or attenuate gene expression and the synthesis of various proteins, to stimulate or inhibit enzyme activities, transport processes, ion fluxes and secretion and to cause dramatic morphological changes (Castagna, 1987; Sha'afi and Molski, 1987). According to these observations, PKC appears to play a role in complex physiologic processes such as tissue homeostasis, wound healing, apoptosis, inflammation, LTP and learning, as well as various diseases. Among the putative pathophysiological functions of PKC, tumour promotion is the most prominent (Marks and Gschwendt, 1995).

1.2.8.6 Cyclic AMP dependent kinase structure and function

PKA is a multimeric protein composed of two catalytic subunits (C) and two regulatory subunits (R). The mammalian PKA family includes four regulatory subunits, two of each type (RIα, RIβ, RIIα, RIIβ) and three catalytic subunits (Cα, Cβ and Cδ), each encoded by a unique gene. These each have a distinct expression pattern across tissues, but notably, all are expressed in neurons. Generally, α subunits are expressed ubiquitously, whereas β subunits show a more restricted pattern of expression (Brandon et al., 1997). RI is expressed in most tissues, but the two RII subunits - RII-α and RII-β, although

similar in sequence, are expressed in a tissue specific manner (Beebe, 1994; Cummings et al., 1996). The RIIβ subunit of PKA is expressed at the highest levels in the nervous system and adipose tissues. The RI isoforms appear to be more sensitive to cAMP activation than RII-PKAs (Cummings et al., 1996). Biochemically, the most important difference between RI and RII is that RII is phosphorylated by the C subunits, whereas RI is not. The phosphorylation slows the rate of re-association of RII with the C subunits and, therefore, may have a role in transcriptional regulation as cAMP has been shown to enhance the DNA binding of the catabolite repressor protein to regulate the transcription of several operons (Kennelly and Krebs, 1991).

As a monomer, PKA has a molecular mass of around 41kDa but at least three isoenzyme forms have been identified. The Cα and Cβ subunits in mammals differ by less than 10% when their amino acid sequences are compared and they seem to be highly conserved between species. Cγ is a catalytic subunit whose expression appears to be specific for the testis (Beebe et al.,1992). Cβ has been found in high levels in the nervous system and encodes at least three different splice variants termed, Cβ1, Cβ2 and Cβ3, which have unique termini sequences and show differential expression across various regions of the brain (Guthrie et al.,1997). Cα appears to be expressed constitutively in most cells. The catalytic core of the subunits also shares homology with other known kinases and includes defined regions for peptide binding, ATP binding and a catalytic core.

The C subunits of PKA are maintained in an inactive state and changes from this conformation to the active state upon dissociation of the C subunits from the complex. Dissociation of the complex occurs in response to binding of cAMP to the R subunits, thereby allowing for control over PKA activity in response to extracellular signals (Scott, 1991). Binding of cAMP to the R subunits alters their conformation such that they can no longer bind to the catalytic subunits resulting in the release and activation of the catalytic subunits. Although cAMP is an absolute requirement for PKA activation, other regulatory mechanisms exist to dictate precisely where and when pools of the kinase are activated in response to specific stimuli. Although many extracellular signals use the same second messenger system, the opposing actions of AC and phosphodiesterases generate localised gradients of cAMP which will have greatest influence where concentrated pools of PKA are co-localised in an inactive conformation.

The functions of PKA are diverse. PKA is involved in the regulation of metabolism of glycogen, lipids and sugars. In glycogen metabolism, for example, hormone-induced increases in intracellular cAMP levels result in PKA mediated phosphorylation of glycogen synthetase and phosphorylase kinase, thereby blocking glycogen synthesis and enhancing the net breakdown of glycogen. Substrates of PKA may be other PKs as well as enzymes of intermediary metabolism. PKA is also involved in cAMP stimulated transcription of genes that have a cAMP responsive element in their control region (Montminy, 1997).

The cAMP signalling pathway plays a major integrative role in all animal cells, and components of this system are richly expressed in neurons. Many studies link the cAMP signalling pathway to various paradigms of synaptic plasticity such as LTP and LTD. PKA has also been implicated in other forms of neural plasticity, such as adaptions to pharmacological agents and nociception.

1.2.8.7 Cyclic AMP dependent kinase binding proteins

For the most part, targeting of inactive PKA is achieved by a family of proteins, AKAPS which bind PKA through the regulatory subunits. AKAPS are a family of more than 30 proteins that often share little or no sequence homology, but which all bind PKA in the same way. The AKAPs range between 15 and 300kDa and have been detected in a variety of tissues and in several subcellular components (Colledge and Scott, 1999). Up to 75% of the total cellular PKA is believed to be associated with some member of the AKAP family (Rubin,1994). AKAPs bind PKA via its regulatory subunit, RIIα and RIIβ, and activation of PKA after cAMP elevation induces detachment of the catalytic subunits and their translocation to new subcellular sites. AKAPs serve at least two functions; to place the PKA at locations where it can respond rapidly to cAMP production and to favour certain PKA phosphorylation events by placing the enzyme close to a particular group of substrates (Feliciello et al., 2001).

1.2.8.8 Cyclic AMP dependent kinase inhibitors and activators

PKA inhibitors are useful tools to study PKA-dependent signalling pathways. One of the most potent natural kinase inhibitors for PKA is PKI (cAMP dependent PK peptide inhibitor). PKI is a peptide of 76 amino acids originally isolated from rabbit skeletal muscle by its ability to inhibit PKA activity through binding to its free C subunits (Kawakami and Nakanishi, 1991). Despite the ability of PKI to inhibit PKA activity, its physiological roles are not well understood. In adult heart, the level of PKI activity is ~20% of that necessary to inhibit fully activated PKA (Walsh and Long, 1992). Other agents include the H-compounds such as H-7 and H-89 which are not as specific and also bind to PKC with a lower affinity. PKA activators include forskolin which activates PKA through the stimulation of AC, cAMP analogues such as dibutyryl cyclic AMP (dcAMP) and 8-bromo-cyclic AMP (8-Br-cAMP) and the phosphodiesterase inhibitor 3-isobutyl-1-methyl-xanthine (IBMX) (Hidaka and Endo, 1984).

1.2.9 Other protein kinases

Ca^{2+}/calmodulin kinase II (CaMK II) is another PK that is widespread throughout several tissues and is most abundant in the CNS. The brain contains at least six major types of CaMK, each with very different properties. CaMK II contains a regulatory domain which binds to and inhibits a catalytic domain (Nestler and Greengard,1984). The CaMK

cascade includes three kinases: CaMK kinase (CaMKK); and the CaM kinases CaMKI and CaMKIV, which are phosphorylated and activated by CaMKK. Members of this cascade respond to elevation of intracellular Ca^{2+} levels and are particularly abundant in the brain. CaMKK and CaMKIV localise both to the nucleus and the cytoplasm, whereas CaMKI is only cytosolic. Activation of CaMK II has been linked to memory and the learning processes (Braun and Sculman, 1995).

PKs belonging to the MAP kinase cascade are MAP kinase kinase kinase (MEK kinase), MAP kinase kinase (MEK), and MAP kinase (MAPK) (Nishida and Gotoh, 1993). Each member of this cascade is a protein serine/threonine kinase that phosphorylates the one downstream of it. In addition, MAPKs can autophosphorylate on tyrosine residues. MEK is also a dual specific kinase phosphorylating both tyrosine and threonine residues. Thirteen mammalian MAPKs have been identified and classified on the basis of both sequence homology and differential activation by agonists (Cohen, 1997). Transcription factors are the major targets for MAPKs, where MAPKs translocate from the cytoplasm to the nucleus in order to phosphorylate these proteins and thereby stimulate cell proliferation.

1.3. Glial functions in the CNS

The first description of glial cells was made by Rudolph Virchow as early as 1846

(Tower, 1992). He thought that the main function of these cells was to join neuronal elements together, and he referred to them as neuroglia (nerve glue). In early studies of the CNS, neuroscientists were mostly interested in the electrophysiological properties of the brain and paid little attention to non-excitable cells, which today seems paradoxical in light of the fact that glial cells are the most abundant cells in the CNS outnumbering neurons by about 10 to 1 (Kimelberg and Norenberg, 1989). For decades neurons have been regarded as the only cells involved in the generation and control of brain signalling, while the surrounding glia was supposed to provide structural and metabolic support to neuronal function. However, these cells have been shown to have a wide variety of integral functions participating in degeneration, regeneration and growth in the CNS and unlike neurons, retain the ability to divide throughout life (Kimelberg, 1983). Neuroglia belong to three main subdivisions: the oligodendrocytes, whose main function is to provide the myelin which insulates neuronal axons; a morphologically and biochemically heterogenous group known as the astrocytes, and the microglia which are the immune cells of the brain and are apparently active only following physical trauma or disease (Barres, 1991). Of the glial cells, astrocytes are the most predominant cell type.

Ramón y Cajal first used the term astrocyte to describe the distinctive star-shaped morphology of these cells. The understanding of the morphological diversity of astrocytes was advanced by the discovery of glial fibrillary acidic protein (GFAP) in the early 1970s which was then used as a marker to distinguish astrocytes from neurons and other glial

cell types. GFAP is a 50kD intermediate filamentous protein that constitutes a portion of, and is specific for the cytoskeleton of astrocytes. However, it is important to note that many astrocytes in normal CNS tissue, especially the grey matter, are GFAP-negative. GFAP has been shown to be up-regulated in response to CNS damage and is now widely used as a marker for reactive astrocytes (Eng et al., 2000). Because of the difficulties associated with attempting to study the biology of these cells in the intact brain, in vitro studies have predominated in the course of research into astrocytes.

Astrocytes in the brain are not isolated from one another; they form a functional synctium via intracellular gap junctions (Giaume and McCarthy, 1996). By communicating with each other, astrocytes may coordinate their behaviour and thus participate in brain functions in an efficient manner. Astrocytes are intimately connected to neurons both structurally and functionally, indicating important roles for astrocytes in brain function. Most of the proposed functions of glia centre around the ability of these cells to 'sense' neuronal behaviour through changes in their extracellular environment and to respond to these changes in a manner that supports continued neuronal activity. Brain development initially involves generation of neurons and glial cells from progenitor cells in the subventricular zone. In many parts of the brain, such as the cerebral cortex, glia act as guides for developing neurons. Astrocytes have implicated in tropic support of neurons, promoting local neuritic outgrowth, and are thought to influence the passage of information between neurons through their ability to control local ion and amino acid

concentrations (Sontheimer, 1992).

One well known function of the astrocyte is concerned with repair. Injury in the brain often results in reactive gliosis - where subsequent to trauma, astrocytes invariably proliferate, swell, accumulate glycogen and undergo fibrosis by the accumulation of GFAP (Wilson, 1997). Reactive astrocytes have more processes and glial filaments than ordinary ones and have increased metabolic activity. The result of this reaction is often the formation of a glial scar made up of these reactive astrocytes. The glial scar is characteristic of epilepsy and there has long been a conjecture that astrocytes play a part in the disorder. Multiple sclerosis is an example of a disease in which the process of astrogliosis is particularly intense (Lee et al., 1990). Liedtke et al. (1996) demonstrated that transgenic mice deficient in GFAP develop abnormal myelination in the spinal cord, optic nerve, and brain after 6 months. The authors also showed that the astrocytic processes in GFAP-deficient mice were short and clublike, leading to reduced contacts between astrocytes and oligodendrocytes, and between astrocytes and myelin sheaths. White matter was poorly vascularised and the blood brain barrier was structurally and functionally impaired.

Interestingly, astrocytes have the highest predisposition to malignant transformation of any CNS cell type. Astrocytomas are the most common brain tumour arising in the CNS, accounting for 65% of all primary brain tumours (Kimelberg et al., 2000). The majority

of astrocytomas are histologically malignant neoplasms and are the most common neoplasm amongst childhood brain tumours. Also, with increasing astrocyte malignancy there is progressive loss of GFAP production (Duffy, 1982). For this reason GFAP has been considered a reliable marker of differentiation in normal astrocytes and tumours of astrocytic lineage.

In order for the neuronal network to function smoothly, neurotransmitters, afer being released into the synaptic cleft must be removed. In many brain regions, synapses are entirely surrounded by astrocyte processes. Astrocytes in the hippocampus, for example, almost exclusively surround synapses that are rich in docked vesicles (Bezzi et al., 2001). It is likely that this close physical affiliation provides an opportunity for many functional interactions between astrocytes and neurons. Astrocytes are active and integrated participants of neurotransmission and can release a number of growth factors for neurons, such as nerve growth factor which may stimulate the neuron as a whole as well as promoting axonal growth (Travis, 1994). Astrocytes contain transport systems that are capable of removing various neurotransmitters from the synaptic cleft by transporters present in the plasma membrane. One such example is the serotonin transporter which plays an important role in the re-uptake of 5-HT. The presence of mRNA and protein for the serotonin transporter has been established in cultured rat astrocytes (Inazu et al., 2001).

Both GABA and glutamate can be metabolised by glutamine synthase, which is found exclusively in astrocytes, to form the amino acid glutamine (Norenberg, 1979). Rapid removal of glutamate from the extracellular space is required for the survival and normal function of neurons (Vesce et al., 1999). The export of glutamine from astrocytes, and the uptake of glutamine by neurons, are integral steps in the glutamate-glutamine cycle, a major pathway for the replenishment of neuronal glutamate. In this way, astrocytes play a role in providing nourishment to neurons (Kimelberg, 1989). Not only do astrocytes take up and remove synaptically released glutamate thereby ending its stimulatory action and preventing neuronal damage, but also they are able to undergo rapid bidirectional communication with neurons, based on reciprocal glutamatergic signalling. Thus, release of glutamate from synaptic terminals, in addition to postsynaptic neurons, turns on the astrocytes nearby which respond by liberating the same neurotransmitter via a novel Ca^{2+}-dependent mechanism and thereby signal back to neurons (Vesce et al., 1999).

GABA is the major inhibitory neurotransmitter in the mammalian brain. GABA is cleared from the synaptic cleft by specific, high-affinity, sodium- and chloride-dependent transporters, which are thought to be located on presynaptic terminals and surrounding glial cells (Borden, 1996). Battaglioli and Martin (1991) showed that GABA synthesis in brain slices is dependent on glutamine produced in astrocytes. The rate of GABA synthesis was decreased by 65% in slices pre-treated with an inhibitor of glutaminase, and added glutamine did not reverse this effect, which suggests that glutamine produced by

astrocytes is a quantitatively important precursor of GABA synthesis in cortical slices.

Glucose is the almost exclusive energy source of the brain. All CNS function is abolished within minutes in the absence of glucose, transported across the BBB by specific glucose carriers. It has been confirmed that astrocytes are indeed the primary site of glucose uptake in the brain and that the uptake process increases in parallel with neuronal activity (Tsacopoulos and Magistretti, 1996). Glucose metabolism begins with glycolysis which occurs at a very high rate in astrocytes (Hertz and Peng, 1992). Astrocytes contain stores of glycogen whose glycogenolytic products can be exported from astrocytes as glucose to neurons. Astrocytic glucose-6-phosphatase, which catalyses the terminal step of glycogenolysis and gluconeogenesis, may permit astrocytes to modulate the trans-astrocytic flux of glucose to adjacent neurons in response to signals reflecting increased neuronal demand (Forsyth, 1996). In this regard it is possible astrocytes could be thought of as nurturers of neurons. These findings reveal an active participation of astrocytes in synaptic transmission and the involvement of neuron-astrocyte circuits in the processing of information in the brain.

1.3.1 Glial signalling

In the central nervous system, astrocytes form an intimately connected network with neurons, and their processes closely enwrap synapses. The critical role of these cells in

metabolic and trophic support to neurons, ion buffering and clearance of neurotransmitters is well established. However, accumulating evidence suggests that astrocytes are active partners of neurons in additional and more complex functions. In particular, astrocytes express a repertoire of neurotransmitter receptors mirroring that of neighbouring synapses (Vesce et al, 1999). Such receptors are stimulated during synaptic activity and start Ca^{2+} signalling into the astrocyte network. Astrocyte-released glutamate activates receptors on the surrounding neurons and modifies their electrical and intracellular Ca^{2+}. Ca^{2+} signalling in astrocytes is not restricted to single cells, but can cross cell borders via gap junctions, resulting in intracellular Ca^{2+} waves travelling from one glial cell to the next, or the induction of Ca^{2+} responses in neurons (Cornell-Bell et al., 1990; Finkbeiner, 1992). Ca^{2+} signalling may therefore, be a form of glial excitability enabling these cells to integrate extracellular signals, communicate with each other and exchange information with neurons.

The proposal that synaptic transmission may propagate to neighbouring glia and activate them by elevating levels of internal Ca^{2+} prompted many studies. Nedergaard (1994) used Ca^{2+} imaging methods in mixed primary cultures of neurons and astrocytes to monitor astrocyte excitation together with the response from neurons. The author showed that an astrocytic Ca^{2+} wave could induce a large increase in intracellular Ca^{2+} levels in adjacent neurons. Astrocytes, thus directly modulate the free cytosolic calcium, and hence transmission characteristics, of neighbouring neurons. The Ca^{2+} signal in astrocytes was

most likely mediated by glutamate released from pre-synaptic terminals. Neurotransmitters elevate astrocytic Ca^{2+} levels as a result of the release of Ca^{2+} from internal stores. Stimulation with glutamate has been reported to induce an increase in intracellular Ca^{2+} oscillations observed in astrocyte cultures (Dani et al., 1992; Pasti et al., 1995). Innocenti et al (2000) observed that mechanically induced Ca^{2+} waves in astrocyte cultures was accompanied by a wave of extracellular glutamate, as determined by NADH fluorescence, suggesting that a Ca^{2+} wave in astrocytes could result in a release of glutamate into the neuronal microenvironment which may then play crucial roles excitatory synaptic transmission. Activation of mGlu5 receptors in astrocytes yields increases in IP production and transient Ca^{2+} responses (Biber and Laurie, 1999). These propagating waves of Ca^{2+} suggest that networks of astrocytes may constitute a long-range signalling system within the brain.

A role for astrocytes in the activity-dependent modulation of inhibitory synaptic transmission mediated by GABA interneurons was demonstrated by Kang et al. (1998). In hippocampal slices patch clamping recordings showed that current pulses delivered to an astrocyte could result in an increased frequency and amplitude of miniature inhibitory post-synaptic currents in a pyramidal neuron. This implies that astrocytes may participate in or modulate inhibitory synaptic responses. NA, acetylcholine, histamine and dopamine have also been implicated in glial activation since receptors for these neurotransmitters have also been noted (Kang et al., 1998; Kulik et al., 1999). The evidence implies that

transmitters released during synaptic activity spill out of the cleft at a concentration sufficient to bind to and activate receptors located on adjacent glial plasma membranes. This raises the possibility that astrocytes are an integral element of many aspects of synaptic activity.

1.3.2 Glial receptors

Glial cells possess numerous types of receptors that were previously thought only to be found on neuronal membranes. The first description of in vitro receptor responses in non-neuronal cells was the observation in neuron-sparse primary cultures prepared from fetal brain of a marked increase in cAMP levels in response to the β-adrenoceptor agonist isoproterenol (Gilman and Schrier, 1972). Later studies reported that essentially all receptor types could be found on astroglial cells (Murphy et al., 1987; Walz, 1988; Inagaki et al., 1991; Pearce et al., 1985).

Amongst the many receptors present on glia are the adrenoceptors. β_2 adrenergic receptors on astroglia in culture are coupled to stimulation of AC and an increase in cAMP (Hansson, 1995). Hansson et al. (1984) found a higher β-adrenergic dependent accumulation of cAMP in astrocytes derived from cerebral cortex, striatum and hippocampus, than in those derived from brain stem. Results from electrophysiological (Bowman and Kimelberg, 1987), neuroligand binding (Ebersolt et al., 1981) and second

messenger studies (Pearce et al., 1985; Pollenz and McCarthy, 1986) indicate that astrocytes also possess α_1-adrenoceptors. Astrocytes in the adult hippocampus express α_1-adrenergic receptors coupled to increases in intracellular Ca^{2+} concentration (Duffy and MacVicar, 1995). Pearce et al. (1986) demonstrated that in primary astrocyte cultures prepared from newborn rat cerebral cortex, cerebellum and midbrain, possess α_1-adrenergic and muscarinic receptors, which when activated elicit the production of IP.

While most of the information on astrocytes comes from in vitro preparations obtained from immature tissue, the observation that astrocytes from different brain regions exhibit different responses (Kimelberg and Katz, 1986) suggest that cultured cells maintain distinct properties. These cells exhibit most, if not all, of the different receptors known to be associated with central neurons. Astrocytes appear to be heterogeneous with respect to the expression of surface receptors which were not influenced by association with neurons (Lerea and McCarthy, 1990). In a co-culture of astrocytes and neurons from hippocampal tissue these authors showed that astroglia continued to express β_1 and α_1 adrenoceptors. These results suggest that under in vivo conditions, where astroglia normally exist in close contact with neurons, astrocytes may express surface receptors which enable them to sense neuronal activity and to selectively respond to such activity. Together, these findings indicate that receptor expression in vitro may reflect expression in vivo. The presence of receptors on astrocytes in vivo is difficult to ascertain. However, in a few cases it has been possible to demonstrate that mature astrocytes exhibit certain

receptor systems, for example, Shao and Sutin (1992) found that a sub-population of mature astrocytes isolated from the brainstem exhibited α_1-adrenoceptors. Steinhauser et al. (1994) also found that glial cells from an intact tissue express receptors for the most abundant transmitters in the CNS, i.e. glutamate, and GABA.

1.3.3 Glial protein phosphatases and protein kinases

The occurrence of PPs in glia has been noted by many authors. Pahan and co-workers (1998) have shown both PP1 and PP2A to be involved in the regulation of the expression of inducible nitric oxide synthase (iNOS) in rat primary astrocytes and macrophages. The expression of this enzyme suggests the production of nitric oxide in these cells which plays a crucial role in cerebrovascular damage and/or secondary brain damage subsequent to traumatic brain injury. Vinade and Rodknight (1996), through the use of PP inhibitors, concluded that dephosphorylation of GFAP in slices prepared from hippocampi of immature rats was primarily due to PP1. Pei et al. (1997) and Gong et al. (1994) found elevated levels of PP2A and PP2B associated with hyperphophorylated tau in Alzheimer's disease. Daniels and Vickroy (1999) found that glutamate transport into glia could be regulated by an OA-sensitive PP and PKC.

Elevated cAMP, DAG and Ca^{2+}, induced by agonists in a range of cell systems, initiate

a cascade of events via the activation of specific PKs. Some of the events prompted by PKs are extremely rapid such as phosphorylation of ion channels, whereas others may be of longer term, such as the modification of proteins associated with exocytosis or of those associated with the cytoskeleton. CaMK II is of particular interest in the nervous system because of its high concentration in the brain and its proposed role in synaptic transmission (Bennett et al., 1983). Bronstein et al. (1988) found CaMK II-like immunoreactivity in pure neuronal and astrocytic cultures. Inagaki et al. (1996) have shown that vimentin and GFAP are phosphorylated by CaMK II in vitro, and that assembly and disassembly of the filaments are consequently regulated. Phosphorylation of the N-terminal domain of GFAP using PKC or PKA has also been reported (Inagaki et al., 1990), which seems to suggest that phosphorylation may be a regulatory process for intermediate filament assembly-dissembley in intact cells. The activation of PKC increases the phosphorylation of astroglial intermediate filament proteins and induces polygonal astroglia to become process-bearing cells (Mobley et al., 1986). The finding that receptors linked to PKA or PKC have the potential to modulate the morphology of astrocytes suggests that this maybe an important interaction between neurons and astrocytes as small changes in astrocytic morphology could markedly change the volume of the extracellular space and neuronal excitability.

Loffler et al. (1985) reported the type II regulatory subunit of PKA to be predominant in astrocytes. Evidence for PKA function in these cells includes the effects of cAMP on

phosphorylation of GFAP and vimetin (McCarthy et al., 1985) and in the regulation of glycogen turnover (Cambray-Deakin et al., 1988). α_1-Adrenoceptor astrocyte receptors coupled to inositol phospholipid metabolism have been shown to be differentially regulated by PKC (Pearce et al., 1993). In astrocyte cultures, staurosporine induced a dose-dependent increase both in GFAP immunoreactivity and in the activity and protein levels of glutamine synthetase (Kronfeld et al., 1995). Staurosporine also induced a decrease in the expression of PKC-βII and an increase in that of PKC-γ. In addition, staurosporine induced translocation of PKC-ϵ from the membrane to the cytosol, whereas no differences were observed in the distribution of the other PKC isoforms (Kronfeld et al., 1995). Recently, phorbol ester-sensitive PKC isoforms have also been reported to direct the guidance of neurites by astrocyte-derived matrix molecules (Powell et al., 2001).

Other roles for PKC in astrocytes have also been suggested, for example, Neary et al. (1999) suggest that signalling from P2Y purinergic receptors to MAPK involves a Ca^{2+}-independent PKC isoform. Activation of P2Y receptors stimulates proliferation of astrocytes. Pearce et al. (1988) proposed an interaction between PKC and AC in the regulation of astrocyte glycogen metabolism and Jeremy et al. (1987) reported that stimulation of PKC by phorbol ester elicited prostanoid synthesis and release by a process that involves Ca^{2+} influx and the activation of phospholipase A_2. Thus, specific receptor

PKs and PPs, which can phosphorylate or dephosphorylate their targets represent a mechanism for controlling the function of receptors associated with astrocyte functions

1.4 Aims of this research

The main aims of the research described here were:

- to elucidate the involvement of PPs and PKs in cell signalling pathways, specifically receptor mediated phosphoinositide metabolism in response to α_1-adrenoceptor stimulation.
- to characterise PP and PK activities in glial cultures prepared from the newborn rat cerebral cortex.

CHAPTER 2
MATERIALS AND METHODS

2.1. Preparation of primary cultures of cortical astrocytes

Astrocyte - enriched cultures were prepared from newborn (0-3 day old) rat cerebral cortices according to the method of Dutton et al. (1981). Equipment was dry heat sterilised at approximately 180°C or sprayed with 70% alcohol, and procedures were carried out in a laminar hood flow at room temperature. Solutions were sterilised by filtration through a 0.2μm cellulose nitrate membrane filter (Whatman) immediately before use.

Neonates were decapitated and the cerebral cortices were detached from the hind brain. The meninges and vasculature were removed using fine forceps and the cortices were placed in a few drops of dissagregation medium containing glucose (14mM), bovine serum albumin (BSA, Sigma) (3mg/ml) and $MgSO_4$ (1.5mM) in 100ml of Ca^{2+} and Mg^{2+} - free Earle's Balanced Salt Solution (Gibco).

The tissue was finely chopped with a sterile scalpel and transferred to a trypsinisation flask containing trypsin (250μg/ml, Sigma) in 10ml of dissagregation medium and incubated at 37°C with gentle agitation for 15 min. A 10ml solution containing soya bean trypsin inhibitor (SBTI, 192μg/ml, Sigma), deoxyribonuclease (DNase, 6.4μg/ml, Sigma) and $MgSO_4$ (240μM) in dissagregation medium was then added and the resulting suspension decanted into 50ml sterile plastic tubes and centrifuged at 1000rpm for 5s to sediment cell bodies. The supernatant was discarded leaving a pellet which was

resuspended in a solution containing a few drops of dissagregation medium containing SBTI (1.2mg/ml), DNase (40μM/ml) and $MgSO_4$ (1.5mM).

The tissue was then dissociated into a single cell suspension by gentle tituration through a sterile 1.5mm diameter stainless steel canula attached to a sterile syringe and the debris allowed to settle. The supernatant was removed and transferred to a 15ml tube, this step was repeated until all tissue blocks had been dissipated. Cell debris and undisrupted tissue were removed from the final suspension by centrifugation (5 min, 1000rpm) through an underlay of 2ml 4% BSA solution containing $MgSO_4$ (1.5mM) in dissagregation medium. The supernatant was removed and the cell pellet resuspended in an appropriate volume of culture medium containing Minimum Essential Medium with Earle's salts (Glutamine free, Gibco) supplemented with foetal calf serum (10% v/v, Gibco), glutamine (2mM), glucose (33mM) and antibiotic/antimyotic solution (10 units/ml penicillin, 10μg/ml streptomycin and 25ng/ml amphotericin B, Sigma).

Cells were then seeded at a density of approximately 10^6 cells/well onto poly-D-lysine (50μg/ml, Sigma) coated 6 well plates (Nunc) or T60 flasks (Greiner) and maintained in a 5% CO_2 air atmosphere in a humidified incubator at 37°C. Cultures prepared from this method contain approximately 95% protoplasmic astrocytes as revealed by immunoflurescence labelling with an antibody to GFAP. The uptake of a labelled low density lipoprotein shows that the remaining cells in these cultures are mainly microglial cells (Phillips and Pearce, unpublished observations). Growth medium was changed

weekly and cultures were grown to confluence (approx. 14 - 21 days in vitro) before being used in experiments.

2.1.1. Preparation of cell lysates

14 day old astrocyte cell cultures were harvested by scraping cells from six T60 flasks into a 5ml volume of phosphate buffered saline (0.1mM KH_2PO_4, 1mM NaCl and 0.1mM $NaPO_4.7H_2O$). Cells were the then centrifuged at 13,000 rpm for 5 min, the supernatant discarded and the pellet snap frozen in liqiud nitrogen then stored at -80°C for use in PP and PK assays.

The pellet was resuspended in homogenisation buffer: 0.25M mannitol, 100mM Tris HCl (Bio-Rad) pH 7.4 at 4°C, 1mM ethylenediamine tetraacetic acid (EDTA, Sigma), and freshly added protease inhibitors: 1mM dithiothreitol (DTT, Calbiochem), 1mM benzamidine (Sigma), 1μg/ml SBTI and 0.2mM phenylmethylsulphonyl fluoride (PMSF, Sigma). DTT is included as a protectant against oxidative damage to proteins. Only the reduced form of DTT has protective qualities, and DTT in free solution will be completely oxidized by molecular oxygen within a few hours to days, depending on temperature; therefore DTT was always added to buffers on the day of use. The other protease inhibitors are hydrolysed to inactive products in water and were also added to buffers fresh on the day of use. EDTA is used as a heavy metal chelator, it chelates $Ca^{2+}$and so inactivates Ca^{2+} dependent proteases. The presence of EDTA and a mixture

of protease inhibitors in the homogenisation buffer was needed for the preservation of active PPs. Cells were then lysed by freeze/thawing to liberate intracellular proteins and adjusted so that the final protein concentration was between 1 - 3 mg/ml and then diluted as necessary for a particular assay.

2.2. Extraction of [^{3}H]-inositol phosphates

Cultures in 6 well plates were incubated with 1μCi/ml [^{3}H]-myo-inositol (specific activity: 92Ci/mmol, Amersham) in 2ml culture medium for 48h at 37°C in a 5% CO_2 incubator to prelabel membrane inositol phospholipids. The cells in each dish were observed before use to ensure that they were confluent. The culture medium was aspirated and the cells washed twice with Krebs buffer solution containing NaCl (116mM), $NaHCO_3$ (26.2mM), NaH_2PO_4 (1mM), KCl (5mM), $MgSO_4$ (1.5mM), $CaCl_2$ (1.3mM) and glucose (20mM) pregassed with 5% CO_2 in O_2.

Cell cultures were then incubated in this medium for 30 min before LiCl (5mM) was added to the cultures for a further 15 min to inhibit the breakdown of the IPs formed (Berridge et al., 1982). Cultures were then incubated for a further 45 min in the presence or absence of various drugs at 37°C in a 5% CO_2 / air atmosphere.

The reaction was terminated by the removal of incubation medium and addition of 0.5ml ice cold methanol to each well. The cells were harvested and the extracts transferred to

1.5ml tubes to which 0.3ml of chlorofrom and 0.3ml distilled water were then added. The contents were mixed and the phases separated by centrifugation (5 min, 10,000rpm). 500µl of the upper aqueous phase was removed and diluted to 2.5ml with distilled water. This was added to small columns containing 1ml of a 50% slurry of AG-1-X8 anion exchange resin (200-400 mesh, BioRad) in the formate form and was used to bind the IPs.

After washing the resin 5 times with 2.5ml volumes of myo-inositol solution (5mM) to remove free [^{3}H]-inositol, the labelled IPs were eluted from the column with 1ml of ammonium formate (1M) in formic acid (0.1M) into scintillation vials and the radioactivity determined in each by liquid scintillation counting. Aliquots of the lower chloroform phase were also taken for counting as a measure of radiolabel incorporation into phospholipids.

In some experiments separation of individual [^{3}H]-IPs was carried out. Incubations were carried out as before and the aqueous phase added to the resin and the phosphates eluted by addition of formate solution of increasing strengths. Glycerophosphoinositol (GPI) was eluted with 10ml sodium tetraborate (5mM) in sodium formate (60mM). IP_1, IP_2 and IP_3, were eluted with 15ml sodium tetraborate (5mM) in sodium formate (150mM), 10ml formic acid (0.1M) in ammonium formate (0.3M) followed by 5ml formic acid (0.1M) in ammonium formate (0.75M), respectively. Eluates from each wash were collected in 1ml volumes and the radioactivity in each fraction measured by liquid scintillation

counting. Differences in cell densities may lead to variation in results obtained with different batches of cell cultures. To overcome this, the radioactivity recovered in IPs was corrected to a standard incorporation of [^{3}H]-inositol (10^5 dpm) into phospholipids, unless otherwise stated.

2.3. Determination of protein concentration

Protein concentration was measured using the method of Bradford (1976) which uses an absorbance shift in an acidic Coomasie Blue dye solution. Binding of proteins to Coomasie Brilliant Blue G-250 via basic amino acids shifts the absorbance of the dye from 495nm to 595nm. The increase in absorbance at 595nm was used to determine the amount of protein present relative to BSA standard solutions. Coomasie Brilliant Blue dye solution was prepared by dissolving 30mg Coomasie Brilliant Blue G - 250 in 100ml of absolute ethanol. Once this has dissolved 50ml concentrated phosphoric acid was added and the solution diluted to 1l with distilled water. The solution was filtered to remove any undissolved dye and stored at 4°C protected from light.

A standard curve was prepared using known concentrations of BSA. A stock solution of BSA was freshly prepared with an absorbance at 280nm of 0.13, equivalent to 0.2mg/ml protein. Seven solutions of 100µl volume containing from 0 to 6µg BSA in water were prepared. To each solution was added 1ml Coomassie reagent (warmed to room temperature before use). The absorbance of each of the solutions were measured at 595nm

against a blank (Perkin - Elmer UV spectrophotometer) and a calibration curve plotted of [BSA] against absorbance. Small aliquots of unknown protein samples were diluted in a final volume of 100µl water to which 1ml reagent was added. The absorbance was measured and the amount of protein determined from the calibration curve. The dilution of unknown protein was then adjusted so that the absorbance of the sample was within the linear portion of the calibration curve, usually within 0.05 and 0.15 units.

$$\frac{\text{Absorbance of sample}}{\text{gradient from calibration curve}} \times \text{dilution factor} = \text{Protein (mg/ml)}$$

2.4. Protein phosphatase assays

PP activities were determined from the rate of dephosphorylation of a [^{32}P]-labelled substrate.

2.4.1. Preparation of [^{32}P] - phosphorylase a

Radiolabelled phosphorylase a was prepared according to the method of Resink et al. (1983). Phosphorylase b has a lower activity in terms of catalysing glycogen breakdown and can be converted into the more active form phosphorylase a by the enzyme phosphorylase kinase.

$$2\text{ATP} + \text{phosphorylase b} \xrightarrow{\textit{Phosphorylase kinase}} 2\text{ADP} + \text{phosphorylase a}$$

Phosphorylase kinase (Sigma) was resuspended at a concentration of 1,000 units/ml in a buffer B: 100mM Tris HCl, pH 7.4 at 37°C, 0.2mM EDTA , 20% glycerol and 2mM DTT. This was used immediately or snap frozen in liquid nitrogen and stored at -80°C. Phosphorylase b (Sigma) was dissolved at a concentration of 10mg/ml in the same buffer.

PP1 and PP2A are the only PPs in mammalian tissues to dephosphorylate glycogen phosphorylase a (Ludlow et al, 1998), thus PP activity can be determined by the ability of extracts to release [^{32}P] from radiolabelled substrate. A pilot phosphorylation of phosphorylase showed that incubation with phosphorylase kinase and [γ-^{32}P]-ATP (ICN) for 30 min gave maximal labelling with a typical specific activity of 0.48nmol ^{32}P/nmol phosphorylase. The phosphorylation reaction contained buffer B (100mM Tris HCl pH 7.4 at 37°C, 0.2mM EDTA, 20% glycerol and 2mM DTT), ATP solution (0.2mM [γ-^{32}P]-ATP (specific activity 1.5 x 10^6 cpm/nmole phosphate), 1mM non-radioactive ATP (Sigma) and 20mM $MgCl_2$, 0.1mM $CaCl_2$, 5mg/ml phosphorylase b and 100 units/ml phosphorylase kinase in a volume of 0.5ml. After 30 min at 37°C, the mixture was dialysed for 48-72 h at 4°C in 1l of 50% buffer B to remove excess [γ-^{32}P]-ATP with two buffer changes. Amberlite monobed resin (Bio-Rad) was also added to the dialysis buffer to sequester free [γ-^{32}P]-ATP and thereby increase the effective buffer volume. Dialysis was continued until the radioactivity in a trichloroacetic acid (TCA) precipitate of 10μl of the protein solution was at least fifty times greater than from supernatant. Each preparation of substrate is stable at 4°C with sufficient radioactivity for a useable lifetime of 4-6 weeks (2 - 3 half lives).

2.4.2 Protein phosphatase assay

[^{32}P] - labelled glycogen phosphorylase a is a traditional phosphoprotein substrate used to monitor PP1 and PP2A activity. Release of [^{32}P] phosphate from labelled phosphorylase a, prepared as in section 2.4.1, was used to determine the activity of these PPs, according to the assay method of Cohen et al. (1989). Dephosphorylation reactions contained 50mM Tris HCl pH 7.4 at 37°C, 0.1mM EDTA and 1mg/ml [^{32}P]-phosphorylase a. The protein concentration was ascertained as described in 2.3. OA (Calbiochem) stored in 25% DMSO (Sigma) was used at 1nM to inhibit PP2A and at 2μM to inhibit both PP1 and PP2A. Control assays included the DMSO vehicle. Inhibitor - 2 (0.5μM, Calbiochem) is a heat-stable, natural and specific inhibitor of PP1 and was included in some assays.

The assays were incubated at 37°C and 10μl aliquots taken at 4 and 8 min. The aliquots were pipetted into 40μl 25% TCA to stop the reaction, vortexed, then placed onto ice for 5 min. After centrifuging at 13,000rpm for 3 min to separate denatured protein from free phosphate, a 40μl aliquot of supernatant was added to 1ml scintillation fluid and counted for 1 min to measure [^{32}P]. A blank incubation which contained no protein extract was included to measure free [^{32}P] in the substrate. This was subtracted from each test assay. The cpm obtained for each aliquot was converted into nmoles [^{32}P] labelled phosphate released by counting an aliquot of the ATP solution used to phosphorylate the peptide with each assay. Since the phosphate content of the aliquot was known, the cpm/nmole

phosphate could be calculated.

$$\text{Specific activity} = \frac{\text{(total cpm/reaction tube - cpm blank)}}{\text{nmoles of protein bound phosphate}}$$

2.5 Protein kinase assays

Cells were harvested as described in 2.4. A solution of [^{32}P]-ATP was prepared containing 20mM non radioactive ATP, 20mM $MgCl_2$, and [γ-^{32}P] - ATP of a sufficient volume such that on the day the solution was used it had a specific activity of 200- 400 x 10^3 cpm / nmole phosphate. The non radioactive stock ATP was diluted to produce a final concentration of 1mM.

2.5.1 Assay for cyclic AMP - dependent protein kinase

PKA is activated by dissociation of its regulatory and catalytic subunits upon binding of cAMP to the regulatory subunit. The catalytic subunit then phosphorylates serine and threonine residues on substrate proteins.

The method for the assay of PKA was based on that described by Maller et al. (1978). PKA activity was measured as the incorporation of [^{32}P] from [γ-^{32}P]-ATP into the synthetic peptide substrate kemptide (Sigma) which has a reported K_m of 4.7μM for PKA

(Whitehouse et al., 1983). PKA activity in cell or tissue extracts were taken to be the activity that was sensitive to the specific pseudosubstrate inhibitor of PKA (PKI, Sigma) which is highly specific for PKA over other kinases (K_i = 1.7nM). PKA was measured in the absence of cAMP (initial activity) and in the presence of 10μM cAMP (total activity) and expressed as an initial: total activity ratio.

Brains or cells were homogenised 0.1g dry weight in 100μl buffer A: 0.25M mannitol, 100mM Tris HCl pH 7.4 at 4°C, 50mM NaF, 2mM PPi, 1mM EDTA, 1mM DTT, 1mM benzamidine, 1μg/ml SBTI, 0.2mM PMSF, 1mM IBMX (Sigma). The suspension was centrifuged at 13,000rpm for 1 min and the supernatant diluted 1 in 20 in dilution buffer (0.5M Tris HCl pH 7.2 at 37°C, 25mM NaF, 25mM NaCl, 0.5mM EDTA, 10mM IBMX, 20% BSA). Phosphorylation of kemptide was assayed in a final volume of 50μl containing 200μM kemptide, 1mM IBMX, 4mM $MgCl_2$, 0.2mM [γ-^{32}P]-ATP (200- 400 x 10^3 cpm /nmole phosphate) in the presence /absence of 10μM cAMP (Sigma) and /or 50units/μl PKI. Thus for each experimental condition (i.e. per cell extract) 4 assay conditions; ± inhibitor and ± cAMP were used to determine total PKA activity and the proportion of PKA activated in cells or tissues. All reagents were kept ice-cold.

The reaction was started by addition of 10μl 1mM [^{32}P]-ATP. 20μl aliquots were removed at 4 and 8 min intervals during incubation at 37°C and spotted onto phosphocellulose P81 discs (Whatman) which were immersed into a dish containing 77mM phosphoric acid and gently stirred to terminate the reaction. After 10 - 20 min the

acid was changed and the filters washed twice more before a final brief wash with water. The phosphocellulose discs were then transferred into scintillation vials for counting.

The acid protonates the basic amino acids in the [^{32}P]-kemptide peptide so that its positive charge binds to the negatively charged phosphocellulose paper. Negatively charged [^{32}P]-ATP substrate is thus washed away. PKA activity was calculated as the activity resistant to PKI inhibition in the presence or absence of cAMP. The activity ratio of PKA is the degree of dissociation of the C subunit at the time of isolation compared to the total amount of C subunit present and was calculated by dividing the activity in the absence of cAMP by the activity in presence of cAMP.

Calculations:

$$\frac{\text{cpm/ 10}\mu\text{l }^{32}\text{P/substrate solution}}{\text{nmol ATP}} = \text{cpm/ nmol phosphate (Specific activity)}$$

$$\frac{\text{cpm/phosphocellulose disc}}{\text{cpm/ nmol phosphate}} = \text{total nmol incorporated phosphate}$$

$$\frac{\text{nmol phosphate incorporated}}{\text{time of assay}} = \text{nmol/min/assay tube}$$

$$\frac{\text{nmol/min/assay tube}}{\text{mg extract}} = \text{nmol/min/mg extract}$$

$$\text{nmol/min (-PKI, -cAMP) - nmol/min (+PKI, -cAMP)} = \text{nmol/min activated PKA}$$

nmol/min (-PKI, +cAMP) - nmol/min (+PKI, +cAMP) = nmol/min total PKA

$$\frac{\text{nmol/min activated PKA}}{\text{nmol/min total PKA}} \times 100 = \%\text{ activated PKA}$$

2.5.2 Assay for protein kinase C

PKC is a serine / threonine kinase first characterised by Nishizuka (1986) on the basis of its activation in vitro by Ca^{2+}, phospholipid (primarily, PS) and DAG. The most common methods for assaying PKC activity involves measuring the transfer of [^{32}P] labelled phosphate to a protein or peptide substrate such as histone III which can be captured on phosphocellulose filters via weak electrostatic interactions as in the PKA assay described previously.

PKC activity was assayed in cell and tissue fractions in a 50µl reaction mixture containing assay buffer (20mM Tris HCl pH 7.5 at 4°C, 1mM PPi, 50mM NaF, 10% glycerol and 1mM DTT), 3.5mg/ml Type III-S histone (Sigma), 1mM [^{32}P]-ATP in the presence and absence of PS (2mg/ml, Calbiochem) and 0.5mM $CaCl_2$ at 37°C. Some reactions also contained no protein or substrate for controls. The reaction was started by addition of 10µl [^{32}P]-ATP. 20µl of the reaction mixture was spotted onto P-81 phosphocellulose filters at 4 and 8 min and the reaction terminated when placed into a dish of 77mM phosphoric acid and stirred gently. After 10 - 20 min the acid was changed

and this was repeated twice more before a final brief wash with water. The phosphocellulose discs were then transferred into scintillation vials for counting.

Calculations:

$$\frac{\text{cpm/ 10}\mu\text{l } ^{32}\text{P/substrate solution}}{\text{nmol ATP}} = \text{cpm/ nmol phosphate (Specific activity)}$$

$$\frac{\text{cpm/phosphocellulose disc}}{\text{cpm/ nmol phosphate}} = \text{total nmol incorporated phosphate}$$

$$\frac{\text{nmol phosphate incorporated}}{\text{time of assay}} = \text{nmol/min/assay tube}$$

$$\frac{\text{nmol/min/assay tube}}{\text{mg extract}} = \text{nmol/min/mg extract}$$

$$\text{nmol/min (+ PS) - nmol/min (- PS)} = \text{nmol/min activated PKC}$$

2.6 $^{45}Ca^{2+}$ uptake and release assays

For the $^{45}Ca^{2+}$ uptake studies, cultures were grown in 24-well plates (Nunc). Cells were washed three times in Krebs buffer then incubated at room temperature in 0.5ml of buffer containing 0.4μCi/ml $^{45}Ca^{2+}$ (Amersham) for various periods. Uptake was terminated by the addition of 1ml ice-cold normal saline containing 2mM EDTA. Buffer was then removed and the cells digested in 0.5ml 0.1M NaOH, 0.2ml of digest was taken for counting. Subsequent studies examining the effects of various drug additions were carried out over a 2 min incubation period.

For $^{45}Ca^{2+}$ release studies, cultures grown in 6 well plates were incubated for 24h in medium containing 4μCi/ml $^{45}Ca^{2+}$. Cells were washed three times in 2ml volumes of Krebs buffer then incubated for 15 min with OA (0.5μM) or PMA (0.3μM, Affiniti) as required. Radiolabelled Ca^{2+} release was assessed over a 10min period at room temperature, samples being collected each min. At the end of each period, 0.5ml aliquots of incubation medium were taken for counting , the remaining medium was removed and replaced with fresh buffer. The first five periods were to establish basal levels of release, the next 1 min period was in the presence of NA (100μM) or a combination of drugs, the remaining four 1min periods were in drug-free buffer. At the end of the experiment the cells were digested in 1ml 0.1M NaOH and 0.2ml of the digest was taken for counting. The release of $^{45}Ca^{2+}$ in each incubation period was then calculated as a fractional release i.e. the amount released as a percentage of that present in the cultures at the time the fraction was taken.

2.7 Statistical analysis

Statistical comparisons between drug treatments were made using the Mann-Whitney U-test. P-values of less than 0.05 were taken as indicating statistically significant differences.

CHAPTER 3
RESULTS

3.1 Protein phosphatase activities in rat brain and glial cultures

The phosphorylation state of a protein results from the balance of activity of PKs and PPs. The importance of PPs in a variety of cellular processes has been highlighted by the use of cell-permeant PP inhibitors such as OA. OA inhibits PP1 and PP2A with IC_{50} values in the range of 10-15nM and 10-100pM, respectively, PP2B is inhibited with an IC_{50} value of 5μM whereas PP2C is completely unaffected (Cohen et al., 1989). Therefore, suitable dilutions of OA can be used to discriminate between PP species in cell extracts.

In the following assays, extracts were measured for PP activity by their ability to dephosphorylate glycogen phosphorylase which is a traditional substrate used to monitor PP2A and PP1 activity (Ingebritsen and Cohen, 1983). OA was diluted in DMSO (25%), the vehicle was used as a control in all assays. Figure 3 shows that 68% of the total PP activity present in rat brain can be ascribed to PP2A with 31% being of the PP1 subtype and approximately 1% belonging to OA insensitive PPs. Figure 4 shows that 51% of the PP activity present in glial cultures are of the PP1 species with 44% being of the PP2A subtype and less than 5% belonging to other PP species. In rat brain therefore, there is almost two fold the amount of PP2A than PP1 species whereas glial cells appear to have an almost equal proportions of both PPs. Glial cultures were prepared from the cerebral cortex of neonatal rats whereas brain cultures were prepared using whole adult rat brain. Thus, differences in the proportion of PP species may be due to differences in brain area or the age of cell cultures or brains from which the extracts were prepared.

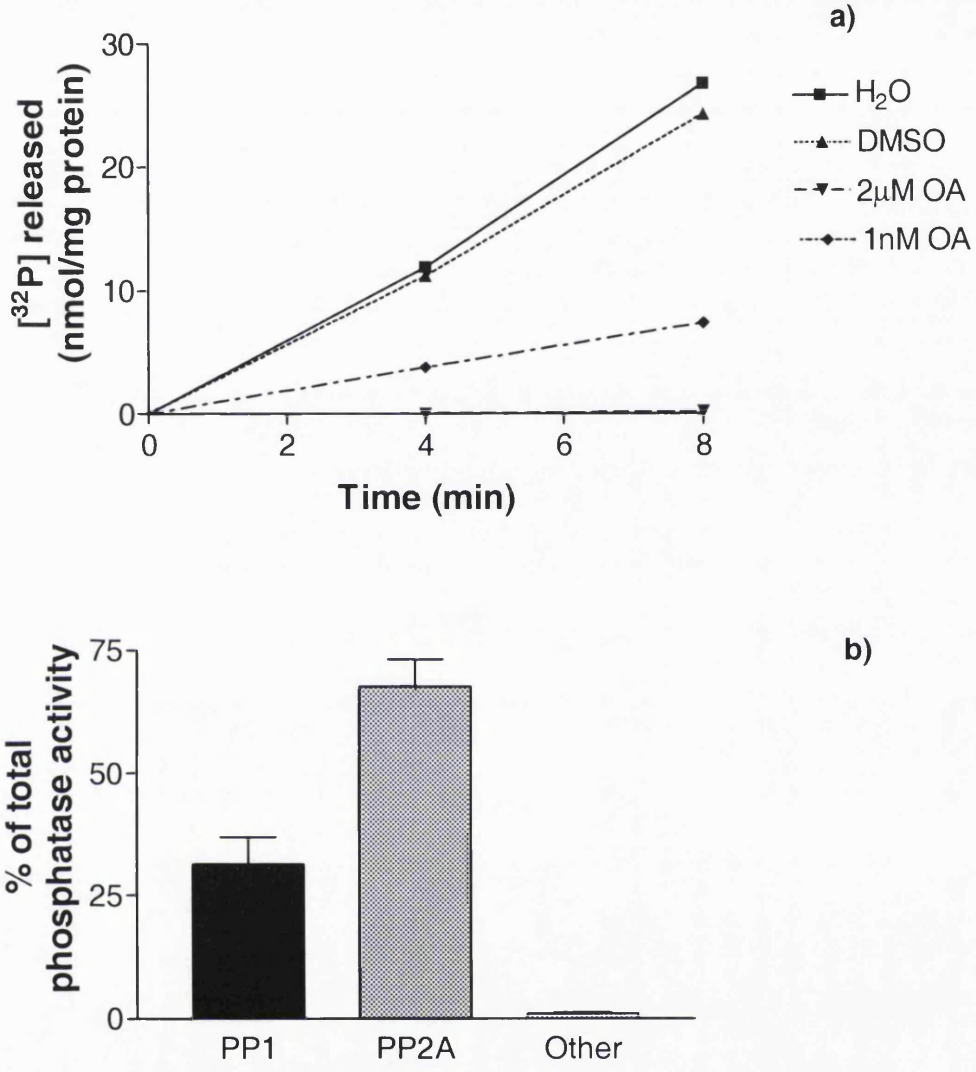

Figure 3. Protein phosphatase activity in whole rat brain.

a) A representative time course showing the ability of rat brain extracts to liberate [^{32}P] from labelled glycogen phosphorylase. Release was monitored at 4 and 8 min. Results are expressed as nmol [^{32}P] released/mg protein. **b)** Proportion of PP species present in rat brain. Total activity was 3.35 ± 0.12 nmoles [^{32}P] released/min/mg protein. Results are means ± S.E.M. for 3 determinations.

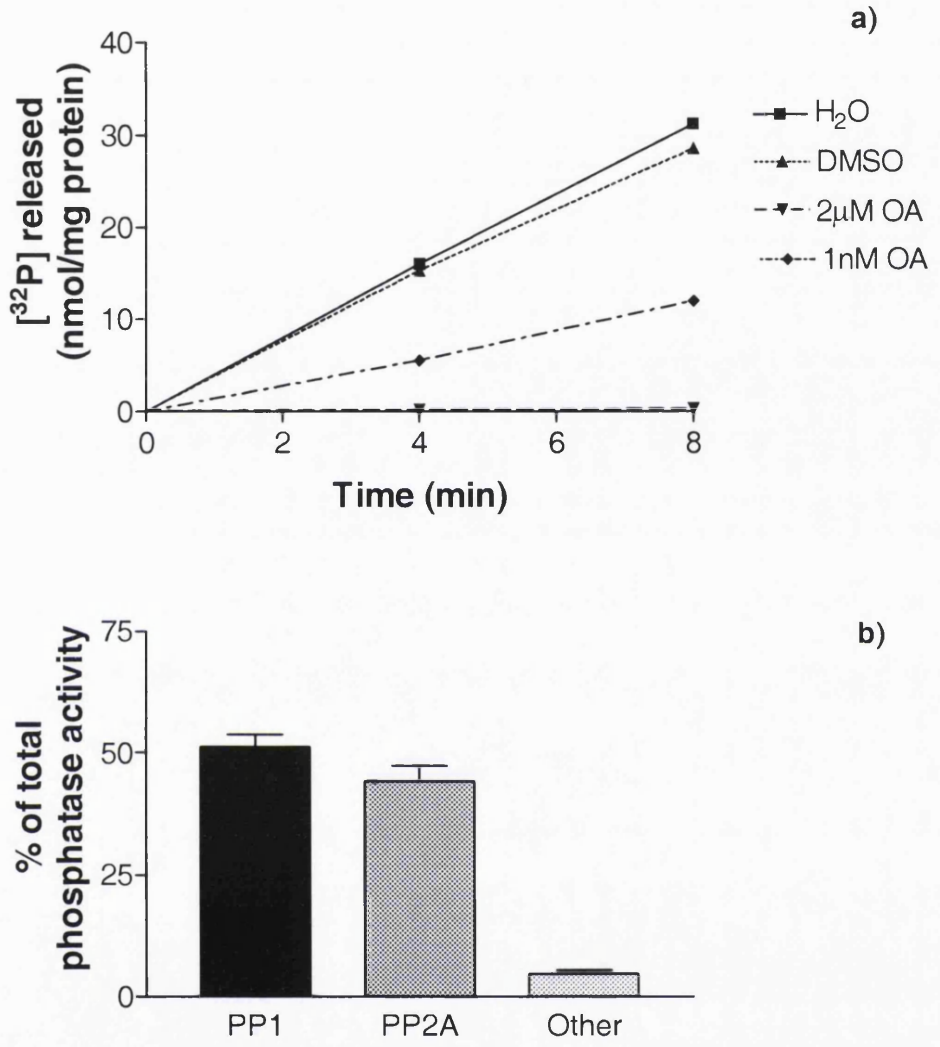

Figure 4. Protein phosphatase activity in glial cultures.

a) A representative time course showing the ability of glial cell extracts to liberate [^{32}P] from labelled glycogen phosphorylase. Release was monitored at 4 and 8 min. Results are expressed as nmol [^{32}P] released/mg protein. **b)** Proportion of PP species present in glial cultures. Total activity was 3.36 ± 0.63 nmoles [^{32}P] released/min/mg protein. Results are means ± S.E.M. for 3 determinations.

Other tissue extracts have also been found to possess varying PP proportions. Gagliardino et al. (1997) found PP1 and PP2A to be present in crude islet homogenates in equivalent proportions of 53% and 47%, respectively. Foulkes and Jefferson (1984) also found the specific activity of PP2A to be equivalent to PP1 in the case of skeletal muscle extracts, but to be approximately 50% higher than PP1 in extracts of liver and heart. This may suggest a degree of tissue heterogeneity in terms of the PP species expressed, a feature which may be linked to the functions of that particular tissue.

Protein phosphorylation and dephosphorylation play a prominent role in signal transduction processes. Having established the presence of both PP1 and PP2A activities in glial cell cultures, the next series of experiments were designed to assess their involvement in receptor-mediated inositol phospholipid metabolism. This signalling pathway involves phosphoinositide metabolism where receptor activation leads to the PLC mediated breakdown of PIP_2 to yield two second messengers, IP_3 which modulates the release of Ca^{2+} from internal stores and DAG which activates PKC. Factors known to stimulate this pathway in glia include agonists such as NA acting via α_1 adrenoceptors.

3.2 Inositol phosphate accumulation in glial cultures

Initial experiments were aimed at characterising NA-stimulated [^{3}H]-IP accumulation in glial cultures and were carried out using cultures which had been pre-labelled with [^{3}H]-inositol for 48h. The agonist NA was found to stimulate an accumulation of [^{3}H]-IP above basal which was time and concentration dependent. NA-induced [^{3}H]-IP

accumulation increased in an essentially linear fashion over the 60 min incubation period whilst basal [^{3}H]-IP formation was virtually unchanged for the duration of the experiment (figure 5a). The ability of NA to increase [^{3}H]-IP accumulation in the cultures was also found to be dependent on the concentration of the agonist. Maximal responses were elicited with agonist concentrations of 100μM and the curve shown in figure 5b gave an apparent EC_{50} value of 5μM which is in accordance with previously published results (Pearce et al.,1986).

Studies of this nature are invariably performed in the presence of Li^+ as this prevents the breakdown of IP_1 to free inositol (Berridge et al.,1982). To confirm this in glial cultures, basal and NA-stimulated [^{3}H]-IP accumulation were observed in the presence and absence of 5mM Li^+. Results show that addition of Li^+ did not affect basal values, whereas NA-stimulated [^{3}H]-IP accumulation is increased 3-fold in the presence of Li^+ compared to its absence (figure 6). Figure 7 shows an elution profile of water soluble [^{3}H] metabolites produced in response to 100μM NA. NA caused a marked increase in the recovery of [^{3}H] in the IP_1 fraction, some 10-fold when compared to basal values. NA also produced a significant accumulation of IP_2, an 8-fold increase as compared to basal values. The radioactivity recovered in the IP_3 fraction, however, was very low and only slightly increased in response to the agonist. Similarly, there was no great NA - evoked increase in [^{3}H]-GPI production with values very close to those of the basal fractions. In general terms, these data are as one might expect for a 45 min exposure to the agonist and are similar to the findings of Pearce et al. (1986).

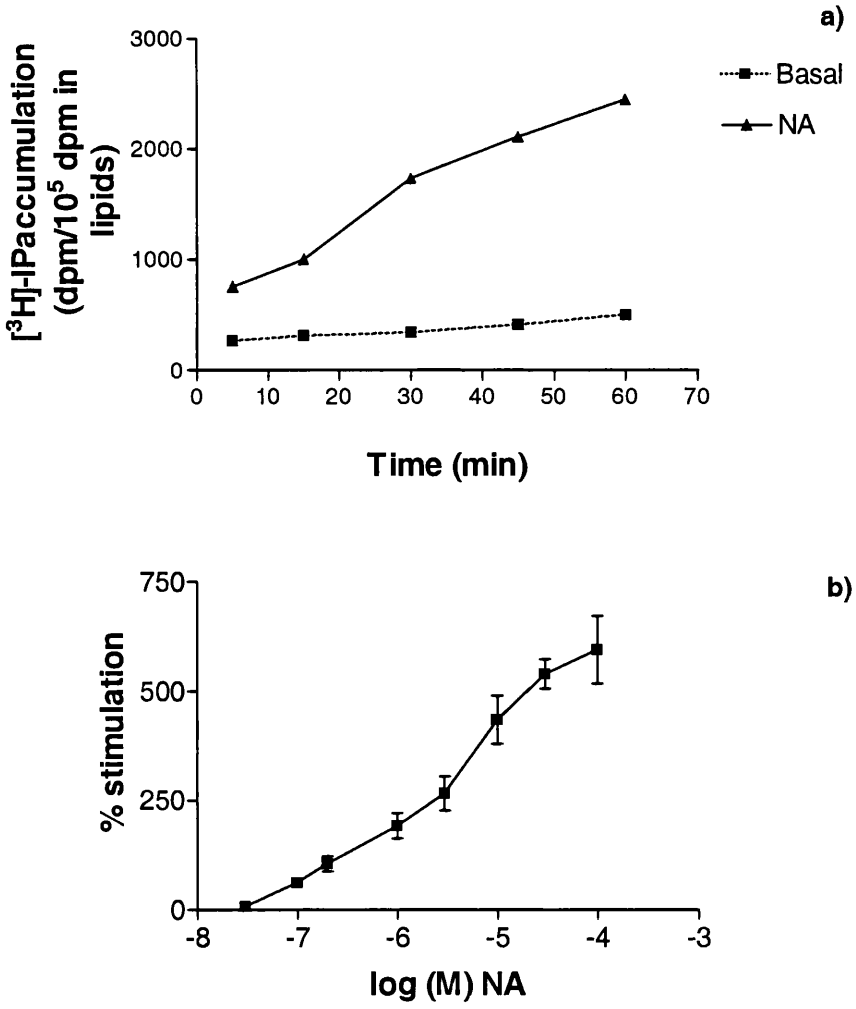

Figure 5. Effect of noradrenaline on [^{3}H]-inositol phosphate accumulation in glial cultures. a) Time course of agonist induced accumulation of [^{3}H]-IP. Glial cultures were incubated with either no additions or 100μM NA for the time periods indicated. Results are means of duplicate determinations. **b)** Dose-response curve for NA-stimulated [^{3}H]-IP accumulation. Glial cultures were incubated for 45 min with various concentrations of NA. Results are expressed as % stimulation above basal values and are means ± S.E.M. for 3 determinations.

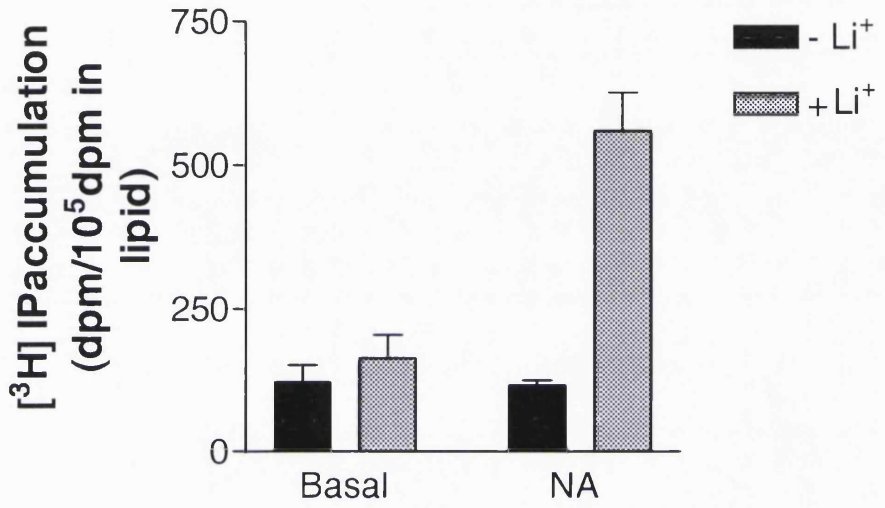

Figure 6. Effect of Li^+ on the accumulation of [^{3}H] inositol phosphates. Cultures were incubated with or without NA (100μM) for 45 min in the presence or absence of 5mM Li^+. Results are means ± S.E.M. from 3 determinations

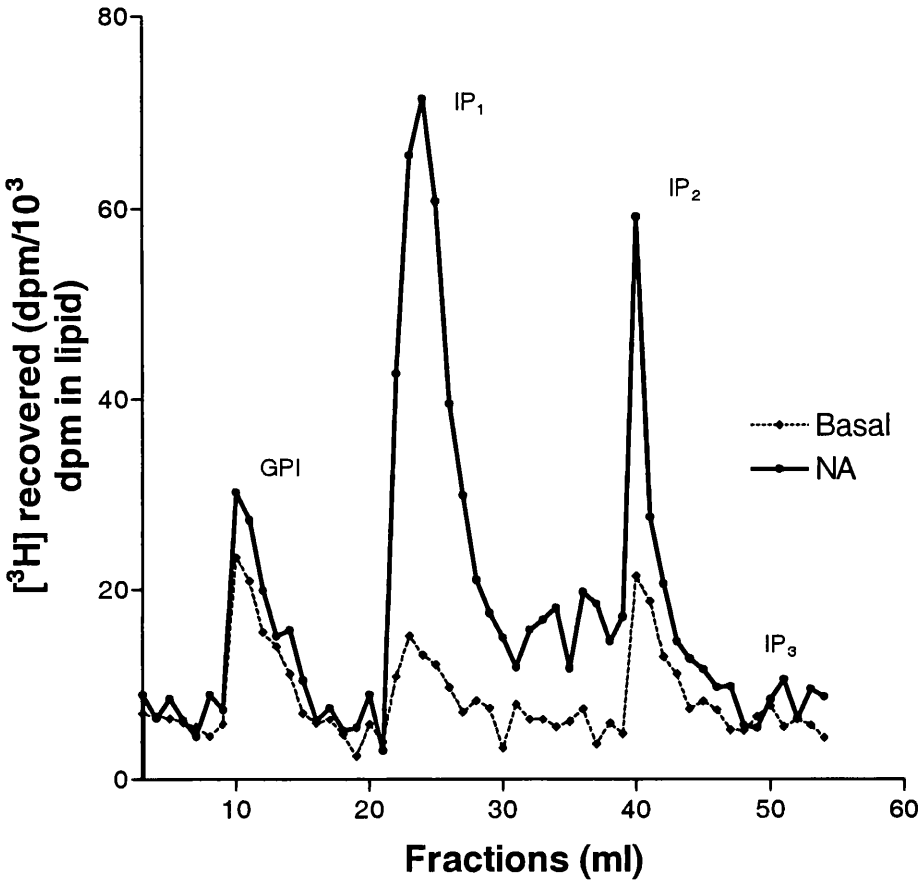

Figure 7. A representative elution profile of the column separation of the [^{3}H]- inositol phosphates formed in response to noradrenaline. Glial cultures were incubated in the presence or absence of 100μM NA for 45 min. The material from 4 cultures was pooled and the [^{3}H] recovered in each fraction was converted to a standard incorporation (10^3 dpm) into lipid.

Previous research has shown that NA-stimulated [^{3}H]-IP accumulation in glial cells is mediated by α_1-adrenoceptor activation (Pearce et al., 1986; Wilson and Minneman, 1990a). However, there are three subtypes of the α_1 adrenoceptor - α_{1A},α_{1B} and α_{1D}. Using selective antagonists, RS17053 which is α_{1A} selective, BMY7378 which is α_{1D} selective and AH11110A which is selective for α_{1B} adrenoceptors (Garcia-Sainz and Torres-Padilla, 1999; Burt et al., 1998), their effects on NA-stimulated [^{3}H]-IP accumulation were assessed. The inhibition curves shown in figure 8 indicate the following rank order of potency (IC_{50} values are in parenthesis): RS17053 (0.03μM) >> AH11110A (6.3μM) > BMY7378 (>10μM), a 50% inhibition was not achieved with BMY7378. This would suggest that NA activates the α_{1A} subtype to promote phosphoinositide breakdown in cultured glia.

To confirm the involvement of α_{1A}-adrenoceptors in NA-stimulated [^{3}H]-IP accumulation, the effect of the selective agonist A61603 was also determined. Both NA and A61603 elicited similar maximal increases in [^{3}H]-IP accumulation, the effect of 100μM A61603 was 96 ± 3% (n=3) of that produced by the same concentration of NA. The dose response curves shown in figure 9 show that A61603 was considerably more potent, approximately 50 - fold, giving an apparent EC_{50} value of 0.06μM compared to 5μM for NA. These results are similar to values obtained by Knepper et al. (1995) who examined the effects of A61603 and NA on phosphoinositide breakdown in fibroblast cells.

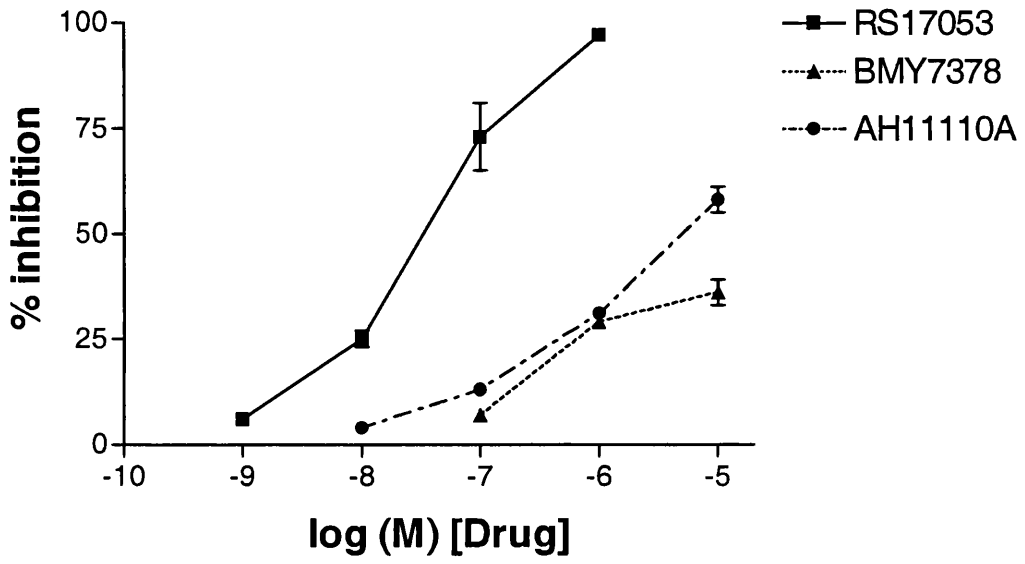

Figure 8. Inhibition of noradrenaline-stimulated [^{3}H]-inositol phosphate accumulation by various α_1-adrenoceptor antagonists. Cells were incubated for 15 min with the antagonist prior to a further 45 min exposure to NA (100μM). Results are expressed as the % inhibition of the NA-evoked response and are means ± S.E.M. (shown when larger than the symbol) from 3 determinations.

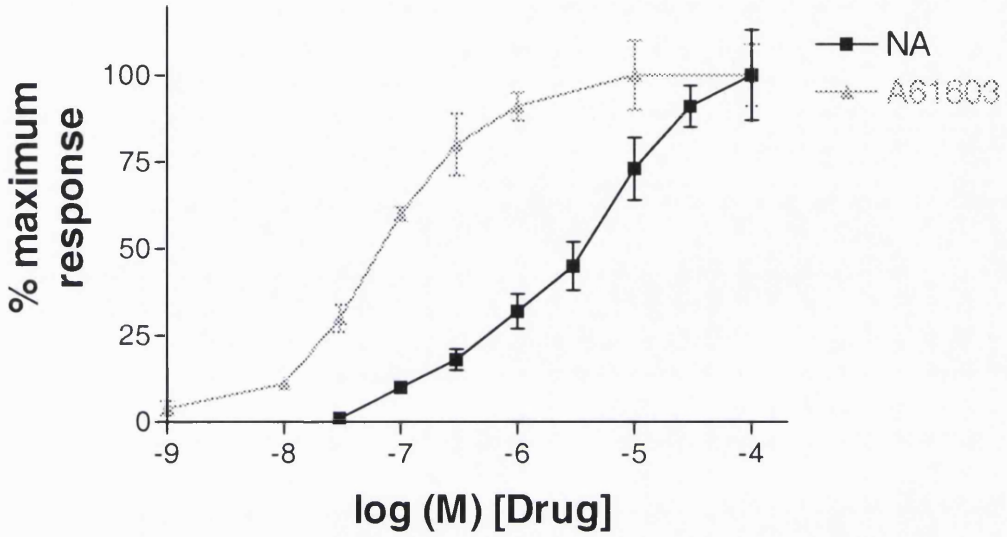

Figure 9. Dose response curves for noradrenaline- and A61603- stimulated [^{3}H]-inositol phosphate accumulation. Cultures were incubated for 45 min with various concentrations of the agonists in the presence of Li^+. Results are expressed as % maximum response elicited by the agonist and are means ± S.E.M. for 3 determinations.

3.3 Effects of Okadaic Acid

Having established that the adrenoceptor involved in the receptor-linked [^{3}H]-IP accumulation in glial cell cultures was of the α_{1A} subtype, the next step was to investigate the possibility that a PP was involved in regulating its response. In order to gain insight into the PPs involved, the effect of the inhibitor OA was tested.

Figure 10 shows the effect of a 15 min pre-treatment with OA (0.5μM) on NA-stimulated [^{3}H]-IP accumulation. It can be seen that the response to NA was reduced by some 50%. When used alone, OA at this concentration elicited a sight increase (138 ± 8% of basal, n=6) in [^{3}H]-IP formation but the vehicle, DMSO (0.5% v/v), was without effect (106 ± 11% of basal, n=4).

The ability of OA to inhibit NA-stimulated [^{3}H]-IP accumulation was found to be concentration dependent. Although complete inhibition was not achieved over the concentration range tested, figure 11a shows that OA had an apparent IC_{50} value of 0.3μM. The time course of the effect of OA on NA-evoked [^{3}H]-IP accumulation was also examined and is shown in figure 11b. Maximal inhibition with 0.5μM of OA was achieved within 30 min of pre-incubation and remained at this level for periods up to 60 min.

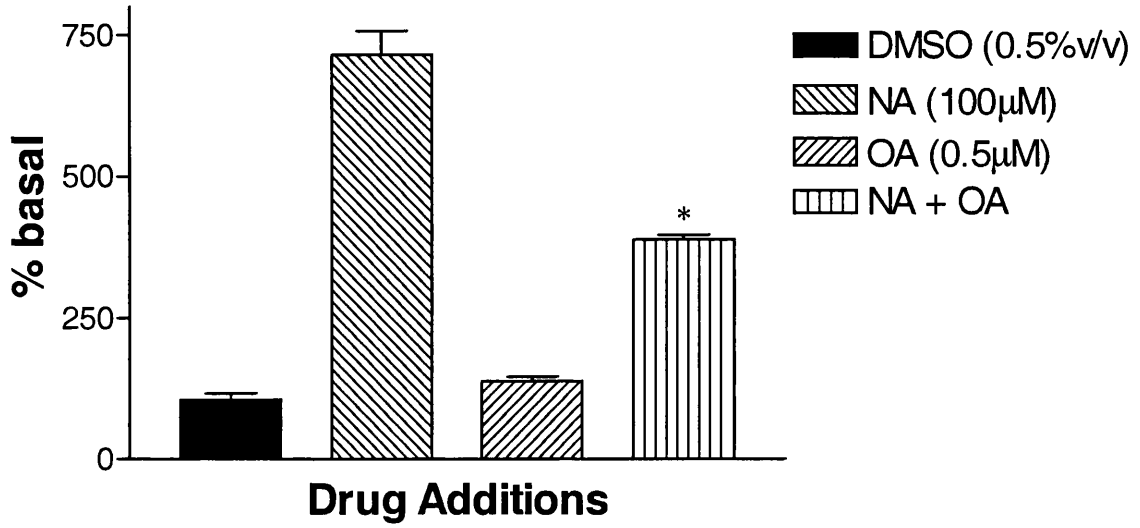

Figure 10. Effect of okadaic acid on noradrenaline-stimulated [^{3}H]-inositol phosphate accumulation. Glial cultures were incubated with the above drugs for 45 min with OA added 15 min prior to NA when used in combination. Results are expressed as % of basal accumulation and are means ± S.E.M. from 4-6 determinations. * indicates statistically significant ($p<0.05$) compared to NA alone.

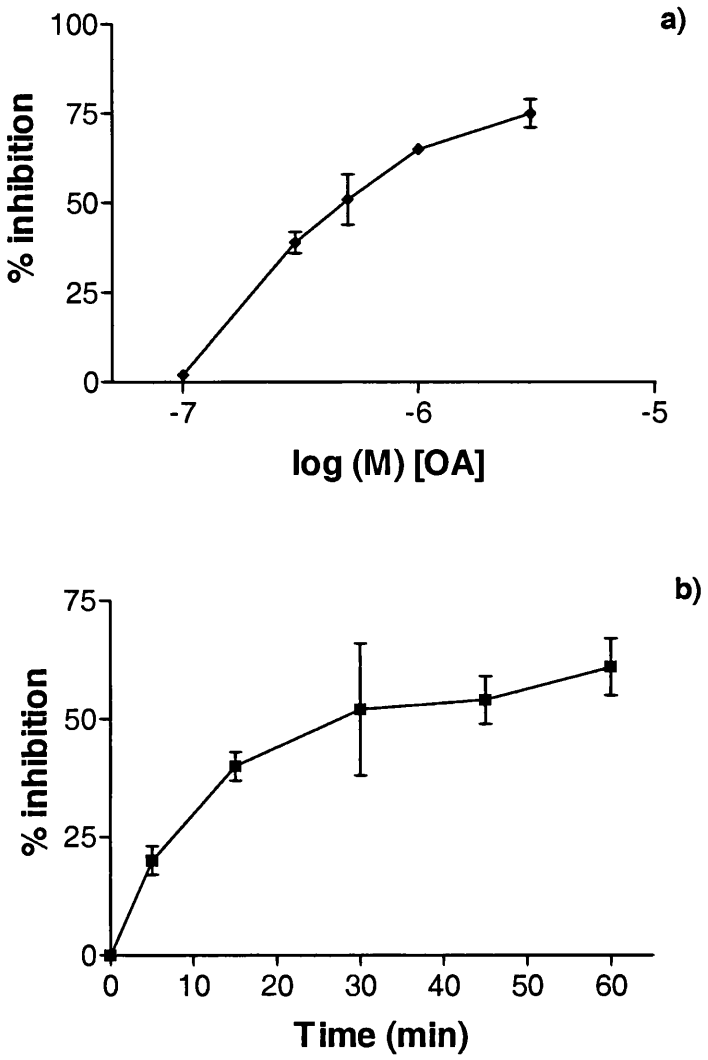

Figure 11. Effect of okadaic acid on noradrenaline-stimulated [^{3}H]-inositol phosphate accumulation. a) Dose response curve showing OA-evoked inhibition of NA-stimulated [^{3}H]-IP accumulation. OA was added at various concentrations for 15 min before addition of 100μM NA for a period of 45 min. **b)** Time course of OA inhibition of NA-stimulated [^{3}H]-IP accumulation. OA (0.5μM) was added to glial cultures for various time periods before the addition of NA (100μM) for a period of 45 min. Results are expressed as a % inhibition of the NA-evoked response and are means ± S.E.M.(shown when larger than the symbol) for 3 determinations.

In figure 12 the elution profile of water soluble [^{3}H] metabolites was repeated, this time in response to NA in the presence of OA. Again results show that the majority of the radiolabel recovered was eluted in fractions containing [^{3}H]-IP_1. There was a 43% decrease in the IP_1 fraction and an 82% decrease in the IP_2 fraction with the addition of OA compared to the effect of NA alone. IP_3 levels were reduced to basal values. OA also inhibited the effect of A61603. Figure 13 shows that the % inhibition of A61603 - evoked [^{3}H]-IP accumulation elicited by OA was almost identical to that found with NA.

Figure 14 shows that the effect of OA on NA-stimulated [^{3}H]-IP accumulation was particularly long lasting. When assessed 3 and 6h after a 15 min pre-treatment, the inhibition of the NA-evoked response was essentially the same (approximately 60% inhibition). An inhibition of the effect of NA was still evident 24h (38%) and 48h (22 %) later, suggesting that OA is not readily removed from cells once it has accumulated intracellularly.

Two other phosphatase inhibitors were also assessed for their ability to modify NA-stimulated [^{3}H]-IP accumulation. Microcystin, IC_{50} 0.1nM, is a non-selective inhibitor of PP1 and PP2A whilst tautomycin has been reported to display more selectivity towards PP1, IC_{50} 0.2nM, than PP2A, IC_{50} 1nM (Takai, 1995; Fujiki and Suganuma, 1999). Figure 15 shows that although microcystin and tautomycin were capable of inhibiting NA-evoked [^{3}H]-IP accumulation, they were slightly less effective than OA when used at the same concentration, however this was not significantly different.

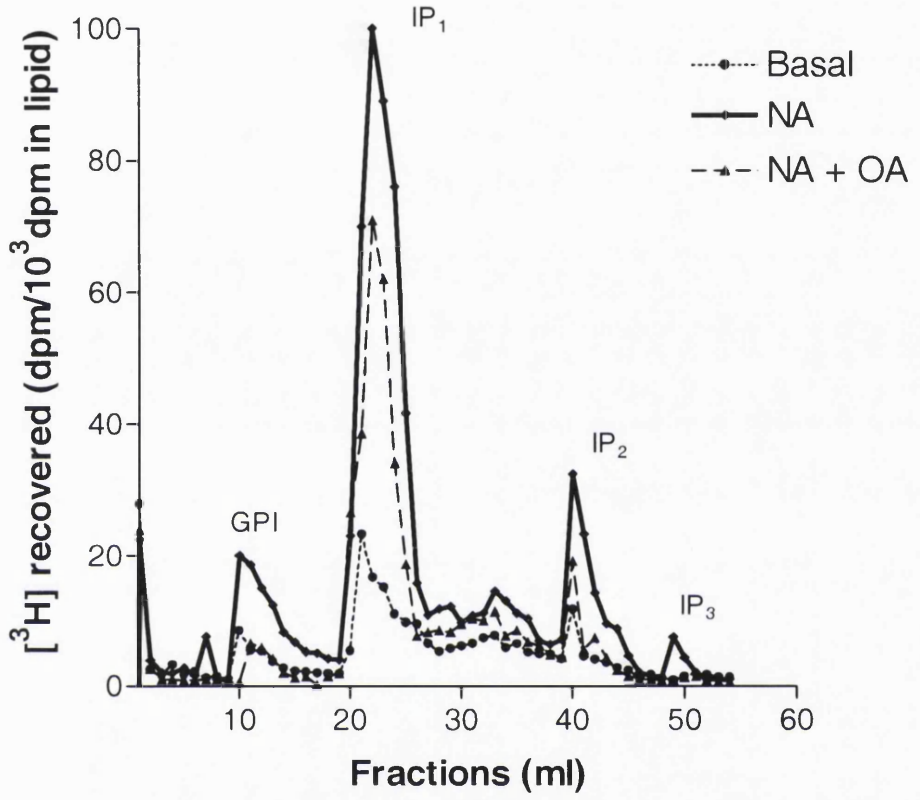

Figure 12. A representative elution profile of the column separation of the [^{3}H]-inositol phosphates formed in response to noradrenaline following pre-incubation with okadaic acid. Glial cultures were pre-incubated with 0.5μM OA for 15 min before addition of 100μM NA for a further 45 min in the presence of Li^+. The material from 4 cultures was pooled and the [^{3}H]-IP recovered in each fraction was converted to a standard incorporation (10^3 dpm) into lipids.

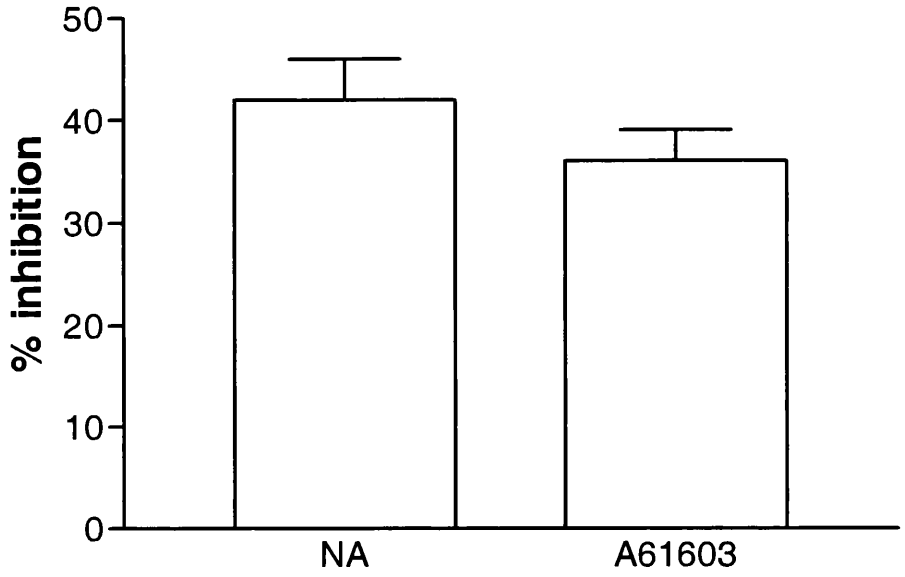

Figure 13. Effect of okadaic acid on noradrenaline- and A61603-stimulated [^{3}H]-inositol phosphate accumulation. OA (0.5μM) was added for 15 min before addition of NA and A61603 both at a concentration 100μM for a period of 45 min. Results are expressed as a % inhibition of the agonist - induced response and are means ± S.E.M. for 4 determinations.

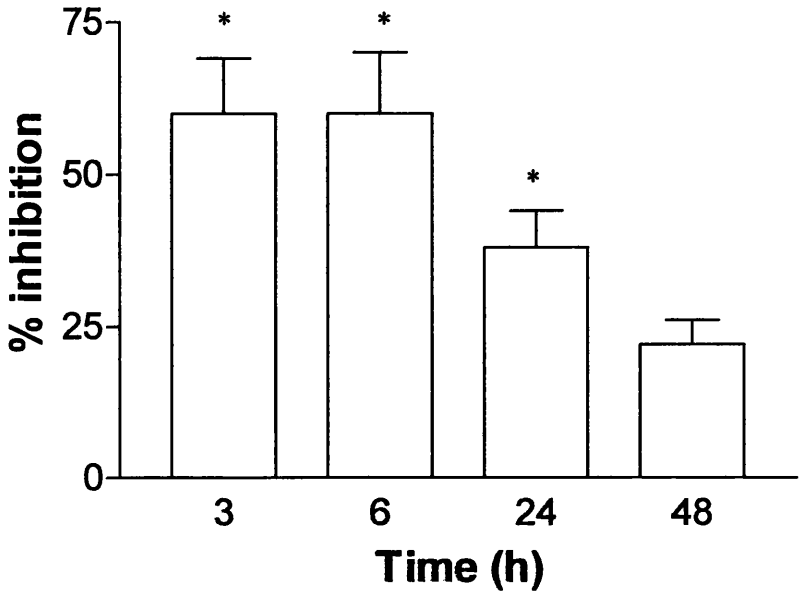

Figure 14. Recovery of noradrenaline-stimulated [^{3}H]-inositol phosphate accumulation following okadaic acid pre-treatment. Glial cultures were incubated with 0.5μM OA for 15 min, washed twice with buffer, and incubated in this medium for 3, 6, 24 and 48h periods before addition of 100μM NA. Results are expressed as a % inhibition of NA-stimulated [^{3}H]-IP accumulation in control incubations and are means ± S.E.M. for 3 determinations. * indicates statistically significant ($p<0.05$) compared to NA alone.

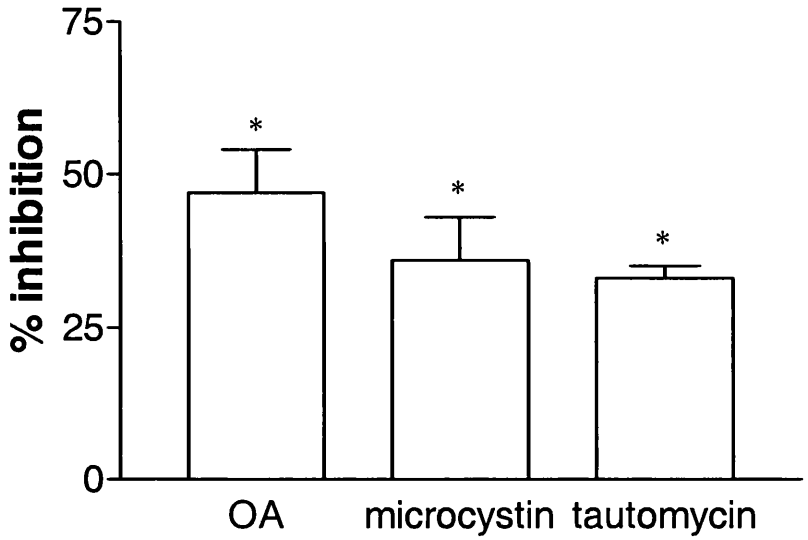

Figure 15. Effect of other protein phosphatase inhibitors on noradrenaline-stimulated [^{3}H]- inositol phosphate accumulation. Cultures were pre-treated for 15 min with either OA, microcystin or tautomycin (all at 0.5μM) prior to a further 45 min incubation with NA (100μM). Results are expressed as % inhibition of the NA - evoked response and are means ± S.E.M. from 4 determinations. * indicates statistically significant ($p<0.05$) compared to NA alone.

3.4 Effects of protein kinase inhibitors

Results so far point to the involvement of a PP in inhibiting NA-stimulated [^{3}H]-IP accumulation perhaps by allowing a PK to act in an unregulated fashion. Staurosporine is a PK inhibitor and figure 16 shows that increasing concentrations of the drug decreased the inhibitory effect of OA on NA - stimulated [^{3}H]-IP accumulation. At a concentration of 1μM, staurosporine achieved almost complete reversal of the inhibitory effects of OA. Staurosporine exerts its effects through its binding to the ATP sites of PKs thereby inhibiting their activity. However, it should be noted that staurosporine is a non selective PK inhibitor with an order of potency PKC >>>PKA>>Ca2+/CaMK II > insulin receptor kinase (Kronfeld et al., 1995).

The myristoylated PKA peptide is a synthetic inhibitor of PKA with a Ki of 2.3nM. It binds to the catalytic subunit of PKA, displacing the regulatory subunit, and mimics the protein substrate (Douglas et al., 1999). The myristoylated PKC peptide is derived from a sequence of the PKCα and PKCβ N-terminal and has an IC_{50} value of 8μM. Both peptides are myristoylated to aid cell permeability. Results in figure 17 clearly show that the PKA inhibitor was unable to reverse the effect of OA, whereas the PKC inhibitor reversed the effect of OA by approximately 60%, thereby suggesting the involvement of PKC in the effect of OA on NA-stimulated phosphoinositide breakdown.

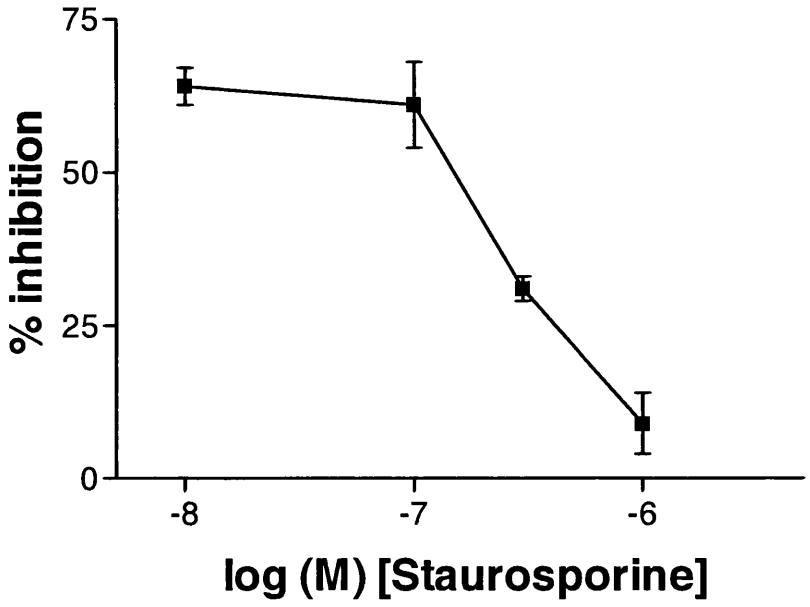

Figure 16. Reversal of the okadaic acid-evoked inhibition of the noradrenaline-stimulated [^{3}H]-inositol phosphate accumulation by staurosporine. Glial cultures were incubated for 10 min with various staurosporine concentrations prior to addition of 0.5μM OA for 15 min, then incubated for a further 45 min with 100μM NA. Results are % inhibition of the NA response and are means ± S.E.M. from 3 determinations.

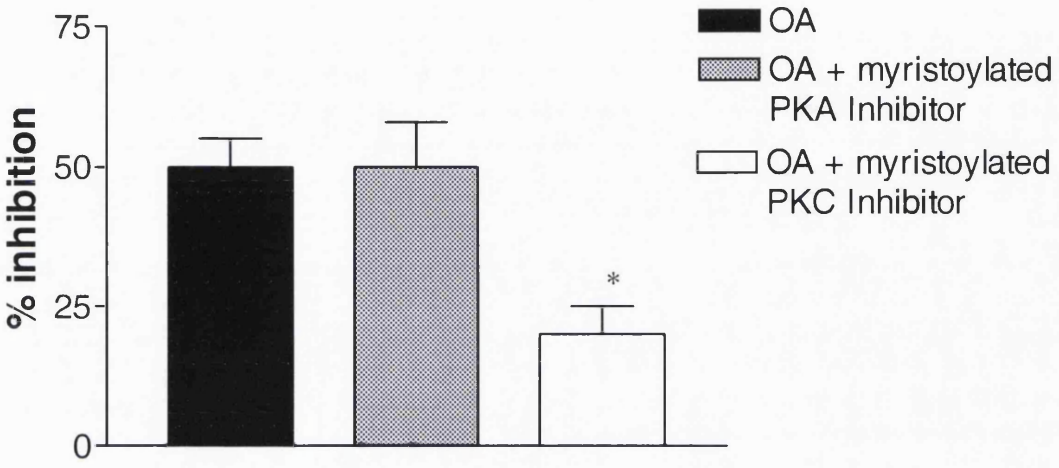

Figure 17. Reversal of the okadaic acid-evoked inhibition of the noradrenaline-stimulated [^{3}H]-inositol phosphate accumulation by myristoylated protein kinase inhibitors.
Glial cultures were incubated for 10 min with either 10μM myristoylated PKA inhibitor or 10μM myristoylated PKC inhibitor prior to addition of 0.5μM OA for 15 min. Cultures were then incubated for a further 45 min with 100μM NA. Results are expressed as the % inhibition of the NA-induced response and are means ± S.E.M. from 3 determinations.
* indicates statistically significant ($p<0.05$) compared to OA alone.

The ability of staurosporine and the myristoylated PKC inhibitor to reverse the inhibitory effect of OA on NA-stimulated [^{3}H]-IP accumulation prompted the next series of experiments in which the phorbol ester PMA was used to activate PKC. Preincubation of PMA alone resulted in an inhibition of NA- evoked responses that were dependent upon concentration, giving an apparent IC_{50} value of 3nM. Incubation of the cultures with various concentrations of PMA in the presence of OA at 0.5μM showed that the degree of inhibition achieved was no greater than that elicited by the more effective agent alone (figure 18). This result indicates that the effects of PMA and OA on NA-stimulated [^{3}H]-IP accumulation were not additive.

Figure 19 shows that PMA at 0.03μM caused almost complete inhibition of the agonist response, an effect which was completely reversed by staurosporine. These results appear to confirm the suggestion of the involvement of PKC in regulating the activity of α_{1A}-adrenoceptors coupled to the IP signalling pathway in glial cells.

Previous work has shown that prolonged phorbol ester treatment causes PKC down-regulation in various cell types (Zhao et al., 1994; Young et al., 1987), including glia (Pearce et al., 1993). This approach was used to determine whether OA-mediated inhibition of NA-stimulated [^{3}H]-IP accumulation was still evident in PKC depleted cells.

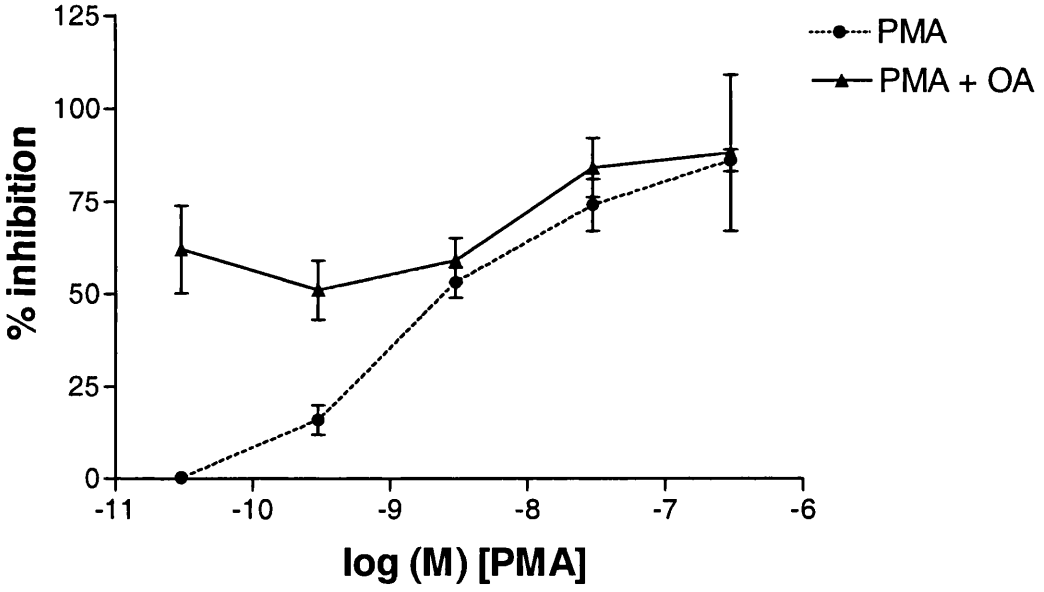

Figure 18. Inhibition of noradrenaline-stimulated [^{3}H]-inositol phosphate accumulation by phorbol 12-myristate 13 acetate in the presence or absence of okadaic acid. Glial cultures were incubated for 15 min with various concentrations of PMA ± 0.5μM OA prior to a further 45 min incubation with 100μM. NA Results are expressed as % inhibition of the NA-evoked response and are means ± S.E.M. from 3 determinations. Incubations with OA (0.5μM) in the absence of PMA produced a 64 ± 3% inhibition (n=3) of the response to NA

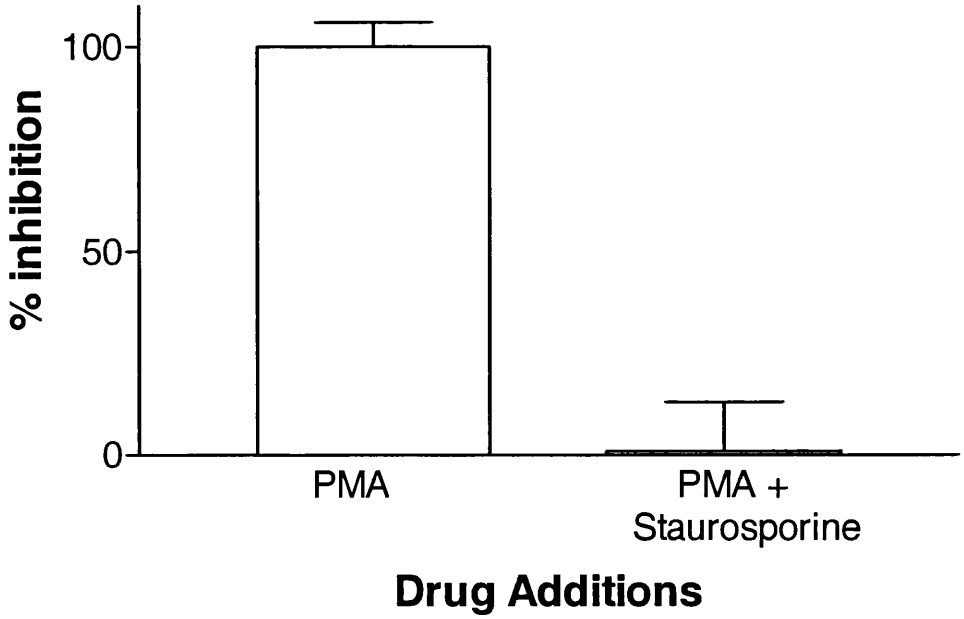

Figure 19. Reversal of the phorbol 12-myristate 13 acetate-mediated inhibition of noradrenaline-evoked [^{3}H]- inositol phosphate accumulation by staurosporine. Cultures were incubated for 15 min with PMA (0.03μM) in the presence or absence of staurosporine (1μM) prior to a further 45 min exposure to NA (100μM). Results are expressed as the % inhibition of the NA-evoked response and are means ± S.E.M. for 4 determinations.

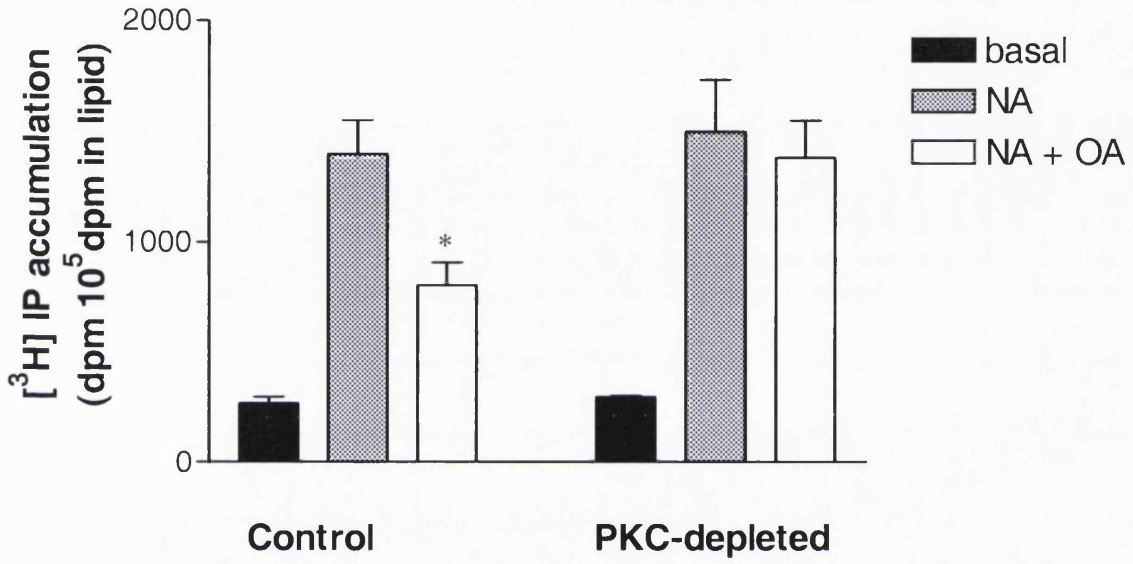

Figure 20. The effect of protein kinase C depletion on okadaic acid-induced inhibition of noradrenaline-stimulated [^{3}H]-inositol phosphate accumulation. Cultures were incubated for 6h in serum-free medium containing PMA (1μM), washed, then incubated for 15 min in buffer containing OA (0.5μM) prior to a 45 min incubation with NA (100μM). Results are means ± S.E.M. from 5 determinations. * indicates statistically significant ($p<0.05$) compared to NA alone.

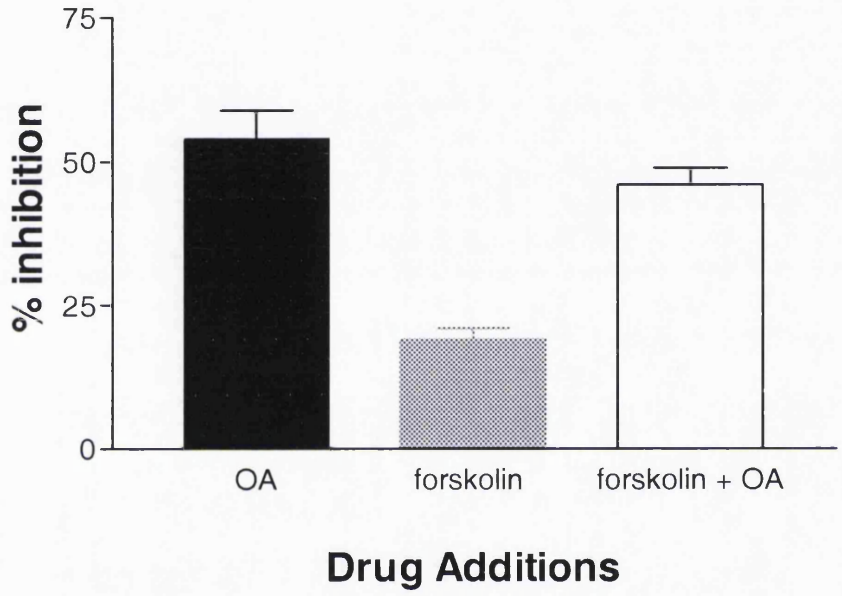

Figure 21. Effect of forskolin (10μM) on noradrenaline-evoked [^{3}H]-inositol phosphate accumulation. Cells were incubated for 15 min with forskolin (10μM) in the presence or absence of OA (0.5μM) prior to a further 45 min exposure to NA (100μM). Results are expressed as the % inhibition of the NA-evoked response and are means ± S.E.M. for 5 determinations.

The results presented in figure 20 show that basal and NA-evoked [^{3}H]-IP accumulation were largely unchanged in PKC-depleted glia compared to controls run in parallel. Whereas OA elicited a 53 % reduction in the response to NA in controls, only an 8 % inhibition was found in those depleted of PKC. Despite the evidence supporting the involvement of PKC but not in PKA in the regulation of α_1-adrenoceptor linked phosphoinositide metabolism in glia, the effect of raising intracellular cAMP levels on the response to NA was also assessed. Cultures were pre-incubated with forskolin, an activator of AC, alone or in addition to OA. At a concentration of 10μm, forskolin produced a modest (~20%) inhibition of NA- stimulated [^{3}H]-IP accumulation but did not alter the effect of OA (figure 21).

To summarise, the main findings so far are as follows:

- NA promotes [^{3}H]-IP accumulation through α_{1A} adrenoceptors.
- Glial cultures possess both PP1 and PP2A activity in almost equal abundance.
- Experiments with the PP inhibitor OA show that a PP, possibly of the PP1 or PP2A subtype, is involved in the regulating receptor-linked IP accumulation.
- The PK inhibitor staurosporine and the myristoylated PKC inhibitor both reversed the inhibitory effects of OA, moreover the effect of OA was not observed in PKC-depleted cultures, suggesting the involvement of PKC in regulating this process.
- The PKC activator PMA inhibited the agonist response and its effects were reversed by staurosporine, also suggesting that PKC is involved in regulating the activation of α_{1A} adrenoceptor coupled to the IP signalling pathway in glial cells.
- Results do not support a role for PKA in the regulation of these receptors.

3.5. Effect of okadaic acid on protein phosphatase and protein kinase activities

In order to further define the PP involved in regulating the activity of α_{1A}-adrenoceptors coupled to the IP signalling pathway, cultures were pre-treated with either OA or PMA and assessed for their ability to liberate [^{32}P] from glycogen phosphorylase. Pre-treated cultures were also assessed for their ability to incorporate [^{32}P] into substrates specific for PKA and PKC activity.

Following OA treatment, a substantial (60%) reduction in total PP activity was observed compared to controls (see figure 4 for control results). Analysis of the PP species revealed that PP activity attributable to PP2A was only some 18%, the majority being due to the activity of PP1 (figure 22).

A reduction in total PP activity compared to control was also found following pre-treatment with PMA, however, the extent of this reduction (25%) was much lower than that found with OA (figure 23). Under these conditions, PP2A activity was reduced by 35% compared to controls. These data point to an inhibition of PP2A activity in glial cultures pre-treated with OA, and to a lesser extent with PMA.

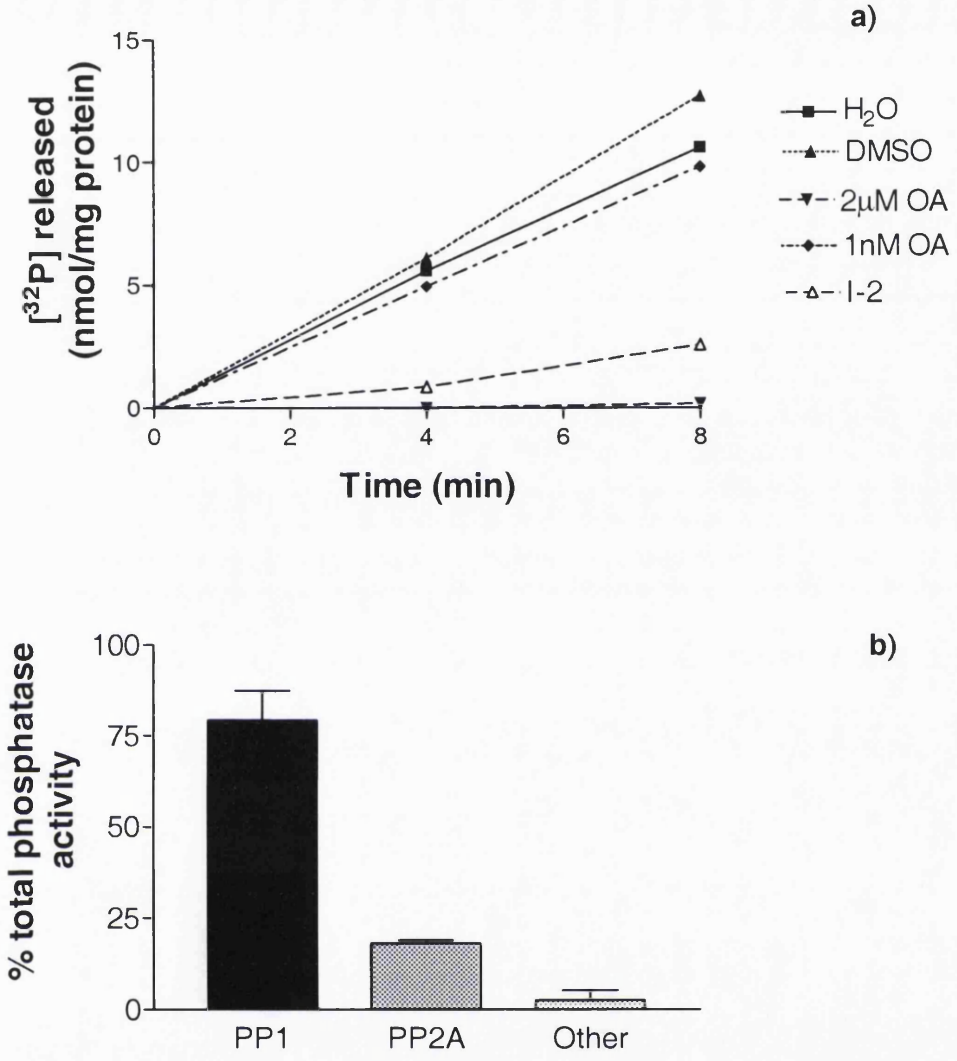

Figure 22. Effect of okadaic acid pre-treatment on protein phosphatase activity in glial cultures. a) A representative time course showing the ability of glial cells pre-treated with OA (0.5μM) to liberate [^{32}P] from labelled glycogen phosphorylase. Cultures were pre-treated with OA (0.5μM) for 15 min. Inhibitor-2 was included at a concentration of 0.5μM and is selective for PP1. Results are expressed as nmol [^{32}P] released/mg protein. **b)** Proportion of phosphatase species present in glial cells pre-treated with OA. Total activity was 1.14 ± 0.07 nmoles [^{32}P] released/min/mg protein. Results are means ± S.E.M. for 3 determinations

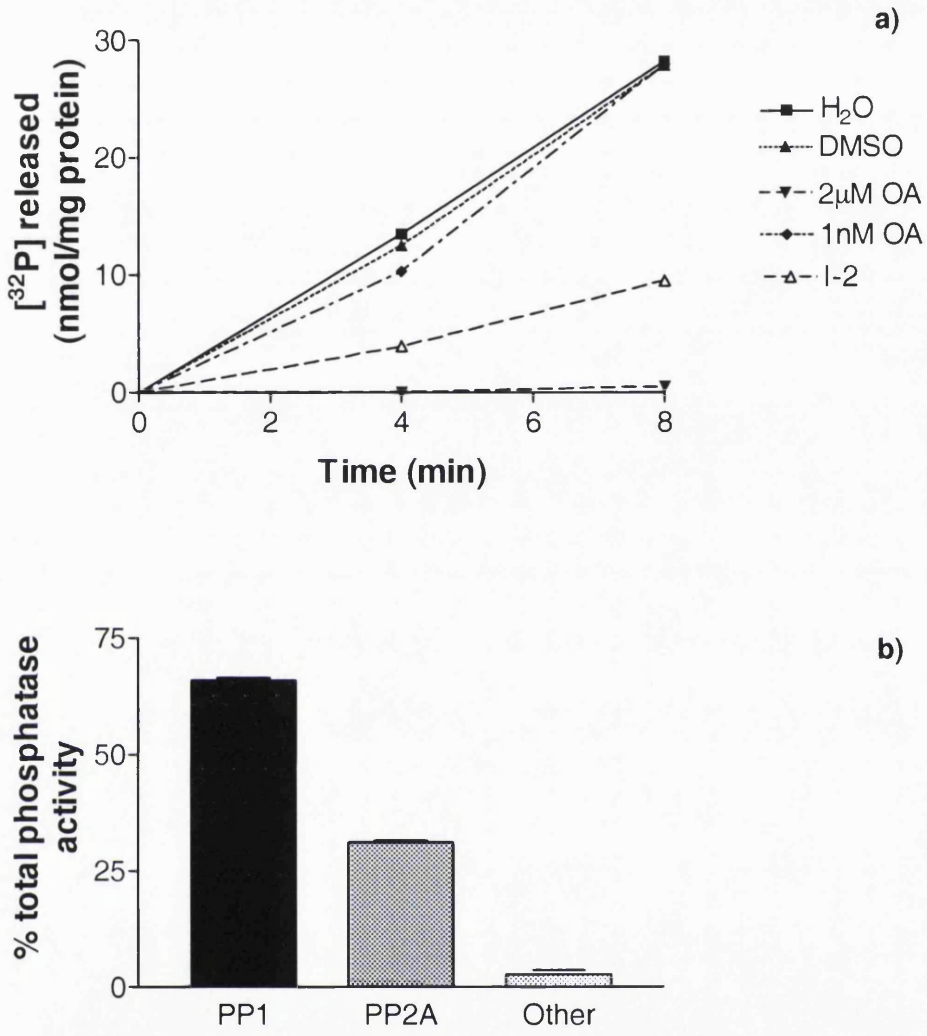

Figure 23. Effect of phorbol 12-myristate 13 acetate pre-treatment on protein phosphatase activity in glial cultures a) A representative time course showing the ability of glial cells pre-treated with PMA to liberate [^{32}P] from labelled glycogen phosphorylase. Cultures were pre-treated with PMA (0.3μM) for 15 min. Inhibitor-2 was included at a concentration of 0.5μM and is selective for PP1. Results are expressed as nmol [^{32}P] released/mg protein. **b)** Proportion of phosphatase species present in glial cultures pre-treated with PMA. Total activity was 2.58 ± 0.05 nmoles [^{32}P] released/min/mg protein (control total activity 3.36 ± 0.63). Results are means ± S.E.M. for 3 determinations.

PKA activity in extracts from whole rat brain and glial cultures was determined by their ability to promote [^{32}P] incorporation into kemptide, a specific substrate for this kinase. Figures 24a, 25a and 26a show that the time courses of radiolabel incorporation into substrate were linear over the 8 min incubation period in extracts prepared from whole brain, control and OA-treated cultures, respectively. In all cases the presence of cAMP in the incubation medium resulted in a higher rate of [^{32}P] incorporation into kemptide compared to that found in the absence of the cyclic nucleotide.

It can be seen in figure 24b that PKA activity was very low in rat brain extracts in the absence of cAMP, with the majority (8-fold) of the PKA activity being cAMP-dependent. The activity ratio (activity in the absence of cAMP divided by that in the presence of cAMP) for these samples was found to be 0.12 ± 0.01. Compared to whole brain, the activity ratio in glial cultures was much higher (0.27 ± 0.04) reflecting higher PKA activity in the absence of added cAMP in these cells, presumably due to increased levels of free C subunit (figure 25b).

In OA-treated cultures, cAMP-stimulated PKA activity was approximately 67% higher than controls (figure 26b). Interestingly, however, this treatment resulted in a considerable increase in PKA activity in the absence of added cAMP, such that the activity ratio was found to be 0.69 ± 0.04. This would suggest a further increase in free catalytic subunit of PKA in these cultures following OA treatment.

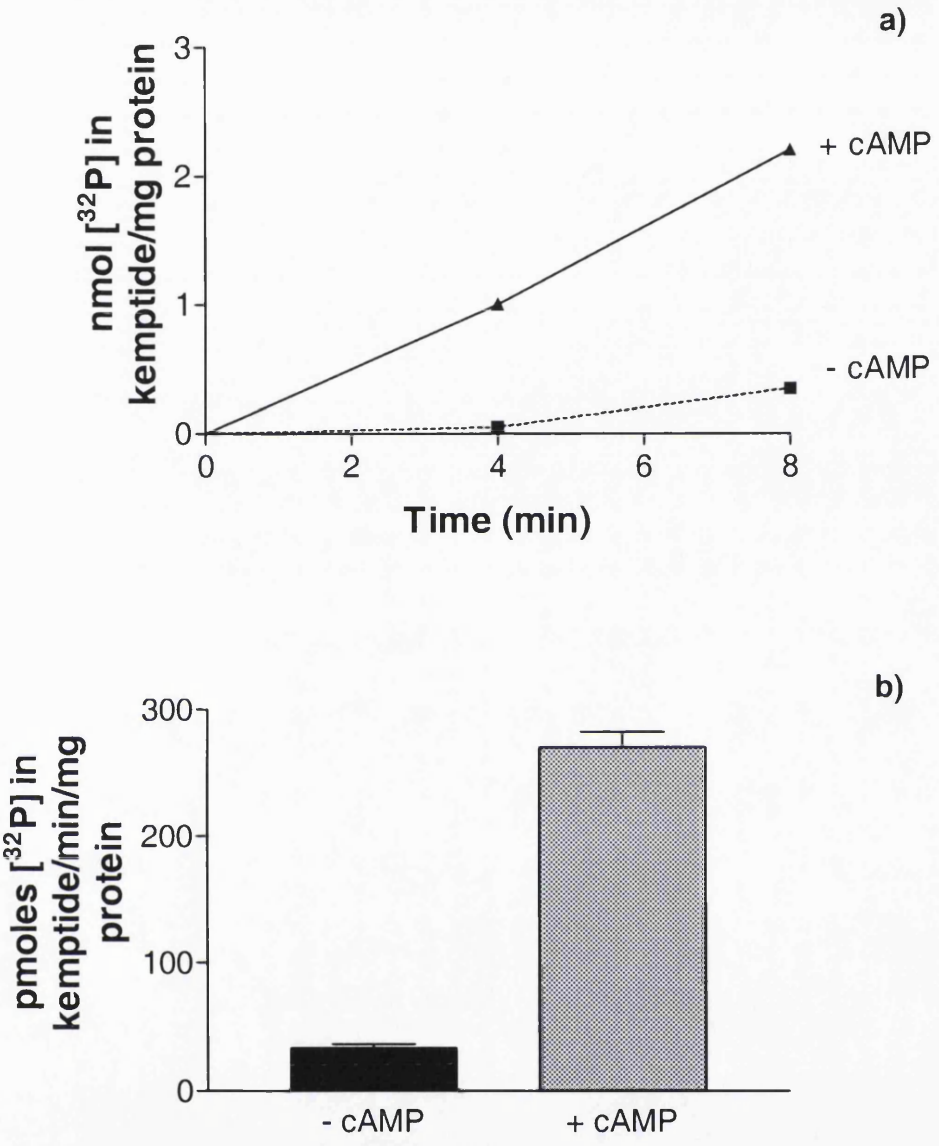

Figure 24. Cyclic AMP dependent protein kinase activity in whole rat brain a) A representative time course showing the ability of rat brain extracts to incorporate [^{32}P] into kemptide. Results are expressed as nmol [^{32}P] incorporated/mg protein. **b)** Rates of [^{32}P] incorporation into kemptide in the presence and absence of cAMP (10μM). Results are expressed as means ± S.E.M. for 4 determinations

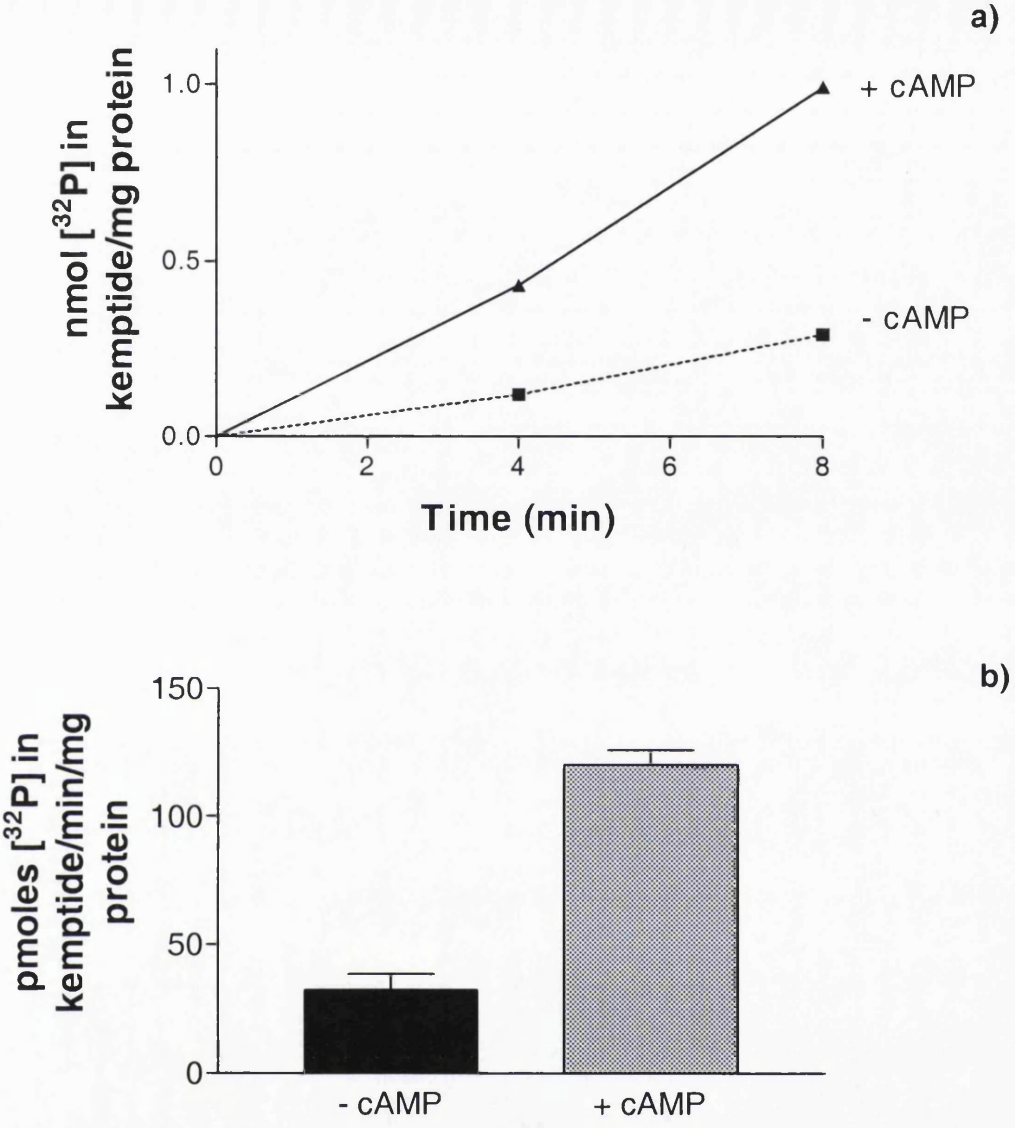

Figure 25. Cyclic AMP dependent protein kinase activity in glial cultures a) A representative time course showing the ability of glial cells cultures to incorporate [^{32}P] into kemptide. Results are expressed as nmol [^{32}P] incorporated/mg protein. **b)** Rates of [^{32}P] incorporation into kemptide in the presence and absence of cAMP (10μM). Results are expressed as means ± S.E.M. for 4 determinations.

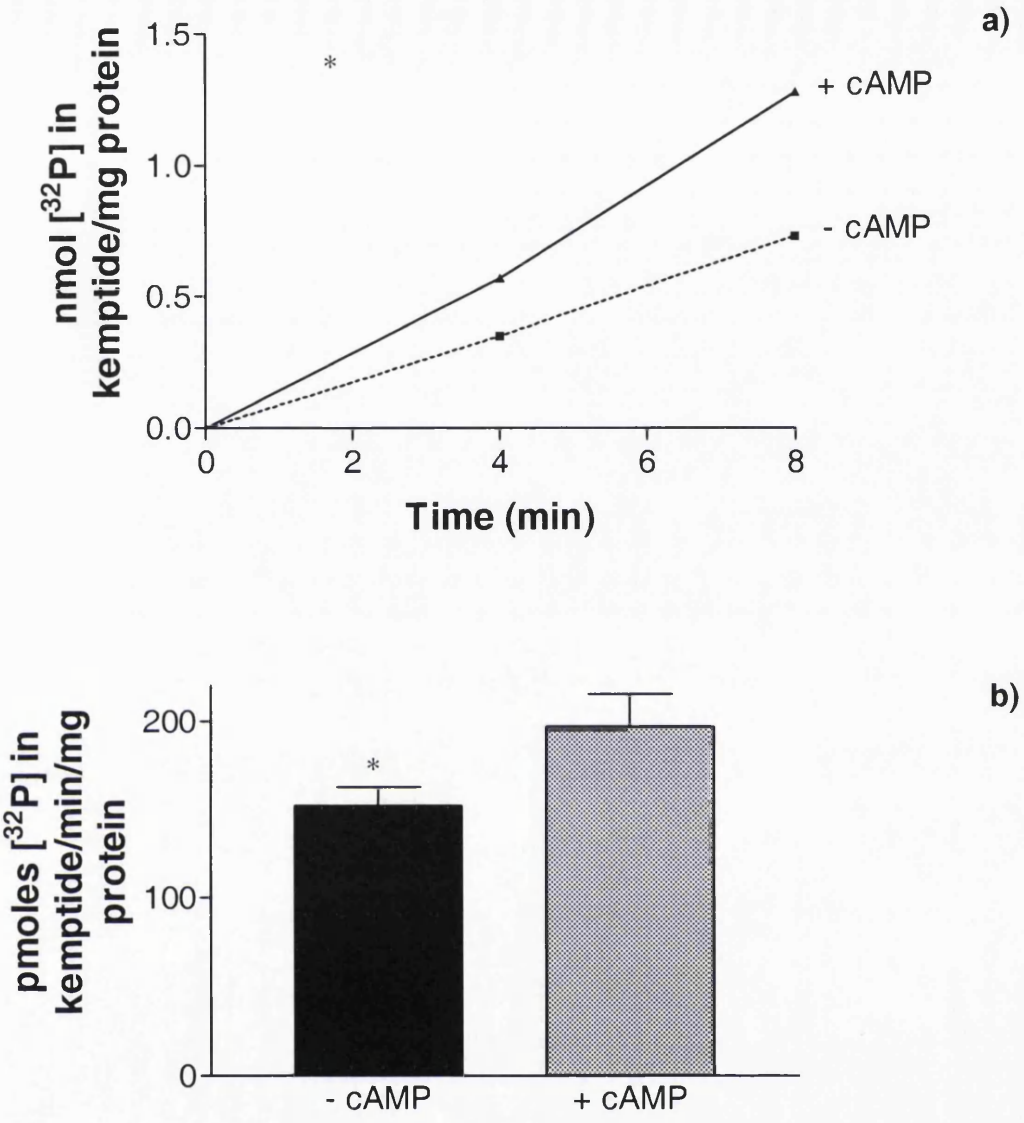

Figure 26. Effect of okadaic acid pre-treatment on cyclic AMP dependent protein kinase activity in glial cultures

a) A representative time course showing the ability of glial cells pre-treated with OA to incorporate [^{32}P] into kemptide. Results are expressed as nmol [^{32}P] incorporated/mg protein. **b)** Rates of [^{32}P] incorporation into kemptide in the presence and absence of cAMP (10μM). Results are expressed as means ± S.E.M. for 6 determinations. * indicates statistically significant ($p<0.05$) compared to the same condition in control cells as shown in fig 25b.

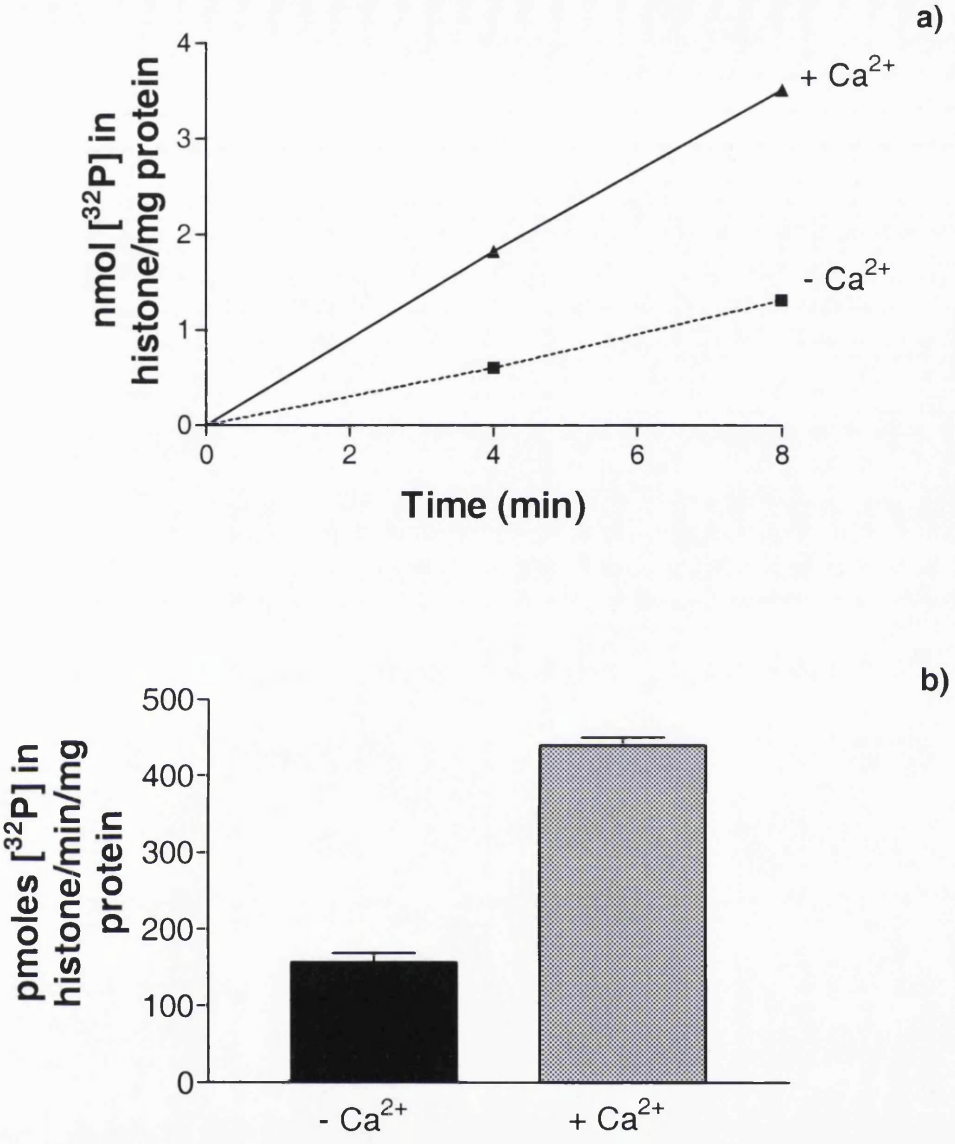

Figure 27. Protein kinase C activity in whole rat brain

a) A representative time course showing the ability of rat brain extracts to incorporate [^{32}P] into histone in the absence and presence of Ca^{2+}. Results are expressed as nmol [^{32}P] incorporated/mgprotein. **b)** Rates of activated PKC activity in rat brain extracts in the absence and presence of Ca^{2+} (0.5mM). Results are means ± S.E.M. for 3 determinations.

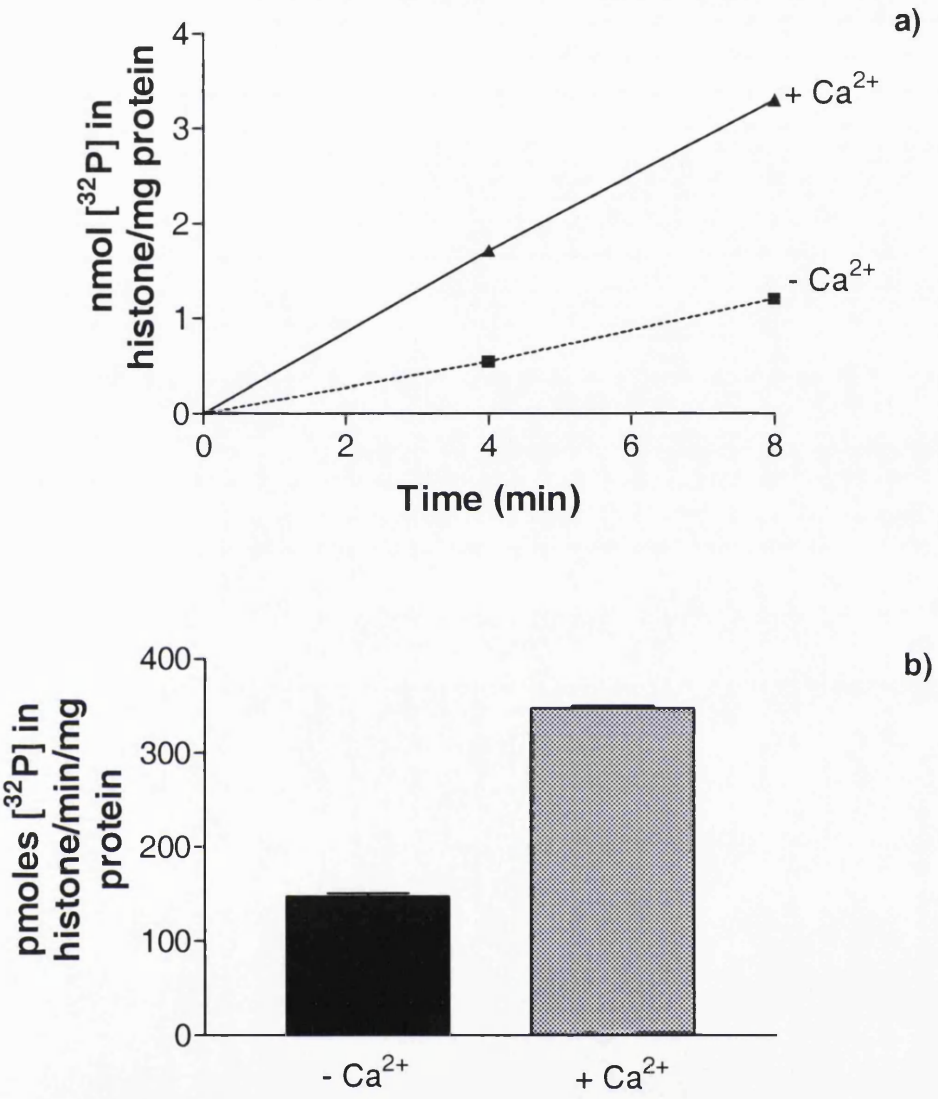

Figure 28. Protein kinase C activity in glial cells. a) A representative time course showing the ability of glial cultures extracts to incorporate [^{32}P] into histone in the absence and presence of Ca^{2+}. Results are expressed as nmol [^{32}P] incorporated/mg protein. **b)** Rates of PKC activity in glial cultures in the absence and presence of Ca^{2+} (0.5mM). Results are means ± S.E.M. for 3 determinations

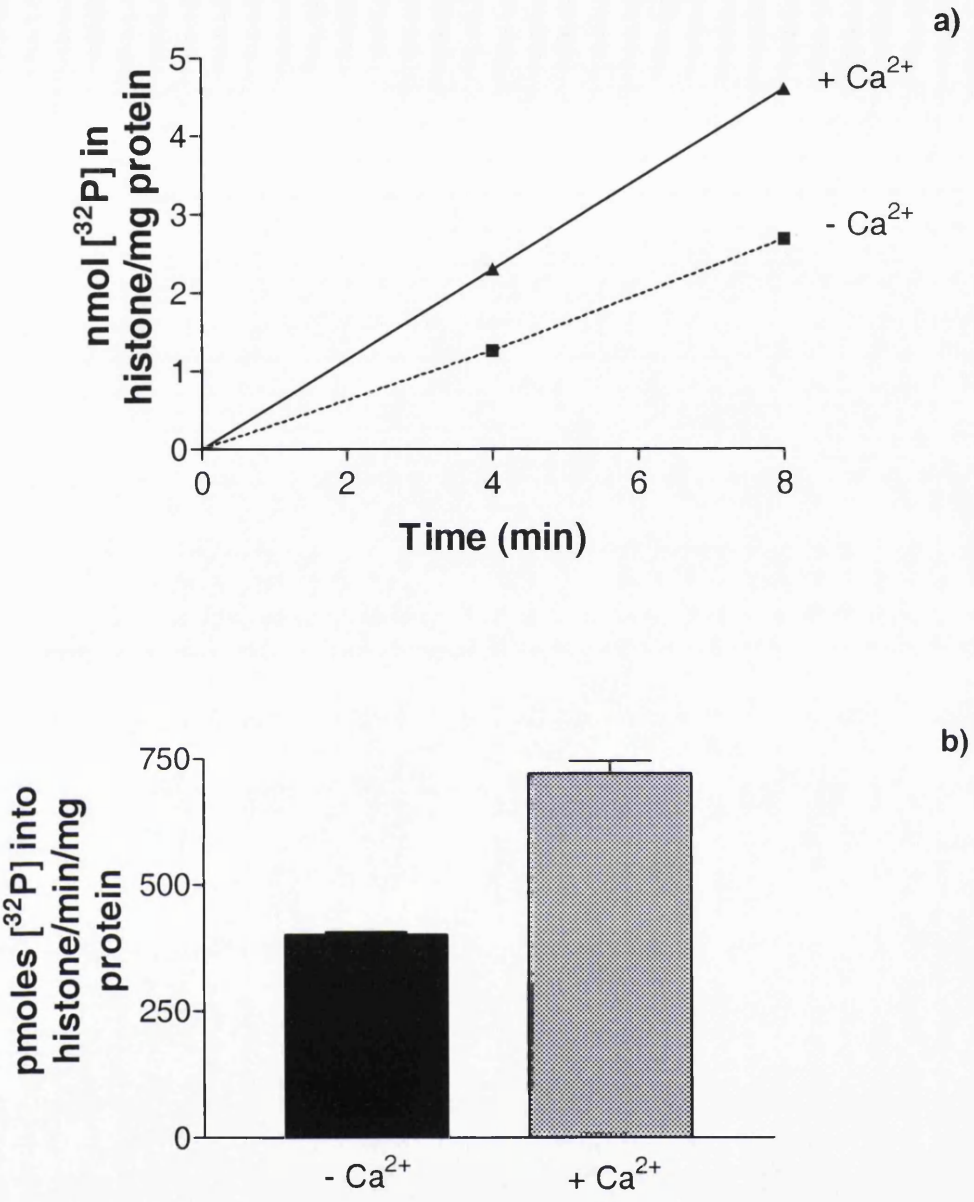

Figure 29. Effect of okadaic acid pre-treatment on protein kinase C activity in glial cultures. a) A representative time course showing the ability of glial cells pre-treated with OA (0.5μM) to incorporate [^{32}P] into histone in the absence and presence of Ca^{2+}. Results are expressed as nmol [^{32}P] incorporated/ mg protein. **b)** Rates of PKC activity in glial cells pre-treated with OA in the absence and presence of Ca^{2+} (0.5mM). Results are means ± S.E.M. for 3 determinations.

PKC activity in extracts from whole rat brain and glial cultures was determined by their ability to promote [^{32}P] incorporation into histone III, a substrate for this kinase. Figures 27a, 28a and 29a show that the time courses of radiolabel incorporation into substrate were linear over the 8 min incubation period in extracts prepared from whole brain, control and OA-treated cultures respectively. In all cases the presence of Ca^{2+} in the incubation medium resulted in a higher rate of [^{32}P] incorporation into histone compared to that found in its absence.

Figure 27b shows that Ca^{2+}-dependent PKC activity in rat brain extracts is 4-fold greater than that which is independent of Ca^{2+}. Similar proportions of Ca^{2+}-dependent and Ca^{2+}-independent PKC activity were also found in glial cell cultures (figure 28b). However, in OA-treated cultures, Ca^{2+}-dependent and Ca^{2+}-independent PKC activities were increased by approximately 2-fold compared to controls (figure 29b).

To summarise, the main findings of this section are as follows:

- OA pre-treatment resulted in a considerable reduction in total PP activity and almost complete abolition of that mediated by PP2A. A similar trend, albeit to a much lesser extent, was also found with PMA pre-treatment.
- Analysis of PKA activity in glial cultures revealed that OA pre-treatment elicited an increase in the activity ratio compared to control suggesting increased levels of free catalytic subunit in these cells.
- Glial cell cultures were found to posses both Ca^{2+}-dependent and Ca^{2+}-independent PKC activity, the latter being approximately half that of the former. OA pre-treatment resulted in increases in both activities.

3.6 Ca^{2+} fluxes.

It has long been known that NA-stimulated [^{3}H]-IP accumulation in glial cells is dependent to a large extent on the presence of extracellular Ca^{2+} (Pearce et al., 1986). Exactly how much Ca^{2+} enters these cells upon receptor activation and which element(s) of the second messenger pathway are influenced, remains to be fully elucidated. However, the involvement of an ion channel of some description, and the subsequent activation of a Ca^{2+}-dependent isoform of PLC cannot be ruled out. The next series of experiments were designed to shed some light on this process and to determine whether the effects of OA and PMA on receptor-stimulated [^{3}H]-IP production can be explained by their ability to modify Ca^{2+} fluxes in these cells.

Figure 30 shows that basal [^{3}H]-IP accumulation was largely unchanged when cells were incubated in Ca^{2+}-free buffer. In contrast, there was a marked reduction (80%) in the response to NA under these conditions, although it should be noted that the effect of NA is not abolished in the absence of extracellular Ca^{2+}. The dependence of NA-evoked [^{3}H]-IP accumulation on extracellular Ca^{2+} was further examined by incubating cells in Ca^{2+}-free medium to which various concentrations of Ca^{2+} were added. Figure 31 shows that NA-evoked increases in [^{3}H]-IP accumulation over basal levels were evident with Ca^{2+} at 1μM and that maximal responses were achieved at 100μM extracellular Ca^{2+} . The EC_{50} value for Ca^{2+} appears to be in the order of 10μM.

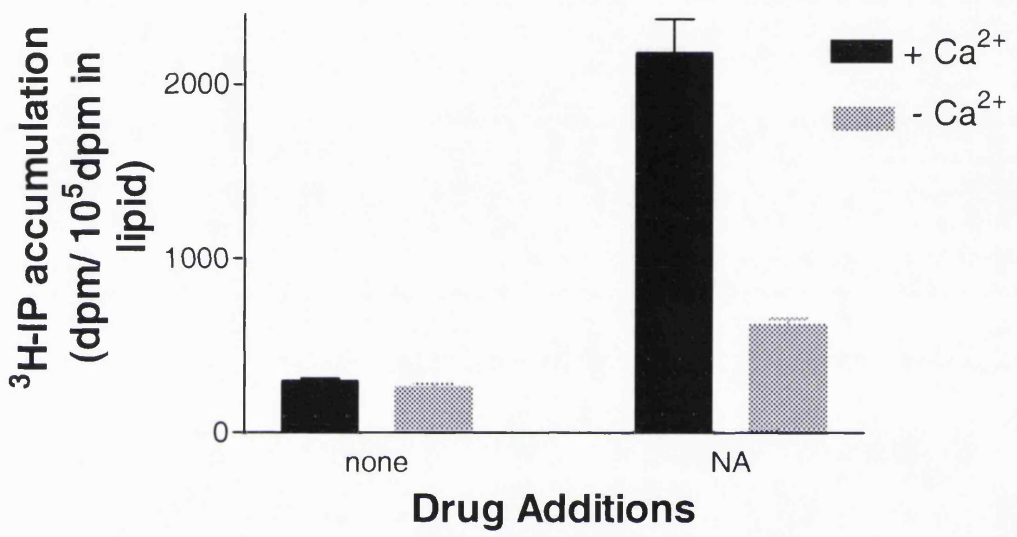

Figure 30. Effect of extracellular Ca^{2+} on noradrenaline-evoked [^{3}H]-inositol phosphate accumulation. Cultures were incubated for 15min in either normal buffer or Ca^{2+}-free buffer containing 0.2mM EGTA. Cultures were then incubated for 45 min in the presence or absence of 100μM NA. Results are expressed as means ± S.E.M. from 3 determinations.

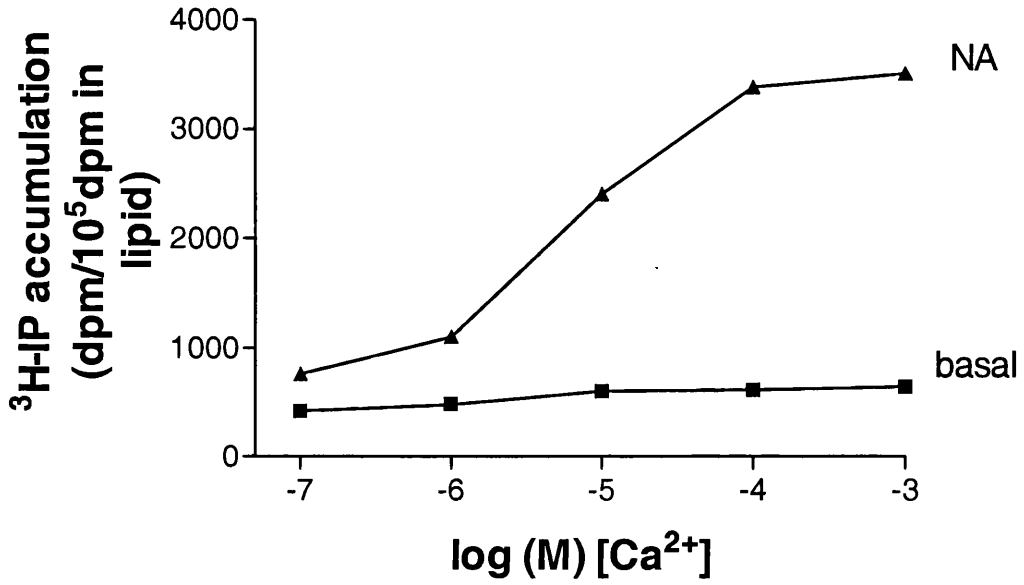

Figure 31. Concentration response curve showing Ca^{2+} dependence of basal and noradrenaline-evoked [^{3}H]-inositol phosphate accumulation. Ca^{2+} was added at various concentrations for 15 min before a further incubation for a period of 45 min in The presence or absence of NA (100μM) . Incubation buffer was made up in HPLC grade water (Ca^{2+}- free). Results are means in duplicate determinations.

In order to elucidate how Ca^{2+} enters glial cells in response to NA, experiments were carried out using various Ca^{2+} channels blockers. Nifedipine is a blocker of voltage operated Ca^{2+} channels, particularly of the L-type. Only at a high concentration of 30μM, did it inhibit the NA response and then by less than 50% (figure 32). The divalent metal cation channel blockers, however, were ineffective at lower concentrations and only managed to overcome competition with Ca^{2+} at higher concentrations. These high concentrations are expected as the cation blockers compete with Ca^{2+} for entry into the cell. The rank order of inhibition for the divalent cations was: $Cd^{2+} > Mn^{2+} >> Co^{2+} = Ni^{2+}$, with Cd^{2+} giving approximately 60% inhibition at 100μM. These results show that Ca^{2+} influx was blocked by divalent cations but not by an inhibitor of voltage - operated Ca^{2+} channels.

The next experiments examined Ca^{2+} uptake more directly by using tracer amounts of $^{45}Ca^{2+}$. A time course of $^{45}Ca^{2+}$ uptake into glial cells is shown in figure 33. Results show that there was a rapid uptake of $^{45}Ca^{2+}$ within the first minute which then reached a plateau by 2 min. The rate of $^{45}Ca^{2+}$ uptake was increased as was maximal uptake in the presence of NA. Both controls and NA-stimulated $^{45}Ca^{2+}$ uptake eventually reaching equivalent levels by 5 min of incubation.

Having established that NA-evoked [^{3}H]-IP accumulation was dependent upon extracellular Ca^{2+}, it was then decided to see if Ca^{2+} uptake was affected by drugs which had been shown to modify the effect of NA. Table 4 shows that NA evoked a 78% increase in $^{45}Ca^{2+}$ uptake over basal values. The Ca^{2+} ionophore A23187 also increased

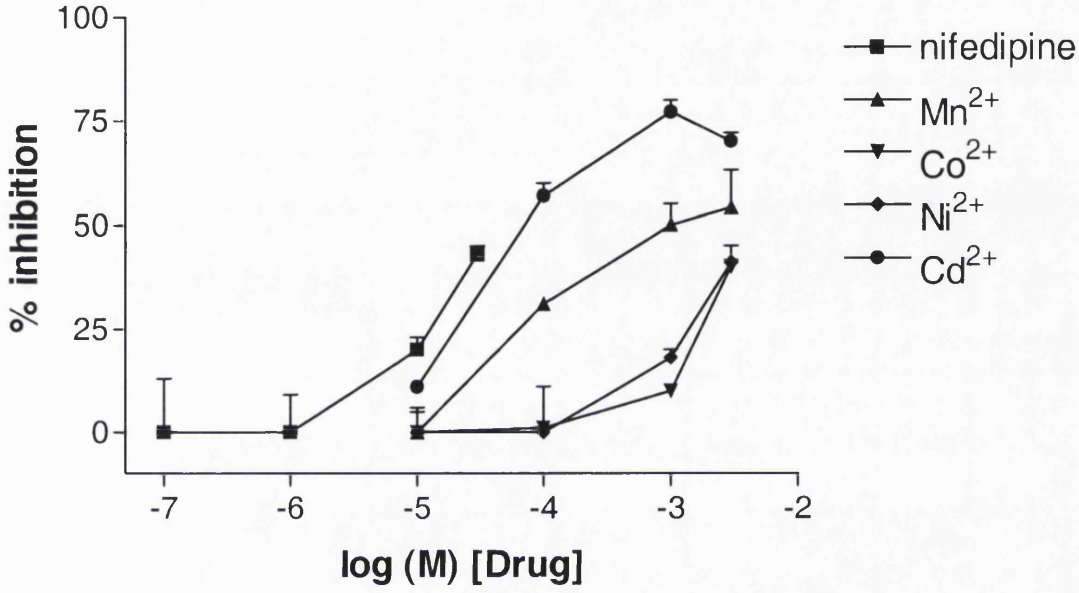

Figure 32. Dose response curves showing the ability of various Ca^{2+} channel blockers to inhibit noradrenaline-stimulated [^{3}H]-inositol phosphate accumulation. Drugs were added at various concentrations for 15 min before addition of 100μM NA for a period of 45 min. Results are expressed as % inhibition of the agonist response and are means ± S.E.M. for 3 determinations.

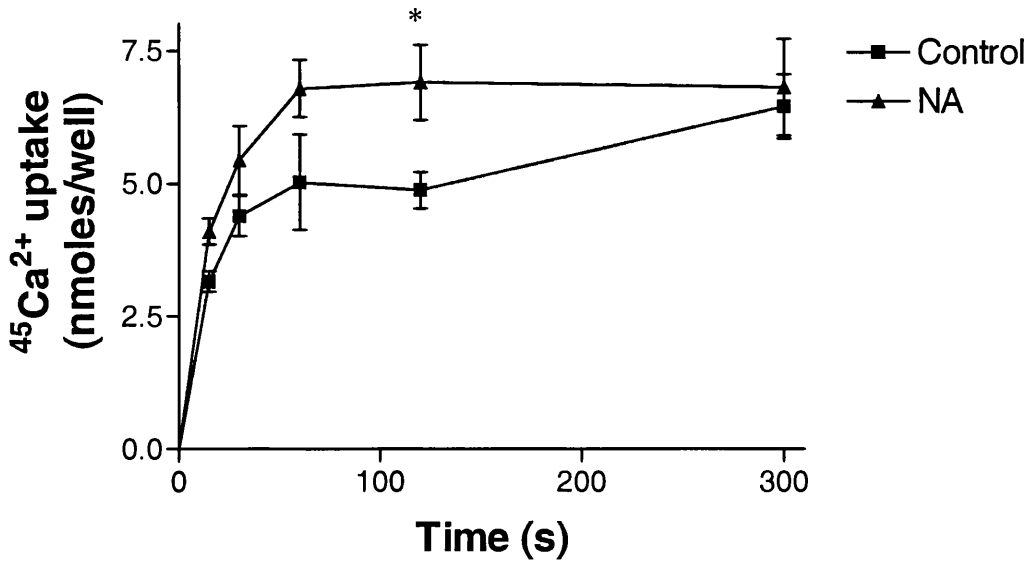

Figure 33. Time course showing $^{45}Ca^{2+}$ uptake into glial cells. Cultures were incubated with either no addition (control) or 100μM NA over a 5 min period. Results are means ± S.E.M. from 6 determinations. * indicates statistically significant ($p<0.05$) compared to control.

Drug additions	$^{45}Ca^{2+}$ uptake (nmoles/min/well)
None	1.52 ± 0.21
NA (100μM)	2.71 ± 0.05 *
NA + RS17503(1μM)	1.71 ± 0.09 †
OA (0.5μM)	1.77 ± 0.17
NA + OA	1.93 ± 0.09 †
PMA (0.3μM)	1.68 ± 0.12
NA + PMA	1.67 ± 0.07 †
A23187 (3μM)	2.27 ± 0.18 *

Table 4 . Effect of various drug additions on $^{45}Ca^{2+}$ uptake. Cultures were incubated for 2 min with various drug additions as indicated. Results are means ± S.E.M. for 6 determinations.* indicates statistically significant ($p<0.05$) compared to no addition, † indicates statistically significant ($p<0.05$) compared to NA alone.

$^{45}Ca^{2+}$ uptake by 49% over basal and was used as a positive control. As NA has previously been shown to activate the α_{1A}-adrenoceptor, the selective antagonist RS17053 was also tested. This drug reversed the NA response by 83% though a complete inhibition was not observed. $^{45}Ca^{2+}$ uptake into OA and PMA pre-treated cultures which were not challenged with NA was no different to controls, however, the ability of NA to promote $^{45}Ca^{2+}$ uptake was reduced by some 65% and 87% in OA and PMA pre-treated cultures, respectively. These results confirm that α_{1A}-adrenoceptor activation stimulates Ca^{2+} uptake into glial cells and that this event can be inhibited by both OA and PMA pre-treated suggesting that receptor-coupled Ca^{2+} influx is regulated by phosphorylation.

The release of $^{45}Ca^{2+}$ from pre-labelled cultures was also examined. In these experiments cultures were pre-incubated for 24h with $^{45}Ca^{2+}$, washed, then subjected to ten 1 min periods of incubation to assess the release of $^{45}Ca^{2+}$ into the bathing medium. The release profiles shown in figure 34 indicate that a steady basal level of release was achieved within 3 min. The subsequent addition of NA for 1 min provoked a 2-fold increase in $^{45}Ca^{2+}$ release, an effect which persisted only for the period of challenge. When experiments were performed on OA-treated cultures, the effect of NA was potentiated. $^{45}Ca^{2+}$ release under these conditions was almost 4-fold over basal. Table 5 summarises these results and those found for other drug additions. It can be seen that, in contrast to OA pre-treatment, pre-treatment with PMA resulted in a 54% reduction in NA-stimulated $^{45}Ca^{2+}$ release. In addition, it is evident that the response to NA was unaffected by the presence of the α_{1A} adrenoceptor antagonist RS17053 but was abolished when performed in the presence of the α_{1B} adrenoceptor antagonist AH11110.

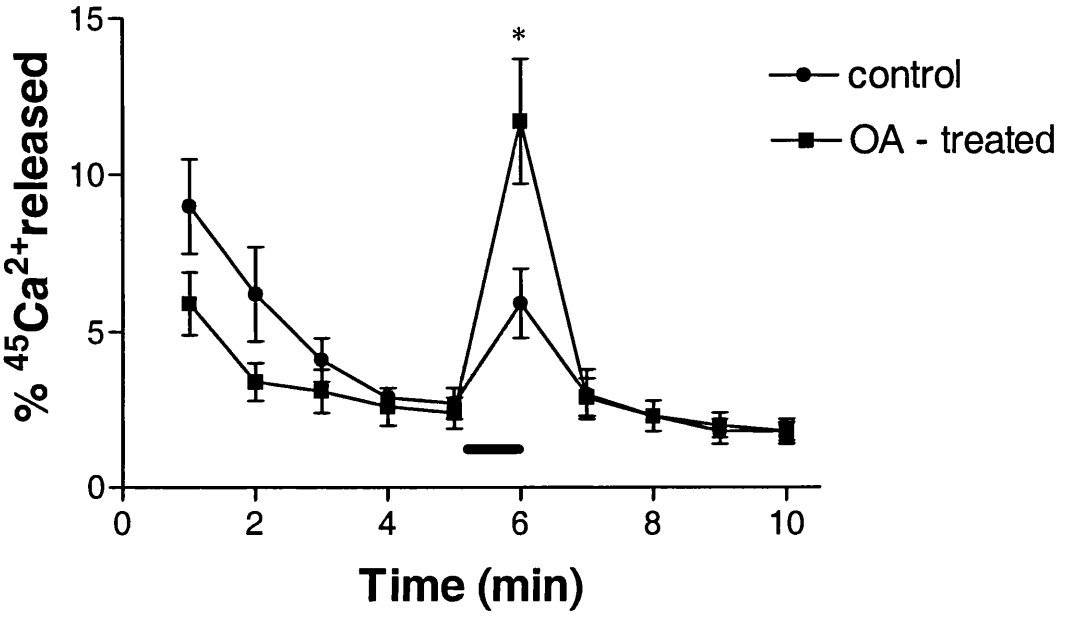

Figure 34. $^{45}Ca^{2+}$ release profiles. Cultures were incubated with 4μCi/ml for 24h, washed, then incubated with or without OA (0.5μM) for 15 min. $^{45}Ca^{2+}$ release was then assessed over a 10 min period with fractions collected each min. The first five periods were to establish basal levels of release, the next period (indicated by the bar) was in the presence of NA (100μM) and the remaining four min periods in drug free buffer. The release of $^{45}Ca^{2+}$ in each incubation period was calculated as the amount released each min as a % of that present in cultures at the time the sample was taken. Results are means ± S.E.M. for 4 - 6 determination. * indicates statistically significant ($p<0.05$) compared to control.

Drug additions	$^{45}Ca^{2+}$ release (% basal)
NA (100μM)	219 ± 41
NA + RS17503(1μM)	190 ± 6
NA + AH11110 (1μM)	88 ± 7 *
NA + OA (0.5μM)	394 ± 21 *
NA + PMA (0.3μM)	158 ± 8

Table 5. Effect of various drug additions on noradrenaline-stimulated $^{45}Ca^{2+}$ release. The $^{45}Ca^{2+}$ released into the bathing medium in response to NA in the presence or absence of various drug additions was calculated as a % of that released in the preceding basal fraction. Results are means ± S.E.M. for between 3-6 determinations.* indicates statistically significant ($p<0.05$) compared to no addition.

In summary, the main findings of this section are as follows:

- NA-evoked increases in [^{3}H]-IP accumulation are largely dependent upon extracellular Ca^{2+}.
- NA-evoked [^{3}H]-IP accumulation was blocked by divalent metal cations, particularly Cd^{2+} and Mn^{2+}, but not by an inhibitor of voltage-operated Ca^{2+} channels.
- $^{45}Ca^{2+}$ uptake into glial cells was stimulated by α_{1A}-adrenoceptor activation and was blocked in OA and PMA pre-treated cells suggesting that receptor-coupled Ca^{2+} influx is regulated by phosphorylation.
- NA-stimulated $^{45}Ca^{2+}$ release was potentiated by OA pre-treatment but reduced by pre-treatment with PMA.
- Agonist-evoked $^{45}Ca^{2+}$ release was unaffected by an α_{1A}-antagonist but abolished by the presence of an α_{1B} antagonist suggesting a possible involvement of α_{1B}-adrenoceptors in this mechanism.

CHAPTER 4
DISCUSSION

4.1 Receptor stimulation increases [^{3}H]-IP accumulation in glia

The hydrolysis of a membrane phospholipid by a specific PLC is one of the earliest key events by which an extracellular signalling molecule can regulate functions of target cells. Results presented here show that NA stimulated phosphoinositide metabolism, as determined by [^{3}H]-IP formation, above basal values in a time and concentration dependent manner in glial cultures. This also demonstrates that exposing the cultures to NA over periods up to 1h did not result in any detectable receptor desensitisation as the NA response did not diminish over this time period. Maximal responses to NA were achieved at a concentration of 100μM and concentration response relationships gave an EC_{50} value of 5μM. Similar results have been obtained by others in both glial cultures (Pearce et al., 1985; 1986; Ritchie et al., 1987; Gonzales et al., 1985) and in brain slices (Brown et al., 1984).

Various IPs are formed in response to receptor-stimulated phosphoinositide metabolism which include IP_1, IP_2, and IP_3. In this study, analysis of the individual metabolites of inositol phospholipid metabolism formed in response to receptor stimulation revealed that following a 45 min exposure to NA in the presence of Li^+, the major [^{3}H]-IP accumulated was [^{3}H]-IP_1. NA also provoked an increase in [^{3}H]-IP_2 accumulation under these conditions. The finding that the bulk of the [^{3}H] recovered was present in IP_1 was not unexpected considering that Li^+ was present in the incubation medium. Berridge et

al. (1982) previously showed that Li^+ prevents the breakdown of IP_1 to free inositol, thereby allowing [^{3}H]-IPs to accumulate in cells. This occurs by the uncompetitive inhibition of inositol monophosphatase, which lowers the level of myo-inositol and could lead to a decrease in the concentration of PI when reincorporated. Work by Batty and Nahorski (1985) proposed that Li^+ also blocks the dephosphorylation of IP_2 to IP_1, this may account for the observed increase in [^{3}H]-IP_2 accumulation reported here. No agonist stimulated [^{3}H]-IP_3 accumulation was found in these cultures following a 45 min incubation. It seems likely that NA action results in a majority of the [^{3}H]-IP_1 isoform accumulated as a result of an initial formation of IP_3. Other studies investigating the individual IP isoforms formed in response to agonist stimulation show corresponding similarities. Pearce et al. (1986) showed that the major [^{3}H]-metabolite accumulated under these conditions was IP_1 but that at least some of this was due to the initial formation of IP_3. An increased accumulation of [^{3}H]-IP_3 was noted within 1 min of incubation suggesting that the dephosphorylation of [^{3}H]-IP_3 in glial cells cultures is rapid. Steinberg et al. (1989) also illustrated that agonist stimulation produced individual IPs in slices of rat cortex and detected distinctive isomers of IP_3 and IP_2 that were prevailing over others, with (1,4,5)-IP_3 predominating over (1,3,5)-IP_3 and (1,3)-IP_2, (3,4)-IP_2 and (4,5)-IP_2 isoforms which respectively accounted for about 22%, 8% and 3% of total IP_2 in the extracts. Although individual isomers were not ascertained in the work described here, a report by Oliva et al. (1997) suggests that IP_3 is phosphorylated via 3-kinase in astrocytes and that in neurones dephosphorylation of IP_3 via 5-phosphatase is

the pathway that predominates which produce (1,3,4,5)-IP_4 and (1,4)-IP_2, respectively.

4.2 Noradrenaline-evoked [^{3}H]-IP accumulation is stimulated via α_{1A}-adrenoceptors

The formation of [^{3}H]-IPs in response to NA in glial cells appears to be mediated by α_1 adrenoceptors since prazosin (α_1 selective) was 40 fold more potent than yohimbine (α_2 selective) in blocking the NA response (Pearce et al., 1986; Ritchie et al., 1987; Wilson and Minneman, 1990b). Studies involving saturation, time course, and competition binding experiments identified α_1-adrenoceptors on immunocytochemically defined astroglial cells where approximately 66% of cortical polygonal astroglia were found to express detectable numbers of α_1-adrenoceptor (Lerea and McCarthy, 1989). The authors also found that astroglia maintained in the presence of neurons, continue to express α_1-adrenoceptors (Lerea and McCarthy, 1990).

Three subtypes of the α_1-adrenoceptor (α_{1A}, α_{1B} and α_{1D}) have been characterised and selective ligands can be used to distinguish between them (Graham et al., 1996). This study shows that the α_{1B} antagonist (AH11110A) and the α_{1D} antagonist (BMY7378) failed to inhibit fully the response to NA, whereas the α_{1A} (RS17053) antagonist was able to completely abolish the NA response at a concentration of 1μM. At this concentration the α_{1B} and α_{1D} selective antagonists produced merely a 20-25% inhibition of the NA response. Thus there was a clear rank order of antagonist potency (RS17053 >>

AH11110A > BMY7378) indicating that α_{1A}-adrenoceptors are the predominant subtype expressed by cultured cortical glia. Wilson and Minneman (1990a) also suggested this subtype to be predominantly activated by NA in cultured glia basing their observations on the adrenoceptor's requirement for extracellular Ca^{2+}. The presence of α_{1A}-adrenoceptors on these cells was confirmed by the use of the selective agonist A61603 which was as effective but considerably more potent (50-fold) than NA in increasing [^{3}H]-IP accumulation. Knepper et al. (1995) found that A-61603 stimulated phosphoinositide hydrolysis, approximately 200-fold more potently than NA in fibroblast cells transfected with α_{1A} -adrenoceptors. It may be that the α_{1A}-adrenoceptor is the subtype present on glia in the intact brain. Autoradiography and electrophysiological studies show the existence of α_1-adrenoceptors on both acutely isolated cortical glia (Shao and Sutin, 1992) and hippocampal glia in situ (Duffy and MacVicar, 1995), moreover, it is interesting to note that mRNA for the α_{1A} subtype appears to be particularly abundant in these brain regions (Graham et al., 1996).

Receptors can be regulated through a number of processes such as phosphorylation and dephosphorylation. Phosphorylation of proteins is a dynamic event involving both PKs and PPs which serve as molecular switches in signal transduction pathways. In general, phosphorylation leads to desensitisation of the receptor and is mediated by PKs. PKC mediated phosphorylation has been shown to subsequently desensitise histamine receptors. Zamani et al. (1995), for example, showed that acute stimulation of H_1

receptors leads to the desensitisation of histamine-mediated Ca^{2+} mobilisation, manifested as a concurrent reduction in both the magnitude of the Ca^{2+} transient and of the sustained phase. Their results demonstrated the presence of both a PKC-dependent mechanism and PKC-independent mechanism are involved in the H_1-receptor desensitisation cascade. PKC phosphorylation on the GluR2 subunit using biochemical and molecular techniques have also been reported (McDonald et al., 2001), suggesting that phosphorylation may be important in the regulation of excitatory synaptic transmission. Phosphorylation of receptors by PKs is a process that can be reversed by PPs which are characterised by the amino acid residues that they can dephosphorylate. Both transmitter receptors and those for second messengers are regulated by phosphorylation. Serine/threonine PPs such as PP1 have been shown to mediate receptor dephosphorylation. Zhao et al. (1998) investigated the possibility that PP1 was targeted to the ryanodine receptor of both skeletal and cardiac muscle by the direct isolation of PP1-binding proteins on PP1-sepharose affinity columns. Their results showed that the ryanodine receptor of both extracts bind to this affinity column. The ability of PKA to phosphorylate the ryanodine receptor was also demonstrated.

4.3 Involvement of protein phosphatases in the regulation of receptor-linked [^{3}H]-IP formation in glia

Many studies confirm the presence of PPs in brain and glial cultures. Sim et al. (1994)

found that the specific activity of PP1 and PP2A in the particulate fraction of rat forebrain was much lower than that of the cytosol and of the particulate fractions of other tissues. That the majority of PP1 and PP2A in rat forebrain are associated with membrane structures, but in a low activity state, suggests that regulatory mechanisms exist that have considerable and unique potential for activation of protein dephosphorylation. PP isoforms, PP1γ, PP1δ, PP1α and PP2A (α and/or β), have been immunohistochemically localized in the rat cerebellum (Hashikawa et al., 1995). By immunohistochemical analyses of rat brain, Strack et al. (1999) found the isoforms PP1β and PP1γ1 to have widespread cellular expression that was quite distinct from one another. PP1γ1, for example, was additionally found to be highly enriched in dendritic spines and presynaptic terminals of rat brain. These results show that PP1 isoforms are targeted to different neuronal cytoskeletal compartments with a high degree of specificity, presumably by isoform-specific association with regulatory/targeting proteins. Furthermore, the authors propose that the synaptic localization of PP1γ1 indicates that it is this isoform that is involved in the regulation of synaptic phosphoproteins such as neurotransmitter receptors and ion channels implicated in synaptic plasticity.

The presence of PPs have also been demonstrated in glia. Vinade and Rodknight (1996) used PP inhibitors to study the phosphatase activity involved in the dephosphorylation of the astrocyte cytoskeletal protein GFAP in slices prepared from hippocampi of immature rats, with results indicating that GFAP dephosphorylation in intact tissue is

primarily due to PP1. Daniels and Vickroy (1999) demonstrated that glutamate transporters in glia of the adult rat forebrain may be regulated through reversible phosphorylation cataylsed by the opposing actions of PKC and an OA-sensitive PP. Hyperphosphorylated tau, which is the major protein of the neurofibrillary tangles in AD brain, is most probably the result of an imbalance of tau PK and PP activities in the affected neurons. Many reports propose that a deficiency of PP2A may be involved in abnormal phosphorylation of tau in AD as PP2A was able to dephosphorylate this protein (Gong et al., 1994; Saito et al., 1995) These studies found that selective inhibition of PP2A by OA induced an Alzheimer-like hyperphosphorylation and accumulation of tau and concluded that down-regulation of PP2A activity could hence lead to Alzheimer-like abnormal hyperphosphorylation of this protein. Pei et al (1997) has since reported elevated protein levels of PP2A and PP2B in astrocytes of Alzheimer's disease temporal cortex. Pahan et al. (1998) proposed another role for OA sensitive PPs in rat primary astrocytes and C6 glial cells where it was found that OA and microcystin stimulated the lipopolysaccharide- and cytokine-mediated expression of inducible nitric oxide synthase and production of nitric oxide. Thus the involvement of PPs in the regulation of inducible nitric oxide synthase could play an important role in the pathophysiology of stroke and other neurodegenerative diseases where inducible nitric oxide synthase is produced in response to proinflammatory cytokines.

To date, however, there is no information regarding the activities of particular PP

isoforms in glial cells. Results presented in this thesis show that cultured cortical glia possess the PPs PP1 and PP2A and, in terms of their ability to dephosphorylate glycogen phosphorylase a, are present in approximately equal abundance. Results also show adult rat brain to have a greater amount of PP2A (68%) compared to PP1 species. The amount of PP2A in brain extracts has been reported to be the highest of all tissues investigated, and there is approximately three times as much PP2A as PP1 (Ingebritsen et al., 1983).

An interesting link between PP1 and PP2A is their shared sensitivity, presumably as a consequence of their shared homology, towards the OA class of compounds. These compounds are potent, competitive inhibitors of both PP1 and PP2A and include OA, tautomycin and the microcystins. The structure of OA is very hydrophobic, it is therefore cell permeant and appears to inhibit PPs in a manner that can discriminate between PP species in intact cells (Hardie et al., 1991). This quality offers distinct advantages over other PP inhibitors, such as I-1 and I-2, which are not membrane permeant. Pre-treatment of cultured glia with OA resulted in a reduction of NA-stimulated [^{3}H]-IP accumulation which was time dependent. Maximal inhibition (~55%) of the NA response was achieved within 30min of pre-incubation and remained at this level for periods up to 1h. The ability of OA to inhibit NA-evoked [^{3}H]-IP accumulation was also concentration dependent (IC_{50}, 0.3μM) and higher concentrations of up to 3μM achieved 75% inhibition of the NA response. In the presence of OA, an additional [^{3}H-]IP elution profile experiment showed that the metabolism of IPs was considerably reduced. OA

treatment reduced the agonist-stimulated elevation of IP_1 and IP_2 fractions by almost half. OA also inhibited α_{1A} agonist (A61601)-evoked [^{3}H]-IP accumulation to an extent essentially identical to that of NA. This suggests the presence of a PP in these primary astrocyte cultures that is involved in the NA-stimulated phosphoinositide metabolic pathway, since the effect of OA is assumed to be due to the inhibition of PP action. The inhibition of induced IP formation by OA has also been reported in other cell types such as platelets (Walker and Watson, 1992) and hepatocytes (Higashi et al., 1994). In these reports a PK is proposed to inhibit PLC and to be reversed by PP1 and/or PP2A suggesting that receptor-mediated PLC activation is subject to multiple controls by phosphorylation-dephosphorylation. Alcantara-Hernandez et al. (2000) demonstrated that OA increased α_{1B} receptor phosphorylation in a time- and concentration-dependent manner in transfected fibroblasts by altering the adrenoceptor-G protein coupling, as evidenced by a small decrease in NA- stimulated [^{35}S]GTPγS binding. Other PP inhibitors such as calyculin A and tautomycin also mimicked this effect, demonstrating that inhibition of an OA sensitive PP is involved in adrenoceptor regulation.

Being hydrophobic and cell-permeable, the differential inhibition of PP1 and PP2A activity in intact cells by OA has made this marine toxin a valuable research tool for examining PP-mediated processes and interactions. OA rapidly stimulates protein phosphorylation in intact cells, and behaves like a specific PP inhibitor in a variety of metabolic processes. In this study, results show that following a 15 min pre-incubation,

OA inhibited the NA response for up to 48h. Thus it appears that OA is metabolised very slowly and that the binding of OA to PPs is tight and not easily removed. After 48h there was a gradual increase in IP accumulation which suggests recovery of the PPs that were inhibited by OA.

The inhibiting effects of OA were further substantiated by the action of the related PP inhibitors tautomycin and microcystin, which in glial cultures reduced NA-stimulated [^{3}H]-IP accumulation by 36% and 33% respectively. Both inhibitors act on PP1 and PP2A with tautomycin being more selective towards PP1 (Fujiki and Suganuma, 1999). Mackintosh and Klump (1990) found that OA prevented the binding of tautomycin to PP2A in mammalian, protozoan and plant extracts, indicating a common binding site for both inhibitors. Takai et al. (1995) confirmed this finding using ligand binding methods and proposed that OA, and the other inhibitors, bind to PP2A in a mutually exclusive manner. The PP inhibitors may, therefore, share the same binding site on the PP2A molecule.

Although the identity of the PP involved in the regulation of α_{1A}-adrenoceptor coupled phosphoinositide metabolism in glia could not be ascertained, it is likely to be either PP1 and/or PP2A as PP2B is inhibited by high micromolar concentrations of OA whilst PP2C is resistant to the drug (Wera and Hemmings, 1995). Glial cell cultures which were pre-treated with OA showed a substantial decrease of about 60% in total PP activity.

Analysis of the proportion of PP1 and PP2A mediated activity in these cultures revealed PP2A was only about 18% of the total amount. PP1 activity was also reduced in these cultures but to a lesser extent (25%). Most of the activity in these pre-treated was attributable to PP1 with only 3% due to non OA-sensitive PPs. These studies suggest that PP1 and PP2A play an important role in the regulation of phosphorylation in glial cells. The discovery of novel PPs that are sensitive to low concentrations of OA complicates the interpretation of these results. Several groups have discovered additional PP2A-like PPs that, although they represent only a small fraction of the total cellular serine/threonine PP activity, also show sensitivity to OA (Cohen, 1989; Schonthal, 1995). Although detailed dose-response studies have not been reported for native PP5, PP6 and PP7, studies with PP4 and recombinant PP5 indicate they are sensitive to OA (Andreeva and Kutuzov, 1999). OA was found to inhibit PP4 in vitro with an IC_{50} value comparable to that for PP2A whereas PP5 was inhibited at IC_{50} values of 1-10 nM. Like PP2C, PP7 was apparently insensitive to inhibition by OA (Huang et al., 1998). Thus the resulting effects of OA treatment, even at low concentrations, cannot easily be ascribed to a single PP and functions currently ascribed to PP2A could possibly be due to PP4 or PP5. In light of this, it is more accurate to propose that a PP2A-like PP is the subtype involved in receptor linked responses in glial cells. Novel PPs have not, as yet, been reported in glial cultures although some novels PPs such as PPG (Brady et al., 1997) and PPV (Becker et al., 1994) have been reported to dephosphorylate phosphorylase, the substrate used in the studies reported in this thesis. Together these results suggest that an OA sensitive PP is

involved in inhibiting NA-stimulated [^{3}H]-IP accumulation. This leads to the suggestion that a PK is also involved in this response.

4.4 Involvement of protein kinases in NA-evoked [^{3}H]-IP formation in glial cells

The OA-mediated inhibition of NA-stimulated [^{3}H]-IP accumulation suggests the involvement of a serine/threonine PK in this event as the inhibition of a PP will theoretically allow a PK to act unopposed. This appears to be the case as staurosporine, a non-selective PK inhibitor, completely reversed the inhibitory effects of OA on NA-stimulated [^{3}H]-IP accumulation. Alcantara-Hernandez et al. (2000) also showed that staurosporine and the PKC inhibitor Ro 31-8220 blocked the effect of OA on α_{1B} receptor phosphorylation which suggests that PKC could perhaps be involved in the phosphorylation of this receptor.

Results presented here using myristoylated PKA and PKC inhibitors do not support a major role for PKA in regulating α_{1A}-adrenoceptor linked IP accumulation. Although phosphorylation sites for PKA have been reported on the α_{1B} -adrenoceptor (Alonso-Llamazares et al., 1997), only one report shows evidence for PKA phosphorylation sites on the cloned α_{1A} receptor (Graham et al., 1996). The fact that the myristoylated PKA inhibitor was unable to reverse the effect of OA, whereas the myristoylated PKC inhibitor reversed its effect by about 60% suggests that PKC could be involved in regulating α_{1A}-

adrenoceptors coupled to the IP signalling pathway in these cultures. Studies were conducted to establish further that PKA was not involved in the regulation of the α_{1A} adrenoceptor in glial cultures. The ability of forskolin to inhibit the NA response was also assessed. Forskolin is a well established activator of AC (Seamon et al., 1981) and has been shown to increase cAMP levels in glial cultures (Murphy et al., 1991). Results show that although the drug inhibited the NA response by some 20%, it did not effect the inhibitory effect of OA on NA- evoked [^{3}H]-IP accumulation, further confirming that PKA is not involved in the regulation of α_1-adrenoceptor linked phosphoinositide metabolism in these cultures.

To establish PKC involvement in the regulation of NA-stimulated phosphoinositide breakdown, PMA, a PKC activator, was shown to inhibit NA- evoked responses in a concentration dependent manner with an apparent IC_{50} value (3nM) similar to that reported for the inhibition of muscarinic receptor-coupled phosphoinositide breakdown in these cells (Pearce et al., 1988). Several reports implicate PKC in the down-regulation of receptors coupled to inositol phospholipid metabolism in a number of tissues including brain (Lee and Fain, 1991) and glial cells (Pearce et al., 1988), where acute phorbol ester treatment resulted in a reduced accumulation of IPs in response to agonist stimulation. Previous work by Orellana et al. (1985) showed that PMA blocked agonist-stimulated IP formation in cultured astrocytoma cells. These authors also found that PMA pre-treatment caused a reduction of guanine nucleotide stimulated IP accumulation and

concluded that PMA induced receptor down-regulation could be the result of an inhibition of G -protein activity. Other studies report changes in receptor affinity and/or number in response to phorbol ester treatment as explanation for the observed receptor down-regulation (Chen et al., 1995; Turner et al., 1996). Phosphorylation is an important step in receptor regulation, and the fact that OA effects were not additive with PMA, as results presented here demonstrate, suggests that OA and PMA influence the same component in this process. Results also show that the effect of PMA on NA-evoked [^{3}H]-IP accumulation was reversed completely by staurosporine. Although this evidence strongly indicate that PKC is the PK involved in regulating these receptors, confirmation was sought by the use of PKC depleted glial cultures. Young et al. (1987) have shown that prolonged phorbol ester treatment causes down regulation of PKC. The authors demonstrated a progressive loss of PKC as a result of chronic PMA exposure of rat glioma cells and that the consequence of this was an increased rate of degradation of the polypeptide. Consistent with that observation, they were also unable to detect any change in the amounts of mRNA for PKC. Pearce et al. (1993) also showed that prolonged PMA treatment results in PKC down-regulation in glia. Results presented here show the effect of OA was not observed in PKC depleted glial cultures which, having demonstrated that the effects of OA on NA-stimulated [^{3}H]-IP accumulation are reversed by a PKC inhibitor, suggests that PKC is involved in this response.

Results presented here show that glial cells pre-treated with PMA demonstrated a loss of

PP activity mediated by PP2A, which is comparable to that of OA-treated cultures. This substantiated the idea that many PKs are themselves regulated by reversible phosphorylation (Millward et al., 1999). PP2A may be required for dephosphorylation of PKC substrates or, indeed, PKC itself. Ricciarelli and Azzi (1998) have shown that the regulatory subunit of PP2A can be phosphorylated by the α isoform of PKC. PP1was also found to activate PKCα at low concentrations.

PKC is known to exist in a number of isoforms which can be grouped according to their enzymic activity. The conventional isoforms (α, β and γ) are Ca^{2+}-dependent and activated by phorbol esters, the novel isoforms (δ, ϵ, θ, and η) are also activated by phorbol esters but are Ca^{2+}-insensitive whilst the atypical PKCs (ζ, and ι) are both phorbol ester- and Ca^{2+}-insensitive (Mellor and Parker, 1998). The presence of PKC activity in glial cultures was indicated by the marked increase in [^{32}P] incorporation into the histone substrate. Results show that in adult rat brain extracts and glial cell cultures there was a 2-3 fold higher proportion of Ca^{2+}-dependent PKC activity compared to that in the absence of Ca^{2+}. Glial cultures pre-treated with OA showed a 2-fold increase in both Ca^{2+}-dependent and independent activities which reflect a higher rate of PKC activity in these cultures. The increased activities observed may be predicted if PP2A were to exert some inhibitory effect on PKC. These results point to the presence of both conventional and novel subtypes in glial cultures and appear to suggest that the conventional isoforms of PKC are perhaps in greater abundance. Although the identify

of the PKC isoforms present in glial cultures was not determined here, others have shown through Western blot analysis that similar cultures express all but the γ isoform of PKC (Slepko et al. 1999). It is not possible therefore, to attribute α_{1A}-adrenoceptor down-regulation in these cells to a particular isoform of PKC. Slepko et al. (1999) suggest, however, that particular examples of the conventional (α) and novel (ϵ) isoforms are enriched in astrocytes, the predominant cell type (~ 90%) in our cultures, compared to microglia which constitute the remaining cells and have been reported to contain few if any α_1-adrenoceptors (Norris and Benveniste , 1993).

PKCα is involved in the regulation of bradykinin receptor-linked phosphoinositide breakdown in these cells (Chen et al., 1995). It should be noted, however, that receptor phosphorylation was not examined in the study described here, though it is possible that the observed effects of OA and phorbol ester on receptor-stimulated phosphoinositide metabolism are indirect and mediated by other proteins, such as G protein-coupled receptor kinases. There are currently seven isoforms of G protein-coupled receptor kinases which specifically interact with the agonist-activated form of G protein-coupled receptors to affect receptor phosphorylation and desensitization (Penn et al., 2000). The type -5 and -6 isoforms of this kinase are implicated in α_{1B} -adrenoceptor down-regulation (Diviani et al., 1996) and cannot be ruled out.

It is interesting to note that the α and ϵ isoforms of PKC have been implicated in the

phorbol ester-induced stimulation of AC activity in cultured astrocytes (Slepko et al., 1999), a finding which would therefore support the observation of increased PKA activity in response to OA treatment. Cellular cAMP levels are determined by the activities of ACs which catalyse cAMP synthesis and phosphodiesterases which hydrolyses it. Many extracellular signalling molecules work by controlling cAMP levels and do so by altering the activity of AC rather than the activity of phosphodiesterases. A family of membrane bound ACs, at least 10 of which have been isolated and characterised, converts ATP into cAMP (Chern, 2000). All mammalian AC isozymes are detected in the CNS and many brain areas express multiple AC isozymes; a certain physiological function therefore might be regulated by multiple ACs in a rather complicated manner. Physiologically, activities of ACs are regulated by various G-protein coupled receptors, however, different AC isozymes are tightly governed by other modulators including a variety of PKs and PPs. PP2A has been reported to stimulate at least one isoform (VI) of the enzyme (Chern, 2000).

The proportion of active PKA in cell cultures can be measured by its ability to incorporate [^{32}P] into a specific substrate such as kemptide (Kemp et al., 1977). In this study, data shows that in adult rat brain extracts the majority of PKA activity was cAMP dependent. This is also the case for glial cultures, although the activity ratio reflects a higher proportion of basal PKA activity in these cells. In OA-treated cells however, results show approximately two- and four fold increases in total and basal PKA

activity respectively, which suggests a higher rate of dissociation of the catalytic subunits from their regulatory counterparts. This might be predicted if a PP exerts some inhibitory control over PKA . It is suggested that PP2A forms complexes with PKs such as PKA and PKC which are both inhibited by PP2A (Sim and Scott, 1999; Millward et al., 1999). The removal of this inhibitory effect may explain the increased PKA activities observed in cultures pre-treated with OA. As OA treatment has been shown to inhibit a PP2A like PP in glial cultures the enhanced level of free PKA catalytic subunit in these cells may therefore not be due to increased cAMP production by AC. Some AC isozymes are expressed dominantly in specific areas (ACIII in olfactory cilla; ACV in basal ganglia), while others (ACII, ACVI, and ACVII) are more widespread in the brain (Chern, 2000). PKC can stimulate certain AC isoforms (II, V and VII) and results here show that OA treatment elicited increases in both Ca^{2+}-dependent and Ca^{2+}-independent PKC activity in cultured glia. Phosphorylation and dephosphorylation of AC isozymes are likely to serve as fine-tuning mechanisms of cAMP formation and, therefore, also likely to play important roles in various CNS functions.

Phosphorylation of adrenergic receptors is implicated in the regulation of their activity and has raised the possibility that PKA and/or PKC might play an important role in this event (Garcia-Sainz et al., 2000; Alcantara-Hernandez et al., 2000). These authors propose that direct activation of PKC by the use of phorbol esters, leads to receptor desensitisation/internalisation associated with phosphorylation. Garcia-Sainz et al. (1999)

have shown that the inhibitory actions of phorbol esters were blocked by PKC inhibitors and also by overnight treatment with phorbol esters, a treatment that leads to PKC downregulation. Blockade of the receptor function is very rapid and is consistent with receptor phosphorylation and uncoupling from G-proteins. The authors concluded that activation of PKC with phorbol esters, such as PMA, blocks agonist stimulated α_1-adrenergic activity, which is associated with receptor phosphorylation. This action of PKC seems to be physiologically relevant in that it causes desensitisation of the α_1 adrenergic activity (Garcia-Sainz et al., 1986). In short, these data indicates that inhibition of PPs increases the phosphorylation state of the α_1-adrenoceptor and appears to involve PKC.

It is clear that the findings presented in this thesis differ in a number of respects from those described by Vasquez-Prado and Garcia-Sainz (1996) who reported that in rat fibroblasts phorbol ester pre-treatment either abolished, partially reduced or was without effect on phosphoinositide breakdown in response to α_{1D}-, α_{1B}- and α_{1A}-adrenoceptor activation, respectively, suggesting differential sensitivities to PKC. This group showed that α_{1A}-adrenoceptors were phosphorylated by PKC to a much lesser extent than α_{1B}-adrenoceptors, but that similar levels of phosphorylation could be achieved if chimeric α_{1A}-adrenoceptors possessing the carboxyl-terminal tails of α_{1B}-adrenoceptors were produced (Vasquez-Prado et al., 2000). They also found the carboxyl terminus of the adrenoceptors to be the main domain for receptor phosphorylation. These results however

were obtained from studies on recombinant receptors transfected into cell lines. This is in contrast to our studies where we have shown that native α_{1A}-adrenoceptors expressed in cultured cortical glia are regulated either directly or indirectly by PKC and PP2A. One reason for this discrepancy might be that, unlike native receptors, transfected receptors do not assemble in the host cell with the appropriate accessory/regulatory proteins. Indeed, it is becoming increasingly clear that the targeting of PKs and PPs to particular domains within cells is a key requisite for the control of intracellular signalling pathways (Sim and Scott, 1999; Sontag, 2001).

Pools of PKs and PPs are maintained in specific subcellular locations by anchoring and targeting proteins (Scott and Pawson, 2000). The intracellular organisation of PKA is controlled through association with AKAPs. Accumulating evidence suggests that AKAP-mediated organisation of PKs and PPs is particularly important for the transduction of signals to the cytoskeleton. Keryer et al. (1993) characterised AKAP350 and revealed that this molecule had binding sites for PKA. Docking sites were also found for PP1, PP2A and the immature non-phosphorylated form of PKCϵ (Takahashi et al., 1999). AKAP350 is present in the centromeres of a variety of cell types (Schimdt et al., 1999; Takahashi et al., 1999). Another AKAP also present in centromeres is pericentrin which anchors PKA. The presence of two different AKAP molecules at the centromere raises the possibility that within a single cellular compartment, distinct anchoring proteins might target PKA, as well as other signalling enzymes, to precise sites to allow the

phosphorylation of specific substrates. Studies also show that the anchoring protein AKAP79 binds PKC α, βI and βII isozymes in the presence of Ca^{2+} and PS at a site distinct from the PKA binding site (Klauck at al., 1996). This anchoring protein has also been shown to bind PP2B and at least two AKAPs have been shown to bind and target PP1 (Coghlan et al., 1995). Thus PKA and PP1, both of which are broad signalling enzymes with opposing actions that catalyse changes in the phosphorylation state of cellular proteins, often share the same substrates. AKAP250 is expressed selectively in endothelial cells where it plays a role in adhesion and binds both PKA and PKC. AKAP220 has been shown to maintain a scaffold of both PKA and PP1 (Schillace, 1999). Targeting of these enzymes near the substrate is proposed to enhance phosphorylation-dependent modulation. Another anchoring protein called Yotiao, an NMDA receptor-associated protein, simultaneously binds to both PKA and PP1 in order to modulate NMDA receptor activity (Westphal et al.,1999). The PP is bound to Yotaio in its active state, and electrophysiological experiments suggest that PP1 limits the channel activity until PKA becomes active through the generation of cAMP. PKA activation leads to phosphorylation of the NMDA receptor and a concomitant increase in ion flow. Thus, Yotiao serves as a scaffold protein that physically attaches both a PK and PP to their substrate.

4.5 Ca^{2+} Fluxes

In many cells activation of α_1-adrenoceptors causes formation of IP_3 which promotes Ca^{2+} release from intracellular stores. However, receptor-mediated increases in intracellular Ca^{2+} levels can also be caused by influx from the extracellular fluid. Previous work has shown that the effect of NA on [^{3}H]-IP formation in glial cultures is dependent to a large extent on the presence of extracellular Ca^{2+} (Pearce et al., 1986; Wilson and Minneman, 1990b). Results herein support these observations showing that some 80% of NA-stimulated [^{3}H]-IP accumulation in glia was found to be dependent upon extracellular Ca^{2+}, and was dependent upon the extracellular Ca^{2+} concentration. Maximal responses to NA were achieved with 100μM Ca^{2+} and were evident with a concentration of just 1μM extracellular Ca^{2+}. This is similar to results obtained in brain slices (Kendall and Nahorski, 1984) where agonist-evoked IP metabolism showed a clear dependence on extracellular Ca^{2+}, and that different receptors mediating phosphoinositide breakdown in rat cortex have quantitatively different Ca^{2+} requirements. Wilson and Minneman (1990b) reported the α_{1A}-adrenoceptor to be dependent upon extracellular Ca^{2+}, unlike the α_{1B}-adrenoceptor. Results here show that a remaining 20% of the NA response was unaffected by the absence of Ca^{2+} which would suggest that this portion of the response was not mediated by α_{1A}-adrenoceptors but by the α_{1B} subtype.

Wilson and Minneman (1990b) showed that removal of extracellular Ca^{2+} did not effect

the NA response in hepatocytes, which possess only α_{1B}-adrenoceptors (Han et al., 1990), but reduced the NA response in renal cells by about 67% which was similar to the percentage of α_{1A} receptors found in these cells (approximately 60:40, α_{1A} to α_{1B}). The authors therefore concluded that α_{1A} receptor subtype mediates the influx of Ca^{2+}, whereas the α_{1B} subtype does not. The α_{1B}-adrenoceptor subtype selectively activates a Ca^{2+} insensitive PLC causing the hydrolysis of PIP_2 into IP_3 and DAG, whereas the α_{1A} subtype appears to involve a different pathway, possibly a Ca^{2+} influx stimulated hydrolysis of PI or PIP. These conclusions were based upon the observation that responses of IP isoforms were formed in response to NA stimulation in renal cells were slow whereas receptor-mediated hydrolysis of PIP_2 is usually very rapid (Wilson et al., 1984). Wilson et al. (1984) suggested that PIP_2 is preferentially hydrolysed in the absence of Ca^{2+}, whereas higher concentrations of Ca^{2+} are required for hydrolysis of other phospholipids, for example PIP and PI. Tsujimoto et al. (1989) proposed that α_{1A}-adrenoceptors might control the influx of extracellular Ca^{2+}, whereas α_{1B}- adrenoceptors control the release of stored intracellular Ca^{2+}. This finding has been supported by other studies (Han et al., 1987; Hanft and Gross, 1989) who found that the two subtypes of α_1-adrenoceptors caused responses through different molecular mechanisms in smooth muscle and cerebral cortex, respectively. The Ca^{2+} ionophore A23187 has also been shown to stimulate [^{3}H]-IP accumulation in a concentration-dependent fashion in glial cells (Pearce et al., 1986; Wigginton and Minneman, 1991). Moreover, the effect of the ionophore was dependent on the presence of extracellular Ca^{2+}.

Cells distribute Ca^{2+} among three functional compartments: extracellular space, cytosol and Ca^{2+} stores (Putney, 1986). There is a large electrochemical gradient favouring Ca^{2+} influx into cells as the inside is negatively charged with respect to outside and the extracellular Ca^{2+} concentration is much higher (10^{-3}M) than that inside (10^{-7}M). Ca^{2+} influx is thought to occur through either voltage sensitive Ca^{2+} channels (VOCs) which open and close following membrane voltage changes, or receptor-operated channels (ROCs) which are activated by extracellular ligands such as neurotransmitters. The mechanisms responsible for increased intracellular Ca^{2+} are achieved differ somewhat between excitable cells like neurons and non-excitable cells such as glia (Charles, 1994). In non-excitable cells, receptor mediated changes in cytoplasmic Ca^{2+} concentration occur either through release from intracellular store operated Ca^{2+} channels (SOCs) or the opening of channels in the plasma membrane, allowing influx of Ca^{2+} from the extracellular fluid. These intracellular Ca^{2+}stores can interact with channel-mediated Ca^{2+} entry to give rise to locally confined Ca^{2+} increases, or responses such as Ca^{2+} spikes or waves. In excitable cells such as nerve and muscle cells, Ca^{2+} influx occurs primarily through VOCs which can be regulated by both receptor mediated and intracellular messenger systems. VOCs have also been described in astrocytes (MacVicar, 1984). Second messenger operated Ca^{2+} channels (SMOCs) also exist and are activated by some intracellular second messengers, the Ca^{2+} activated K^+ and Cl^- channels in salivary acinar cells are examples of these channels (Peterson, 1986).

Ca^{2+} channels can be phosphorylated by many PKs, including CaMKs, PKA and PKC (Mackrill, 1999). For example, the amino acid sequence of the L-type Ca^{2+} channel contains several potential sites for phosphorylation by PKA and PKC. An effect of phosphorylation of the L-type Ca^{2+} channel is to convert a channel which is not functional to one which can be activated by depolarization or to increase the time that a functional channel is in the open state (Davere et al., 2000). The effects of such phosphorylation depend on the channel subtype under study, the PK, and other signalling pathways that may be active. In addition to Ca^{2+}, many ROCs admit other divalent cations. Knowledge of ion selectivity and the action of blocking ions provides a potential means of defining different ROC types. These ions may block Ca^{2+} inflow by binding tightly to Ca^{2+} binding sites in the channel pore and thereby occluding Ca^{2+} entry or in the case of Mn^{2+}, by moving through the channel at a substantially lower rate than Ca^{2+}(Hallam et al., 1989).

In an attempt to elucidate the mechanism underlying the influx of extracellular Ca^{2+} necessary to promote [^{3}H]-IP accumulation, the effects of organic and inorganic Ca^{2+} entry blockers were examined. Results show that nifedipine was the least effective in the sense that a high concentration (30μM) of the drug was required to inhibit the NA response, and then only at 50%. Wilson and Minneman (1990b) also found that nifedipine had no effect on the NA response up to a concentration of 10μM. Walz and Wilson (1986) additionally found that Ca^{2+} influx was not sensitive to the organic

nifedipine but that Cd^{2+} and Co^{2+} blocked the flux in millimolar concentrations. This suggests Ca^{2+} entry into glial cells operates through a different Ca^{2+} channel. Nifedipine has been shown to bind to VOCs, in particular the L-type channel (Triggle, 1990). Davare et al (2000) recently implicated PKA phosphorylation of the L-type Ca^{2+} channel through recruitment via an AKAP. It is likely therefore, that a PP may be associated with the channel to effectively balance phosphorylation by channel-bound PKA. The authors further reported that dephosphorylation of this site is mediated by PP2A and conclude therefore that PP2A and PKA determine the phosphorylation level of serine /threonine residues and thereby channel activity.

Co^{2+}, Ni^{2+} , Mn^{2+} and Cd^{2+} are commonly used as Ca^{2+} channel blockers. The divalent cations inhibited NA-stimulated [^{3}H]-IP accumulation at high concentrations, with Cd^{2+} producing approximately 70% inhibition at a concentration of 100μM. This is similar to that of Wilson and Minneman (1990a) who found that addition of 1mM Cd^{2+} blocked the NA response by 56%. Other studies, such as that of Parekh et al. (1993), also found the Ca^{2+} influx pathway to be much more sensitive to Cd^{2+} than other divalent ions. It is noteworthy to mention that these cation channel blockers were ineffective at lower concentrations and only managed to overcome competition with Ca^{2+} at higher concentrations since elevated concentrations obstruct the passage of other ions such as Ca^{2+}. Considered collectively, results suggest that Ca^{2+} entry in glial cells occurs through a mechanism other than VOCs and is more likely to act through SOCs or some Ca^{2+}

release activated channel. Fasolato et al. (1994) propose that Ca^{2+} influx in glial cells occurs through SOCs since they observed an increase of cytosolic free Ca^{2+} as a consequence of Ca^{2+} mobilisation from stores and influx through the plasma membrane. Indeed Mn^{2+}, which produced a 50% inhibition here, is a particularly good marker for SOCs as it has been shown to enter these channels quite readily (Lui and Gylfe, 1997).

$^{45}Ca^{2+}$ is a useful tracer for monitoring Ca^{2+} fluxes in cultured cells. A time course using this tracer showed that $^{45}Ca^{2+}$ uptake was extremely rapid in both control and NA-stimulated cultures and had reached a steady plateau by about 2 min. The rate of NA-stimulated $^{45}Ca^{2+}$ uptake was higher than in control cultures as was the maximal uptake. However, after an incubation period of about 5 min both cultures had reached equivalent levels of $^{45}Ca^{2+}$ uptake. These results are comparable to those of Walz and Wilson (1986) who reported rapid Ca^{2+} uptake for both agonist stimulated and control cultures which was also blocked by Cd^{2+}. In an experiment where A23187 was used as a positive control, $^{45}Ca^{2+}$ uptake increased by 49% over basal values. These studies demonstrate that agonist stimulation causes a rapid Ca^{2+} influx into glial cell cultures which substantiates earlier evidence by Tsujimoto (1989) that α_{1A}-adrenoceptors might control the influx of extracellular Ca^{2+}.

Earlier work presented here showed that many agents are able to modify NA-evoked phosphoinositide metabolism including OA and PMA which inhibited the effects of NA.

As the agonist response was modified by these drugs, the question arose as to whether their addition would affect $^{45}Ca^{2+}$ uptake in these cultures and perhaps affect the regulation of Ca^{2+} stores. NA stimulated $^{45}Ca^{2+}$ uptake by 78% over controls whereas the α_{1A} antagonist RS17053 reversed this response by 83%. Although a complete inhibition was not observed these results consolidate previous findings that NA primarily stimulates α_{1A}-adrenoceptors and that activation incites $^{45}Ca^{2+}$ uptake into glial cell cultures. Furthermore, there is still almost 20% of the response which was not inhibited by the α_{1A} antagonist, and therefore not dependent upon extracellular Ca^{2+}, implying that other adrenoceptor subtypes are involved in mediating $^{45}Ca^{2+}$ uptake into glial cells. Previous experiments did show an approximate 50% inhibition of NA-evoked [^{3}H]-IP accumulation with the α_{1B}-adrenoceptor antagonist at higher concentrations (IC_{50} 6.3μM) though this was 100-fold less potent than the α_{1A}-adrenoceptor antagonist RS17053. Hence it seems feasible that NA evokes $^{45}Ca^{2+}$ uptake through a combination of both α_{1A} and α_{1B}-adrenoceptors. Whether NA evoked Ca^{2+} release through α_1-adrenoceptors subtypes in the same proportion as in Ca^{2+} uptake was also investigated in these cultures.

Receptor-mediated generation of IP_3 initiates Ca^{2+} release from intracellular stores and the subsequent activation of store-operated Ca^{2+} influx which serves to refill the internal Ca^{2+} stores (Putney, 1990). Because the release of Ca^{2+} via the IP_3 receptor (IP_3R) is sensitive to both Ca^{2+} and IP_3, the released Ca^{2+} initiates further release of Ca^{2+} called Ca^{2+}-induced Ca^{2+} release. IP_3 is metabolised within seconds by 5-phosphatase and 3-

kinase, yielding IP_2 and IP_4, respectively. Some studies have suggested that IP_4 controls Ca^{2+} influx in combination with IP_3 (Irvine, 1992). More recently, Hermosura et al. (2000) found that IP_4 inhibited IP_3 metabolism in mast cells through its inhibition of IP_3 5-phosphatase thereby facilitating store-operated Ca^{2+} influx. IP_4 was found to act as an antagonist at the IP_3R inhibiting IP_3-mediated Ca^{2+} mobilisation if its levels were 10-fold that of IP_3 which could theoretically aid Ca^{2+} pools to reload. Ca^{2+} influx in response to internal Ca^{2+} store depletion can, however, occur through other mechanisms, for example, capacitative Ca^{2+} entry (Putney,1990). In this scheme, the ER Ca^{2+} stores act like a capacitor in that they inhibit Ca^{2+} entry when they are full but as they discharge they begin to open Ca^{2+} release activated Ca^{2+} channels (CRACs). The mechanism by which Ca^{2+} stores modulate CRACs in the plasma membrane is unclear. One suggestion is that the empty Ca^{2+} stores release a messenger, Ca^{2+} influx factor (CIF), which diffuses to the membrane and opens CRACs (Clapham, 1993; Jaconi et al., 1997). An alternative model, known as conformational coupling, suggests that IP_3Rs can sense the Ca^{2+} content of the ER stores and can transmit this information via a direct protein-protein interaction to the channels in the plasma membrane (Sharp et al, 1999). Once Ca^{2+} has carried out its signalling functions, it is rapidly removed from the cytoplasm by various pumps and exchangers.

CIF, as a diffusible messenger for the activation of capacitative Ca^{2+} entry pathways, has been studied in xenopus oocytes where pretreatment with thapsigargin depletes Ca^{2+}

stores. It was found that at low levels this factor can sensitise Ca^{2+} entry (Thomas et al., 1996) and that OA treatment abolished CIF activity (Thomas and Hanley, 1995). Randriamampita and Tsien (1995) reported that CIF degradation occured in cell-free homogenates of lymphocytes and astrocytoma cells and was prevented by PP inhibitors as well as lowering of free Ca^{2+} concentrations, which could be a feedback mechanism to enhance Ca^{2+} influx when cells are depleted of Ca^{2+}. The authors reported that CIF, which is proposed to open membrane Ca^{2+} channels when intracellular Ca^{2+} stores are depleted, possess a phosphate group and the authors suggest that PPs could therefore dephosphorylate and thus alter the activity of this protein. This could explain the decrease in NA-evoked $^{45}Ca^{2+}$ uptake observed here following treatment with both OA and PMA suggesting that receptor-coupled Ca^{2+} influx is somehow regulated by phosphorylation. This differs to studies using other preparations. Marriot and Mason (1996) for example, demonstrated that inhibition of an OA sensitive PP inhibited the capacitive Ca^{2+} entry pathway in rat thymic lymphocytes.

To pursue this question further, $^{45}Ca^{2+}$ release from pre-labelled glial cultures was also examined. Results show that NA provoked a 2-fold increase over controls in $^{45}Ca^{2+}$ release which persisted for the period of challenge. These results suggest that in glial cells agonist stimulation results in a release of Ca^{2+} from internal stores. NA-stimulated $^{45}Ca^{2+}$ release was found to be potentiated 2-fold over NA values in OA-treated cells. In contrast, PMA reduced NA-simulated $^{45}Ca^{2+}$ release by approximately 50%. These results

imply that an OA sensitive PP and PKC are involved in inhibiting Ca^{2+} release. Garcia-Sainz and Torres-Padilla (1999) also showed that PMA inhibited the increase in intracellular Ca^{2+} induced by NA in fibroblasts. PKC activation induced an inhibition of IP_3 production which is attributed, at least in part, to participation of PKC in a negative-feedback control of phosphoinositide metabolism and to the desensitisation of the receptor-mediated IP_3 response. Results also show that NA-evoked $^{45}Ca^{2+}$ release was unaffected by the presence of the RS17053, but was abolished when performed in the presence of the α_{1B}-adrenoceptor antagonist AH11110A. Hence NA appears to act primarily through the α_{1B}-adrenoceptor. This is of course a confounding issue as earlier experiments have shown NA to increase [^{3}H]-IP accumulation in these cells through the activation of α_{1A}-adrenoceptors. It may be that in the case of Ca^{2+} release NA acts through a combination of both α_{1A} and α_{1B} subtypes. These results suggest that whereas Ca^{2+} influx occurs primarily through α_{1A}-adrenoceptors, Ca^{2+} release primarily occurs through α_{1B}-adrenoceptors.

Molecular cloning of IP_3R cDNAs from various sources confirm at least three isoforms: IP_3R1, IP_3R2 and IP_3R3 (Furuichi et al., 1994). New isoforms termed IP_3R4 and IP_3R5 have been indicated to belong to IP_3R2 class (Parys et al., 1996). IP_3R1 is the isoform most highly expressed in brain although other forms are present (Sudhof et al., 1991). IP_3R2 has been detected in glia while little IP_3R3 has been found (Yamamoto-Hino et al., 1995; Sharp et al., 1999). Studies have shown that IP_3R functions are finely regulated by

phosphorylation via tyrosine kinases, PKA, PKC and CaMK II (Furuichi et al., 1994; Martini et al., 1994). Cameron et al. (1995) reported that these receptors can also be dephosphorylated by PP2B and FKBP (an FK506 binding protein). IP_3R1 has been reported to contain one or more consensus sites for PKC phosphorylation and also serves as a substrate for this kinase in vitro (Ferris et al., 1991). Furthermore, phosphorylation of this channel protein by PKC, PKA and CaMKII has been shown to be additive occurring at three distinct serine residues. Quinton and Dean (1992) substantiated this report by demonstrating that added PKA directly phosphorylated the IP_3R and still observed phosphorylation of the receptor in its absence. Phosphorylation by PKC and CaMKII provides means whereby Ca^{2+} and DAG, formed during inositol phospholipid turnover, may regulate IP_3R physiology. Willems et al. (1989) demonstrated that phorbol ester treatment caused a reduction in the maximal response of IP_3, therefore affecting its ability to release Ca^{2+} from these stores. In brain microsomes, cAMP dependent phosphorylation leads to a 10-fold reduction in the affinity for IP_3 (Supattapone et al., 1988). A change in the sensitivity of the Ca^{2+} release process to IP_3 may therefore be of a regulatory and physiological significance in intact cells. Communi et al (1999) reported that IP_3 3-kinase is present in astrocytes, is sensitive to Ca^{2+} and is activated by PKC. Thus, regulation of IP_3 3-kinase may be a critical component for Ca^{2+} controlled IP_3 and also IP_4 levels and signalling actions.

4.6 Concluding Remarks

Mounting evidence suggests that the physiological function of the various subtypes of adrenergic receptors is controlled by phosphorylation/dephosphorylation reactions. It seems unlikely that this phenomenon will be limited simply to the adrenergic receptors, since these receptors share transmembrane signalling pathways with a host of other plasma membrane receptors. Different types of PKs appear to be involved. On the one hand, phosphorylation reactions may operate in a classical feedback regulatory sense. Thus, PKC appears to be able to feedback-regulate the function of α_1-adrenergic receptors by phosphorylation. There may also be "cross talk" between the systems. Consequently, PKC, when stimulated by phorbol esters, is able to phosphorylate and desensitise the β-adrenergic receptors. These are examples of one transmembrane signalling system regulating the function of another. Most tissues contain a mixed population of α_{1A} and α_{1B} subtypes so it is possible that there are cooperative effects between receptor subtypes in signal transduction.

In this study we propose that α_{1A}-adrenergic receptors are coupled to phosphoinositide hydrolysis in glia. The study here used a series of PP and PK inhibitors as a means to investigate the role of phosphorylation in agonist-stimulated [^{3}H]-IP metabolism and $^{45}Ca^{2+}$ fluxes in cultured glia. Considered collectively, the results presented here give an insight into the complex nature of the involvement of PPs and PKs to a particular site.

The mechanism underlying the receptor-regulated phosphorylation appears to involve PKC and a PP2A-like PP. Unfortunately, the lack of selective inhibitors for particular PKC isoforms and the existence of OA sensitive PPs, other than PP1 and PP2A, prevents the identification of particular isoforms. Highly specific receptor PKs and PPs, which can phosphorylate or dephosphorylate only the agonist-occupied form of a receptor, represent a potentially elegant mechanism for controlling the function of receptors in a fashion which is linked to their physiological stimulation. How widespread such PKs are, and the actual roles which they play in regulating receptor function, remain to be determined. Also, all of the physiological targets of OA in vivo have not been completely characterised. There may be more PPs that are inhibited by OA or there may be other types of enzymes that are affected by the toxin.

Targeting of PKs and PPs to specific protein residues can alter receptor regulation and thereby specific cellular responses. Protein phosphorylation is a primary means of mediating signal transduction events that control cellular processes. Accordingly, the activities of PKs and PPs are highly regulated. One level of regulation is that the subcellular distribution of several PKs and PPs is restricted by association with targeting proteins or subunits. This mechanism promotes rapid and preferential modulation of specific targets within a defined microenvironment in response to diffusible second messengers. These AKAPs function as scaffolds for a wide range of protein-protein interactions and regulate receptor function in processes such as desensitisation, re-

sensitisation, and sequestration. 36 unique AKAPs have been identified, each of which contains a conserved amphipathic helix responsible for AKAP association with cellular structures (Scott, 1997). Results discussed here suggest a novel model for reversing phosphorylation in which the opposing PK and PP actions are co-localised in signal transduction complex by association with a common anchor protein. Interference in the phosphorylation mechanism, particularly in the way PPs and PKs are targeted through specific AKAPs, could lead to novel therapeutic designs for the control or treatment of CNS disorders.

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