THE
PROTEIN KINASE C
OF GLIA

by

JOHN ANTHONY MURPHY, BSc.

A thesis submitted in accordance

with the requirements for the degree of

Doctor of Philosophy

Department of Biology

University of York

October 1989

CONTENTS

To

Bill and Bridget

- wise parents.

Acknowledgements

Special thanks are due to:

Drs. Martin Rumsby and Tony Suckling for their help and advice throughout this project;

Rebecca Sayner and Jill Chapman for their technical advice concerning tissue culture work;

Dr. Azadeh Freeman for loaning me an hydroxylapatite column and Barbara Durack for her technical expertise in the use of FPLC;

Peggy Innes and Mags Wetherill for the many hours spent producing this thesis in its typed form;

Sarah for her constant help and encouragement, and for producing many of the figures;

Mike Snelling and Linda Jenkinson for their advice and help in all matters concerning the use of laboratory animals;

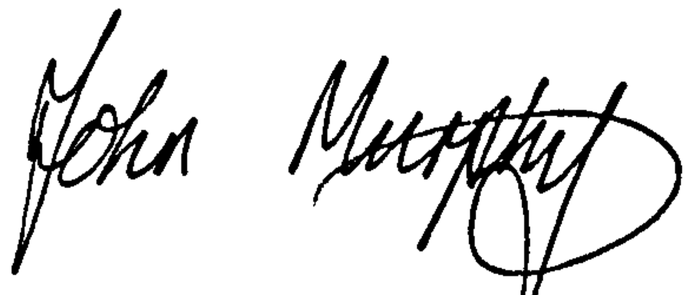
The Wellcome Trust for their generous financial support.

Finally, I should like to thank the many members of the department, past and present, who have contributed to making my three years in York so enjoyable.

Publications arising from this work are inserted in the inside back cover.

DECLARATION

I hereby declare that the work presented in this thesis is the result of my own investigations, and has not been accepted in any previous application for a degree.



John A. Murphy

ABSTRACT

The research work discussed in this thesis concerns the enzyme protein kinase C (PKC) and its biochemistry within the glial compartment of the CNS. Cells of the glial compartment (type-1 and type-2 astrocytes, oligodendrocytes and O-2A progenitor cells) have been examined and their PKC activities measured. An assay for PKC has been developed and the activity of cultured glial cells assayed both in 100,000g cytosolic (supernatant) and particulate (membrane-bound) fractions.

Glial cell supernatants contain an inhibitory factor which is removed by DEAE-cellulose (DE-52) column chromatography. This inhibitor has a phosphatase activity and does not inhibit PKC per se but dephosphorylates the type III-S histone phosphorylated by glial PKC. Thus the phosphatase is an inhibitor of the PKC assay; it is not regulated by either magnesium or manganese but can be inhibited by sodium fluoride.

The specific activity of PKC in glia has been assessed using primary culture, subculture, and complement-mediated cytotoxicity methods. All glial cell types were shown to contain PKC activity. An hydroxylapatite column method was developed to resolve α, β and γ subspecies of PKC, but the glial cultures examined did not contain either of these three subspecies.

In keeping with the characteristics of the enzyme (i.e. calcium- and phosphatidylserine-dependent), phosphatidylserine was the most effective lipid for glial PKC activation; other phospholipids and arachidonate activated PKC less effectively.

Phorbol ester treatment of cultures led to a translocation of PKC from cytosol to membrane. A similar effect was seen with platelet-derived growth factor. Analysis of cell proliferation and morphology following treatment with phorbol ester, PDGF or calcium ionophore

implicated glial PKC in activation of DNA synthesis with a resulting massive cell proliferation. TPA-treated cells appeared to lack a regulatory factor to control cell growth which was present in PDGF-treated cells. Such regulation is probably cAMP-mediated, but may also reflect activation of tyrosine kinase.

Possible roles of PKC in glial cell function are discussed.

ABBREVIATIONS

A23187	calcium ionophore
A2B5	ganglioside Gq
AA	arachidonic acid
As	arsenic
ATP	adenosine trisphosphate
Ba	barium
cAMP	cyclic adenosine monophosphate
Cd	cadmium
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
cpm	counts per minute
CNTF	ciliary neurotrophic factor
Cu	copper
DABCO	1,4-diazobicyclo-(2,2,2)-octane
DAG	diacylglycerol
DMEM	Dulbecco's modified Eagle's Medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol bis (β-aminoethylether) N,N,N',N'-tetraacetic acid
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
GC	galactocerebroside
GFAP	glial fibrillary acidic protein
H-7	isoquinolinesulphonamide
HAM	Ham's F-12 medium
HBSS	Hank's balanced salt solution

Hg	mercury
i.d.	internal diameter
IgG	immunoglobulin G
IP_3	inositol 1,4,5-trisphosphate
IP_4	inositol 1,3,4,5-tetrakisphosphate
kDa	kilodalton
MBP	myelin basic protein
Mn	manganese
mRNA	messenger ribonucleic acid
NaF	sodium fluoride
NF	neurofilament protein
Ni	nickel
O-2A	oligodendrocyte-type-2 astrocyte progenitor cell
OLDEM	oligodendrocyte-defined medium
PA	phosphatidic acid
Pb	lead
PBS	phosphate buffered saline
PC	phosphatidylcholine
PDGF	platelet-derived growth factor
PI	phosphoinositide
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A (cAMP-dependent protein kinase)
PKC	protein kinase C
PKM	protein kinase M
PLA_1/PLA_2	phospholipases A_1 and A_2
PLC	phospholipase C
PLD	phsopholipase D
PMSF	phenylmethylsulphonylfluoride
PNS	peripheral nervous system
PS	phosphatidylserine

RNA	ribonucleic acid
rpm	revolutions per minute
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TCA	tricholoroacetic acid
TEMED	tetramethylethylenediamine
TPA	12-O-tetradecanoylphorbol-13-acetate
v/v	volume/volume
w/v	weight/volume
Zn	zinc

1. INTRODUCTION

1.1. THE CENTRAL NERVOUS SYSTEM

The brain is composed of specialised cells, part of whose function is to receive sensory stimuli and to transmit them to effector organs, thus regulating the function of the individual as a whole. To carry out these tasks of determining the many aspects of behaviour the central nervous system (CNS) possesses an immense network of interneuronal connections. A striking feature of the nervous system is the high degree of precision with which neurones are connected to each other and to different peripheral tissues. The ordered nature of the connections formed during development is a necessary prerequisite for all the integrative mechanisms the brain contains.

1.1.a. Structure and functions of cells of the CNS

Cells of the CNS are of two main types, impulse-transmitting neurones surrounded by satellite cells. The latter are divided according to anatomical criteria into neuroglial cells in the brain and Schwann cells in the periphery. Neuroglial cells make up almost one-half the volume of the brain, and, from counts of cell nuclei, have been estimated to outnumber neurones by at least 10:1. They comprise astrocytes, oligodendrocytes and microglia.

Neuroglia were first described in 1846 by Virchow who later named them (literal translation = "nerve-glue"). In the subsequent 100 years although neurones were investigated extensively the study of glial cells was practically non-existent. Even today, though the importance of glial cells is stressed frequently, relatively little is known of their full functions.

The cell types described below are shown in figure 1.1.1.

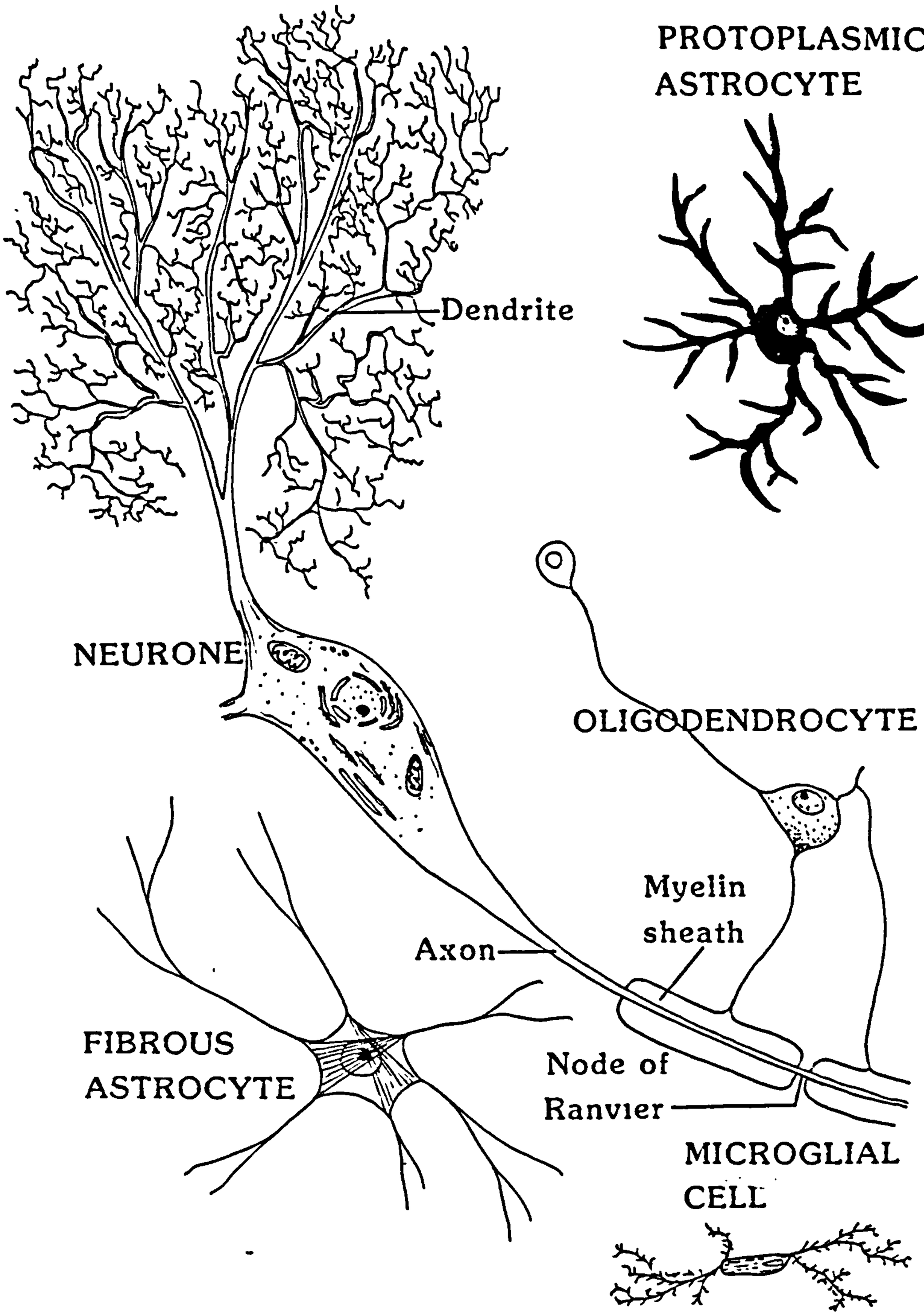


Figure 1.1.1. Glial cells of the central nervous system.

(i) Neurones

Neuronal morphology varies greatly from the simple bipolar cell of the retina to the highly-branched Purkinje cell of the cerebellum. Basically they are typically eukaroytic, having a nucleus enclosing a distinct nucleolus situated within a bulbous cell body, or soma, from which dendrites project. The latter receive information transmitted along the axons, long membrane-enclosed cytoplasmic threads of the neurones which branch at one end. The main function of neurones is the transmission of impulses, over relatively long distances, along the axon surface to facilitate co-ordination within the organism.

In the vertebrate nervous system the larger nerve fibres are myelinated. The myelin wraps itself around the axons during development such that, in terms of dimensions, the myelin occupies 20-40% of the overall nerve fibre diameter. This myelin wrapping, the myelin sheath, increases membrane resistance and decreases membrane capacitance, thus spreading a signal further over a shorter time period. The sheath is interrupted periodically by nodes of Ranvier, exposing patches of axonal membrane. The effect of the myelin sheath is to restrict current flow largely to the node, as ions cannot flow in or out of the high-resistance internodal region and the internodal capacitative currents are very small as well. Therefore, only the restricted portion of the axon membrane at the nodes of Ranvier become involved in impulse propogation and the impulse jumps from node to node. This impulse propogation is known as saltatory conduction.

An additional consequence of myelination is that fewer sodium and potassium ions enter and leave the axon during impulse propogation, as regenerative activity is restricted to the nodes. Hence less metabolic energy is required by the sodium-potassium exchange pump to restore the intracellular concentrations to their resting levels. Myelinated axons not only conduct more rapidly that unmyelinated ones but are also

capable of firing at higher frequencies for more prolonged periods of time.

(ii) Astrocytes

Two types of astrocytes have been identified in the CNS, protoplasmic or type-1 astrocytes and fibrous or type-2 astrocytes (Miller & Raff, 1984). Protoplasmic astrocytes are most commonly associated with the grey matter of the CNS, and are typically stellate with branched processes. Fibrous astrocytes are similar in shape but with finer processes, and are more commonly associated with the white matter of the CNS.

Although a variety of functions have been ascribed to astrocytes, their role is not fully clear. One established function of type-1 astrocytes is a response to injury within the CNS resulting in the formation of "scar" tissue, a process called reactive gliosis (Raff et al., 1987). This cell type is also important in the development of oligodendrocytes and type-2 astrocytes (see section 1.1.b). The latter have processes which surround the nodes of Ranvier in white matter where, in principle, they could help to stabilise local extracellular ion concentrations in the face of repeated nerve impulses (Raff et al., 1987).

Hertz (1981) has implicated astrocytes in potassium homeostasis and metabolism of gamma-aminobutyric acid (GABA) and glutamate, arguing that these processes occur in response to release of the substances by neurones, and that astrocytes and neurones may interact in a number of ways. Astrocytes have also been purported to be involved in the phagocytosis and removal of degenerating myelin (Nathaniel & Nathaniel, 1981) and in the carbohydrate metabolism of the nervous system (Haymaker, 1969). Type-1 astrocytes are also known to attach processes (so-called 'end feet') to the basement membrane of endothelial cells of

the CNS allowing such cells to form the tight low-permeable junctions characteristic of the blood-brain barrier (Bradbury, 1979).

(iii) Oligodendrocytes

First described before the turn of the century, it was not until the famous histological work of del Rio Hortega in 1921 that the "cell with few branches" was so-named the oligodendrocyte. This cell type exists in both white matter and grey matter where del Rio Hortega applied the terms interfasicular and perinueronal respectively.

It is the interfasicular oligodendrocytes which are responsible for the huge burst of membrane synthesis which occurs at myelination and leads to the formation of myelin sheaths around axons in the CNS. The same oligodendrocytes probably maintain the integrity of the myelin sheath throughout life. Perineuronal oligodendrocytes in the grey matter aggregate closely around neuronal cell bodies and may be important in remyelination following axonal damage.

A single oligodendrocyte may be responsible for the myelination of up to fifty nerve fibres (Noble & Murray, 1984). Myelin sheath formation generally occurs around axons close or adjacent to the parent oligodendrocyte, although the distances between the two can be over 30µm (Sternberger et al., 1978). Such formation is dependent upon both the maturity of the oligodendrocytes and the presence of axons (Skoff et al., 1976), but how myelination is initiated and proceeds is unclear. In rats, myelin begins to appear in the optic nerve at postnatal day 6-7, rapidly increasing up to postnatal day 28, by which time 80% of the axons are myelinated (Tennekoon et al., 1980). Once established, the oligodendrocyte-myelin-axon relationship is maintained throughout the lifetime of the organism unless affected by damage or disease.

(iv) Microglia

These cells comprise approximately 4-5% of white matter and 18% of grey matter (Fugita, 1980), and have been implicated to function in a number of diverse processes. These include regulation of astrocyte differentiation and mediation of immunological responses (Fujita, 1980). They may also function as antigen-presenting cells and represent the effector cell responsible for the recruitment of lymphocytes to the brain, resulting in an inflammatory response (Streit et al., 1988). Microglia have also been purported to be one of the CNS targets of the acquired immune deficiency syndrome (AIDS) virus (Gartner et al., 1986).

1.1.b. Origin and development of the neuroglia

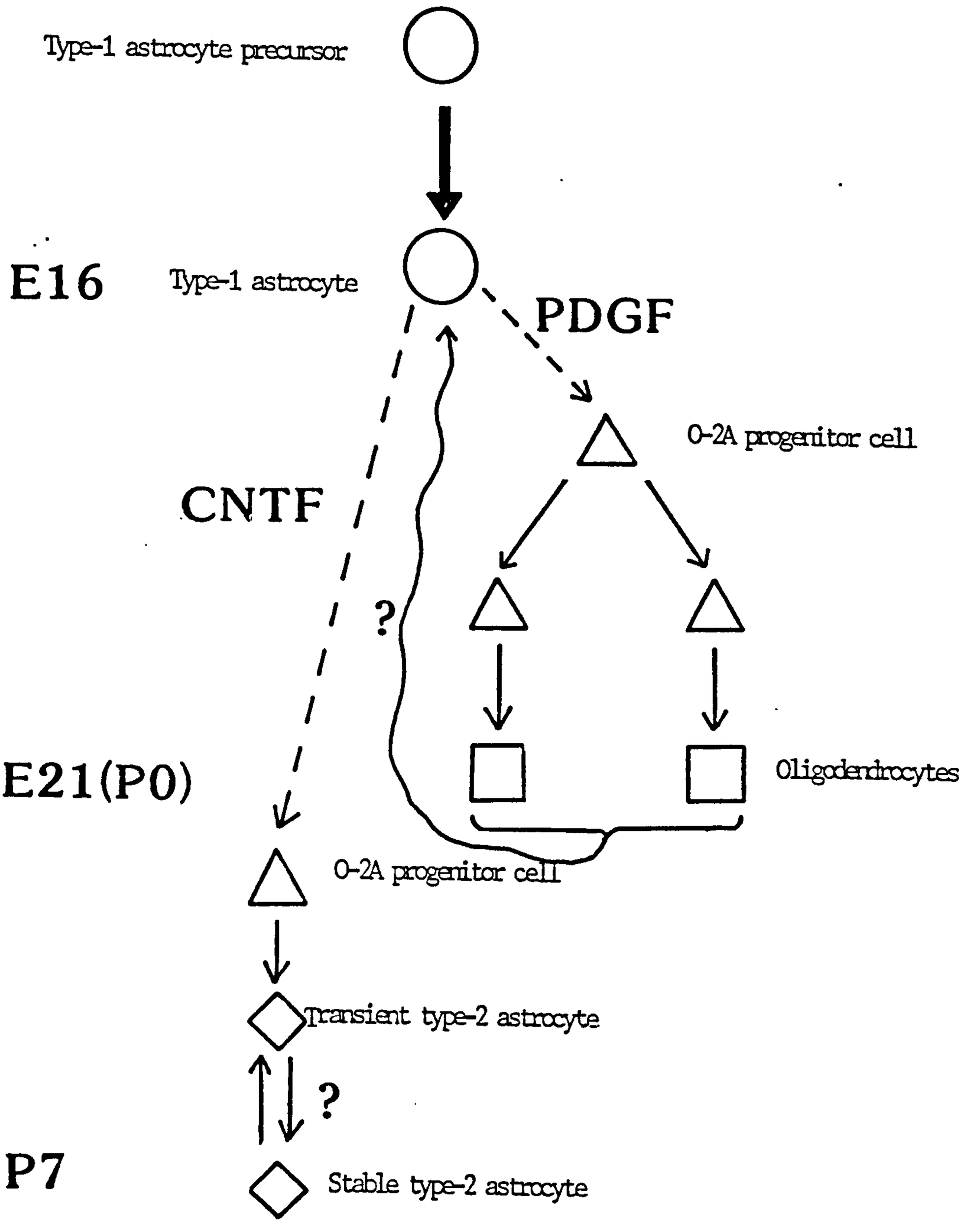
Most of the neurones and macroglial cells in the vertebrate CNS are thought to derive from the neuroepithelial cells that form the neural tube. However, the actual lineage relationships between the different constituent cells of the CNS, the factors determining development into a particular type of neurone or glial cell, and how such decisions are timed are still under investigation. The intense interest in this subject, notably over the last decade, has led to a reassessment of ideas concerning glial cell histogenesis. Much of the recent work has been conducted on short-term cultures of cells taken from developing rat optic nerve, one of the simplest parts of the CNS, notably by Raff and colleagues. This cell system contains only astrocytes and oligodendrocytes, with no neurones present, so allowing investigation of glial cell lineage relationships without any possible interference from other cell types.

In vitro studies using rat optic nerve suggest that the three types of macroglial cells arise by two distinct lineages; oligodendrocytes and type-2 astrocytes develop from a common bipotential progenitor cell (O-2A cell; Raff et al., 1983) whereas type-1

astrocytes develop from a different precursor cell (Raff et al., 1984). See figure 1.1.2. In the rat, type-1 astrocytes first appear at embryonic day 16, oligodendrocytes on the day of birth, and type-2 astrocytes between postnatal days 7 and 10 (Miller et al., 1985). However, although O-2A progenitor cells continue to divide and differentiate, some persist into adulthood where their function is not clearly understood (ffrench-Constant & Raff, 1986; Wolswijk & Noble, 1989).

This strict developmental sequence is disrupted when dissociated optic nerve cells are cultured in a defined medium; all the O-2A progenitor cells stop dividing and differentiate into oligodendrocytes within 48 h. regardless of the age of the animal from which they were derived (Raff et al., 1985). Type-2 astrocytes do not develop in these cultures unless an inducing factor, such as that contained in foetal calf serum, is present (Raff et al., 1983). Correct timing of oligodendrocyte development can be restored in culture by growing embryonic rat optic nerve cells in foetal calf serum (0.5% or less) on a monolayer of type-1 astrocytes, or in astrocyte-conditioned medium (Raff et al., 1985). Thus type-1 astrocytes contain (a) mitogen(s) that can keep O-2A progenitor cells in division, preventing their premature differentiation.

Recent work has shown platelet-derived growth factor (PDGF) to be strongly mitogenic for O-2A progenitor cells in vitro, that cultured type-1 astrocytes secrete PDGF dimers, and that mitogenic activity in astrocyte-conditioned medium is neutralised by anti-PDGF immunoglobulin (Noble et al., 1988; Richardson et al., 1988). Together with other findings, these observations suggest that PDGF is secreted by type-1 astrocytes and so plays a key role in controlling the proliferation and differentiation of O-2A progenitor cells in the developing rat optic nerve (fig. 1.1.2).



(Adapted from Raff, 1989)

Figure 1.1.2. Glial cell lineage in the central nervous system.

Two studies which indicate that the daughter cells of an O-2A progenitor cell both go through the same number of divisions before differentiating into oligodendrocytes have led to contrasting hypotheses. Temple and Raff (1986) suggested that the timing of oligodendrocyte differentiation is controlled by a cell-intrinsic clock that counts cell divisions up to a fixed limit; when this division limit is reached, the cell then automatically differentiates. The second hypothesis contrasts with this postulated permissive role of PDGF in oligodendrocyte differentiation and suggests that PDGF may drive not only proliferation, but also, independently, some other kind of timed cellular process which, when terminated, would trigger differentiation and arrest of division (Raff et al., 1988). A simple explanation for the loss of responsiveness of the O-2A progenitor cell to PDGF, characterised by the cell type dropping out of division and differentiating into an oligodendrocyte, would be the loss of PDGF-sensitive receptors from the cell surface of the progenitor cell. Recent work by Hart et al. (1980), however, has shown that the loss of mitotic responsiveness cannot be explained in this way.

The type-1 astrocyte secretes at least one other growth factor which has a profound effect on cell differentiation. Ciliary neurotrophic factor (CNTF) has been shown, again in the rat optic nerve system, to induce type-2 astrocyte differentiation (Hughes et al., 1988; Lillien et al., 1988). It has been hypothesised that CNTF synthesis is initiated by a feedback signal from differentiated oligodendrocytes, generated when a sufficient number of oligodendrocytes have accumulated (Lillen et al., 1988). The signal would cause type-1 astrocytes to secrete CNTF, so driving new O-2A progenitor cells along the type-2 astrocyte pathway (fig. 1.1.2). CNTF action alone, however, is apparently insufficient to promote stable type-2 astrocyte differentiation. Instead, a transient cell form is produced which

loses its astrocyte-specific marker expression after several days, and the cell apparently becomes an oligodendrocyte (Raff, 1989). This contrasts with the phenotypically stable type-2 astrocyte induced by FCS, suggesting the requirement for (an)other factor(s) to promote the development from a transient to a stable type-2 astrocyte. It has been suggested that PDGF may again be involved here (Anderson, 1989).

Figure 1.1.2 summarises the present conclusions on glial cell differentiation in rat optic nerve.

This work has recently been questioned by McMorris (1989) who believes that oligodendrocytes are indeed induced and that the factor responsible for this is insulin growth factor. He believes that this induction follows the PDGF-driven proliferation of the progenitor cell and would replace the developmental clock proposed by Raff, and that Raff and colleagues mask the effects of the insulin growth factor by always including it in their defined media.

1.1c. Cell-specific markers for glial cell identification

Identification of cells within the CNS used to depend largely upon morphological criteria using light microscopy, a method which can be both laborious and often inaccurate. An alternative approach is to employ cell-specific markers. Each neural cell type expresses a unique pattern of extracellular and intracellular protein and lipid. By raising an antibody against the specific component of a given cell type and linking this to a readily visualised tag (for example, fluorescein, rhodamine, peroxidase, gold), microscopic analysis permits positive identification of cells, even when they occur in small numbers. Cell-specific markers are not only unique for a given cell type but may also be so for a particular developmental stage of that cell. This allows the investigation of origins and lineages of CNS cells. Some commonly used cell-specific markers for neuroglia are listed in the remainder of this section.

Glial fibrillary acidic protein (GFAP), a 51kDa protein constituent of the microfilaments of astrocytes, was initially isolated by Eng et al. (1971) from multiple sclerosis plaques, and is widely used as an intracellular astrocyte-specific marker. A second marker used to identify astrocytes is the enzyme glutamine synthetase which has been demonstrated in frozen sections of rat brain to be localised exclusively to protoplasmic and fibrous astrocytes (Norenberg and Martinez-Hernandez, 1979). S-100 protein and rat neural antigen-2 (ran-2) have also been used as putative astrocyte markers but are not specific to astrocytes alone (Kennedy, 1982).

Both cell surface and intracellular markers for oligodendrocytes have been described. Galactocerebroside (GC), the major glycolipid in myelin (Norton & Autilio, 1966), has been shown to be oligodendrocyte-specific in a variety of CNS cultures (see Raff et al., 1978, for example). Myelin proteins specific to oligodendrocytes include myelin basic protein (MBP) (Sternberger et al., 1978), a useful developmental marker as it is expressed later in the development of oligodendrocytes, myelin-associated glycoprotein (MAG) (Itoyma et al., 1980), proteolipid protein (PLP) (Agrawal et al., 1977) and myelin-oligodendrocyte protein(MOG)(Linnington et al., 1984). Other frequently used oligodendrocyte specific markers include the enzyme 2', 3'-cyclic nucleotide 3'-phosphohydrolase (CNPase) (Nishizawa et al., 1981) and antibodies 01 and 04 (Sommer & Schachner, 1981).

Despite the alleged specificity of these and other antibodies it is important to be aware of the possibilities of cross-reactivity and transient expression. For example, transient expression of GFAP has been demonstrated in developing oligodendrocytes in vitro (Ogawa et al., 1985).

A2B5, an antibody to ganglioside Gq (Kasai and Yu, 1983) has played an important role in the elucidation of glial cell lineage as its

antigen is expressed only by the O-2A progenitor cell and type-2 astrocyte (Raff et al., 1983). A marker similar to this is monoclonal antibody LB_1, which binds to GD_3 ganglioside of a glial progenitor cell type in postnatal rat cerebellar cultures (Curtis et al., 1988) similar to the O-2A progenitor cell of rat optic nerve.

1.2 SIGNAL TRANSDUCTION MECHANISMS

The regulation of different characteristic and specialised functions in cells is usually controlled by the binding of agonists to protein receptors located within the plasma membrane but accessible at the cell surface. A number of mechanisms exist by which a cell communicates with its environment, and not all are clearly understood. Information generated outside the cell arrives to effect a cellular response which involves at least three components: a receptor to recognise the hormone growth factor or neurotransmitter; an enzyme system to liberate messenger molecules into the cytoplasm; a third component which acts to couple the receptor to this messenger-generating system. The cellular response elicited as a result of the interaction of these components will vary according to the identity of the ligand binding to the cell receptor and the cell type in question, but may range from secretion to contraction to cell division.

1.2.a. Adenylate cyclase

A signal transduction system that has been extensively investigated and documented is the class of receptors that mediates its response through adenylate cyclase, causing an increased production of cyclic adenosine 3', 5'-monophosphate (cAMP). Binding of an agonist

activates one of two distinct guanosine 5'-trisphosphate-binding proteins (G-proteins) leading to either an inhibitory or stimulatory effect on adenylate cyclase activity. The adenylate cyclase serves to convert adenosine trisphosphate (ATP) to cAMP; the latter acts as an intracellular or second messenger, activating cAMP-dependent protein kinase (protein kinase A or PKA) resulting in the phosphorylation and subsequent modulation of a wide variety of enzymes and regulatory proteins within the cell.

1.2.b. The phosphoinositide pathway

It is now apparent, however, that another signal transduction system exists through which extracellular agonists exert their biological effects on cells. This is done by increasing the levels of free calcium ions available in the cytosol, so affecting a wide variety of proteins such as kinases and phosphatases either directly or indirectly. Evidence has accumulated, largely within the last fifteen years, which implicates a specific class of phospholipid, the phosphoinositides, in such a process.

Initial observations of increased turnover of inositol lipids in response to an external signal were made by Hokin and Hokin in 1953 when they incubated pancreatic cells in the presence of acetylcholine. This discovery was investigated no further until work by Michell in 1975. Since then there has been a tremendous amount of interest in this subject area with the literature expanding constantly.

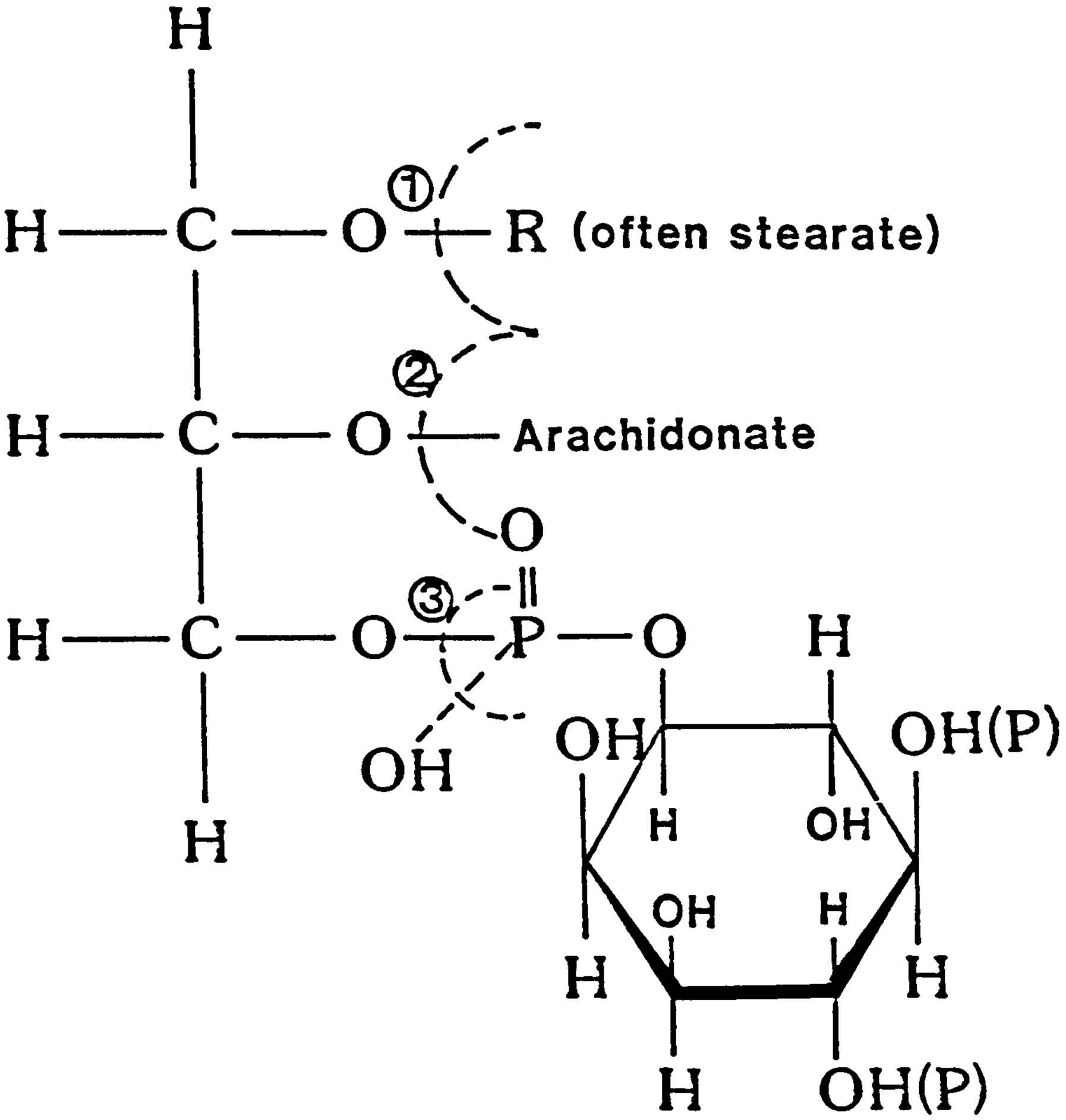
Compounds that cause a biological response via activation of the phosphoinositide (PI) pathway stimulate a PI-specific phospholipase C (PLC), a phosphodiesterase, which breaks down lipids in the plasma membrane (Berridge, 1984). Experimental evidence again implicates the involvement of a G-protein (Joseph, 1985) although it still requires complete characterisation. In contrast to receptor-linked adenylate

cyclase activation which produces a single second messenger, inositol lipid breakdown produces two functionally distinct second messengers, inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG) (Berridge, 1984). The former, hydrophilic in nature, serves to mobilise intracellular calcium ions from an IP_3-sensitive pool associated with the endoplasmic reticulum (Streb et al., 1983) whilst the hydrophobic DAG remains within the membrane and is involved in the activation of a calcium - and phospholipid-dependent protein kinase, protein kinase C (Takai et al., 1979b).

1.2.c. Inositol phospholipid metabolism

The inositol phospholipids represent only about 5-10% of total plasma membrane phospholipids but they are the most metabolically active (Williamson, 1986). The major inositol lipid species is phosphatidylinositol (see fig. 1.2.1) and between 10 and 20% of the phosphatidylinositol pool in the plasma membrane is involved in cell signalling (Martin, 1983). Each of two phosphorylated derivatives, phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP_2) comprises approximately 1% of the total inositol phospholipids.

When an agonist binds to its receptor a conformational change is induced. This activates a G-protein (Joseph, 1985) which in turn stimulates a PI-specific PLC. The latter hydrolyses PIP_2 to form the two second messengers IP_3, a cytosolic component, and DAG which remains in the plasma membrane (Berridge, 1984). Any intracellular signal molecule must be rapidly and specifically metabolised in order to terminate its activity when the stimulus is removed. This is achieved with the inositol phosphates by two separate mechanisms. The first is a simple dephosphorylation of IP_3 by a 5-phosphatase (IP_3 phosphatase) to form inositol 1,4-bisphosphate (IP_2), a compound inactive in calcium ion



(from Majerus et al., 1986)

Figure 1.2.1. Structure of phosphatidylinositol.

The polyphosphoinositides contain additional monoester phosphates in the 4 position (opposite the phosphodiester) or in both the 4 and 5 positions. The breakdown of phosphatidylinositol is initiated by phospholipase C cleavage of bond 3. The diacylglycerol can be further cleaved by lipases with bond 1 preceding bond 2.

release assay (Berridge, 1987). The IP_2 is then directly dephosphorylated to inositol via inositol-4-phosphate. The second pathway involves a 3-kinase which phosphorylates IP_3 to form inositol 1,3,4,5-tetrakisphosphate (IP_4) (Batty et al., 1985), which does not mimic IP_3 but has been suggested to work in concert with it by facilitating calcium ion entry into the cell (Irvine & Moor, 1986). The IP_4 is then dephosphorylated to inositol with inositol 1,3,4-trisphosphate, inositol 3,4-bisphosphate and inositol-3-phosphate as the major intermediates. Additional branch points exist that allow some rather more complex alternatives (for review, see Irvine et al., 1988). Contrasting with this agonist-sensitive formation of inositol phosphates is an agonist-insensitive metabolic pool of phosphates which appear to be linked to the formation of inositol 1,3,4,5,6-pentakisphosphate (IP_5) and inositol hexakisphosphate (IP_6) (Szwergold et al., 1987). IP_5 appears to be synthesised from the inositol 3,4,5,6-tetrakisphosphate precursor; what serves as the IP_6 precursor is not yet known (Downes, 1988). Vallejuo et al. (1987) have demonstrated profound effects of IP_5 and IP_6 when applied to the CNS, suggesting a possible role as neurotransmitters, but work on these two inositol polyphosphates is still in its preliminary stages.

(i) **1,4,5-trisphosphate**

A rise in cytoplasmic IP_3 due to hydrolysis of membrane-bound PIP_2 leads to a release of calcium ions from specialised regions of the endoplasmic reticulum (Berridge, 1984). The calcium will then function to regulate a variety of events including contraction, secretion and various metabolic processes. Some agonists will also trigger an influx of calcium ions across the plasma membrane, possibly via the combined actions of IP_3 and IP_4 (Irvine & Moor, 1986), the former acting to discharge the internal pool and the latter to promote an influx of

external calcium ions.

(ii) Diacylglycerol

DAG is the other immediate product of the phosphodiesteratic cleavage of phosphoinositides. It remains in the plasma membrane only transiently where it activates a calcium - and phospholipid-dependent protein kinase, protein kinase C (PKC) (Kishimoto et al., 1980). Activation of PKC leads to phosphorylation of a vast number of cellular proteins resulting in both long- and short-term effects on the activity of the cell (Nishizuka, 1986). Like IP_3, DAG can be metabolised via two separate pathways (Berridge, 1987). Phosphorylation of DAG by a DAG kinase may occur to form phosphatidic acid which is recycled to reform phosphatidylinositol, or DAG may be hydrolysed by a DAG lipase to form monoacylglycerol which is further hydrolysed to release arachidonic acid. Since the latter is the precursor for the eicosanoids, DAG may give rise to additional messengers which function as local hormones. It may also allow for a subsequent or prolonged activation of PKC as arachidonic acid has been shown to activate a subspecies of PKC (see section 1.3.k). The second messenger roles of IP_3 and DAG are summarised diagrammatically in figure 1.2.2.

A rapid transient accumulation of DAG associated with stimulated phosphoinositide turnover has been demonstrated in various cell types (for example, Bell et al., 1979; Martin, 1983; Cockcroft & Allan, 1984) where it has been derived from either phosphoinositides or phosphatidic acid. Some studies have suggested that only a part of this newly generated DAG is due to PI turnover, the remainder having arisen from other lipids (see Brown et al., 1984a). The potential of alternative pathways of phospholipid turnover in hormone signal transduction has only recently been appreciated. There is an increasing body of evidence which suggests that phosphatidylcholine (PC) is an important source of

UNIVERSITY OF YORK LIBRARY

DAG (summarised in Pelech & Vance, 1989). The phosphatidylcholine cycles for generation of second messengers are presented in figure 1.2.3.

DAG can be generated directly from PC via another PLC. Alternatively it may be generated by the action of phospholipase D (PLD) to yield phosphatidic acid (PA), followed by cleavage by phosphatidic acid phosphohydrolase (PAP) to form DAG. Indeed, this PLD route may be the preferred one (Cabot et al., 1988 a,b). PC hydrolysis by phospholipase A_2 (PLA_2) will produce lyso-PC and arachidonic acid. Lyso-PC re-enters the cycle whilst arachidonic acid is converted to the eicosanoids. Lyso-PC may be re-esterified to PC; alternatively it may be catabolised to glycerophosphocholine (GPC) and further degraded to glycerol-3-phosphate (G-3-P) and choline. G-3-P can be converted back to DAG via PA synthesis.

Much of the work investigating PC cycles has involved the use of tumour-promoting phorbol esters to activate PKC (this is discussed more fully in section 1.3.d). Hence PKC activation is leading to activation of phospholipases to produce DAG. As DAG is the second messenger which activates PKC, this is, in effect, a potential pathway for the generation of a subsequent or prolonged activation of PKC or it possibly may allow regulation of activation of PKC subspecies (see section 1.3.k). In addition, activation of PKC has been shown to enhance PC synthesis via activation of PC cytidyltransferase, the regulatory enzyme controlling the synthesis of PC at the endoplasmic reticulum (Pelech et al., 1984).

In summary, cell-specific responses to an agonist may reflect the phospholipase specificity of the G-protein with which the activated agonist receptor associates, the fatty acid and polar head group composition of the membrane phospholipids, and the particular subspecies of PKC that is expressed in the cell (Pelech & Vance, 1989).

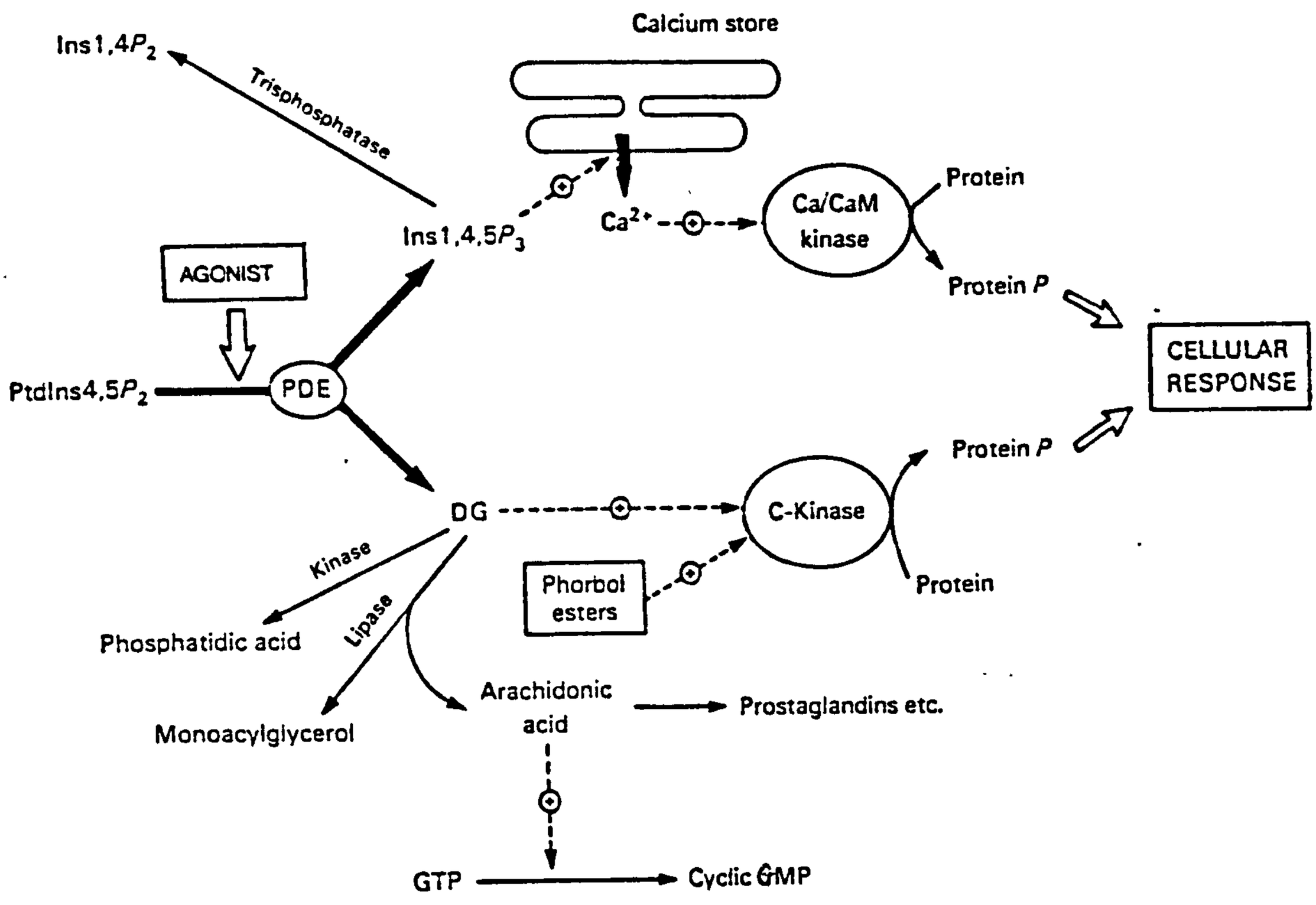
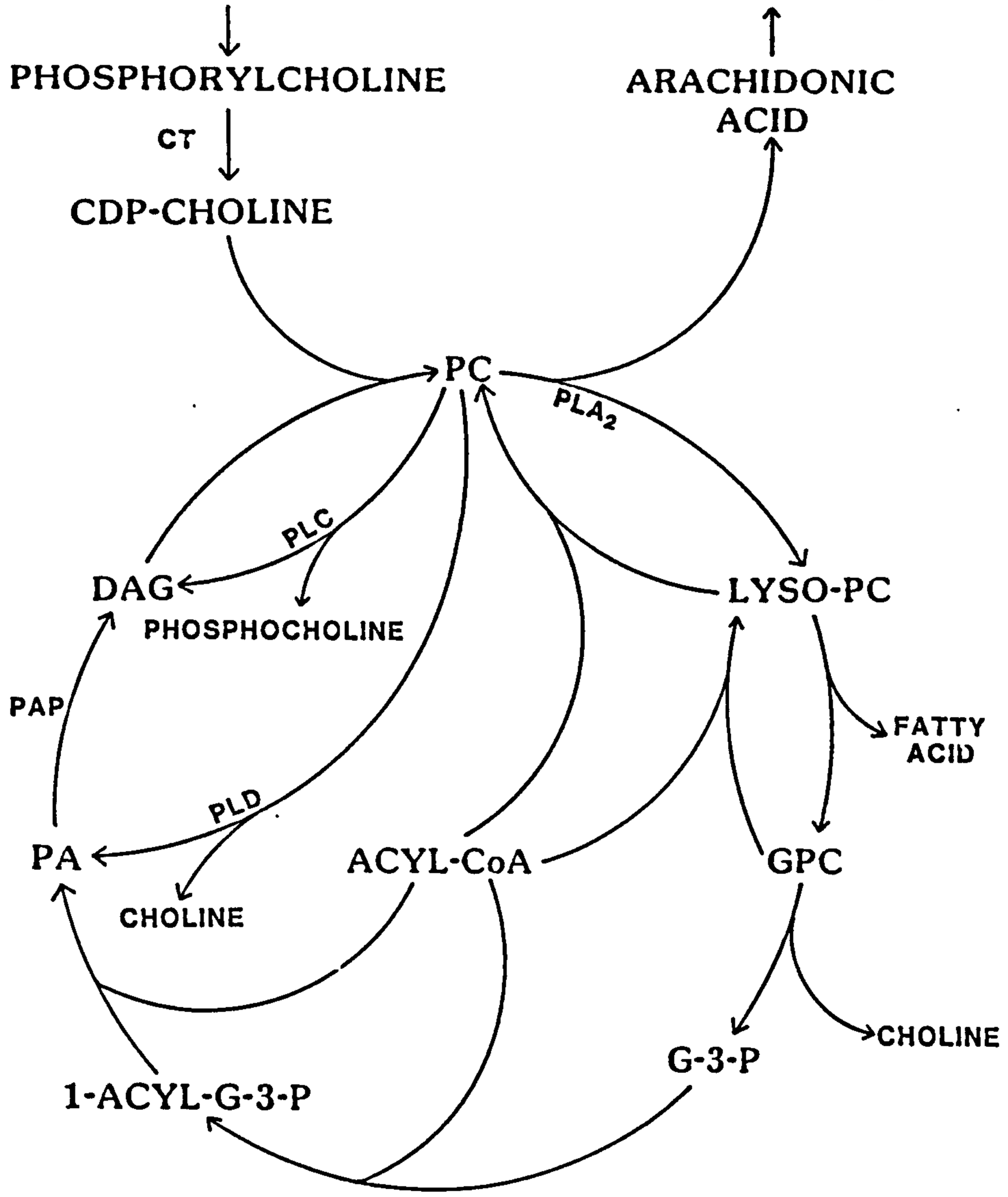


Figure 1.2.2. The second messenger role of diacylglycerol and inositol 1,4,5-trisphosphate.

(CaM = calmodulin; PDE = phosphatidylinositol-specific phospholipase C).



(from Pelech and Vance, 1989)

Figure 1.2.3. Phosphatidylcholine cycles for generation of second messenger.

Whilst the role of PC has been emphasised above, other phospholipids such as phosphatidylethanolamine may contribute towards such a signal transduction process. An interesting observation made recently (Yamada & Kanoh, 1988) concerns the possibility of the existence of multiple species of DAG kinase. Each of these could exist, it may be argued, to inactivate a DAG derived from a different phospholipid source.

1.3 PROTEIN KINASE C

The investigation of the role of protein kinases in the CNS and the identification and characterisation of their specific substrates are active areas of research which have already provided valuable intimation on the mechanisms involved in many neurophysiological processes. Although the properties of the protein kinases of the CNS are generally similar to those of the PNS, there are important differences, especially in their relative concentrations and in their cellular and subcellular distributions. In addition, the brain appears to contain a greater diversity of substrates for most protein kinases. The substrate specificities of protein kinases and protein phosphatases fall into two general categories, those with narrow substrate specificities and these which exhibit broad substrate specificities (Nairn et al., 1985). Into the former group come cyclic guanosine monophosphate (cGMP)-dependent protein kinase, calcium/calmodulin kinase I, myosin light chain kinase and phosphorylase kinase, kinases which presumably are involved in specialised aspects of neuronal function. Those which are likely to be involved in many aspects of neuronal function, and hence have broad substrate specificities, include cAMP-dependent protein kinase, calcium/calmodulin kinase II and protein kinase C.

1.3.a. Occurrence of PKC

PKC was originally isolated as its proteolytic fragment, protein kinase M (PKM), a peptide of approximately 64kDa from frozen bovine cerebellum (Takai et al., 1977). It was subsequently partially purified from the soluble fraction of fresh rat brain, and shown to be activated upon limited proteolysis by a calcium-dependent neutral protease, calpain, present in the same tissue (Inoue et al., 1977). The active protein kinase thus produced is indistinguishable in kinetic and catalytic properties from PKM purified from bovine cerebellum.

PKC is widely distributed amongst the phyla of the animal kingdom (Kuo et al., 1980) and has also been detected in plants (Elliot & Kokke, 1987). The enzyme is present in many tissues and was originally purified to near homogeneity from bovine heart (Wise et al., 1982), but is more abundant in brain, from where it is now more commonly isolated (for example, Kikkawa et al., 1982; Wolf et al., 1984). The enzyme from these preparations exhibits a single protein staining band of approximately 80kDa upon sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), behaves as a monomer upon gel permeation chromatography and has an isoelectric point of approximately 5.6 (Kikkawa et al., 1982; Wise et al., 1982).

1.3.b. Subcellular distribution of PKC

The brain is the richest source of PKC, with a large proportion associated with synaptic membranes. This contrasts with other tissues where the enzyme is present mainly in the soluble fraction as an inactive form (Kikkawa et al., 1982). Upon cell stimulation, the enzyme is apparently translocated to the membrane (Kraft & Anderson, 1983). Precise intracellular localisation is difficult because the enzyme is extracted for assay in the presence of calcium chelators such as ethyleneglycol bis (β-aminoethylether) N, N, N', N'-tetraacetic acid

(EGTA) to prevent its calcium-dependent proteolysis. Hence, any contribution that calcium may give to membrane binding is negated. The enzyme may in fact be loosely bound to the membrane but then dissociates from it upon removal of calcium (Kikkawa & Nishizuka, 1986). The subcellular localisation of PKC is also sensitive to the growth state of the cell; in proliferating cells there is a greater proportion of membrane-bound enzyme than in resting cells which presumably reflects activation of PKC during the process of mitosis (Adamo et al., 1986).

Recent attempts at enzyme localisation have used immunocytochemical analysis, although this has not given any truly definitive results. Shoji et al. (1986) and Kikkawa et al. (1986) were two of many authors who employed this technique, with the latter investigation noting very poor or zero immunoreactivity in the nucleus. Evidence is accruing, however, that PKC may indeed have a nuclear localisation and that this presence is endogenous and not due to a translation event (see Masmoudi et al., 1989). This could easily explain the phosphorylation of nuclear proteins such as topoisomerase II by PKC (MacFarlane, 1986).

An interesting theory concerning PKC localisation has recently been advanced by Bazzi & Nelsestuen (1988). They discovered two populations of membrane-bound PKC; one population was dissociated by calcium chelation but the other was not. The second population appeared to be inserted into the membrane and its activity was independent of calcium. The authors proposed that activation of PKC may lead to insertion into the membrane. Hence, when the second messengers have been degraded the inserted PKC remains active until degraded by the protein turnover processes. This, they believe, may be important for long-term effects within the cell.

1.3.c. Biochemical and physiological activation of PKC

The activation of PKC normally depends upon calcium as well as phospholipids and DAG. However, DAG dramatically increases the affinity of the enzyme for calcium and thus allows activation without a net increase in intracellular calcium concentration (Kishimoto et al., 1980). Obviously the enzyme can also be activated effectively by the simultaneous increase in concentrations of calcium and DAG, but PKC activation, though biochemically dependent on calcium, is physiologically independent of calcium concentration.

Many phospholipids have been tested for their ability to activate PKC in vitro. The enzyme appears to have an almost absolute requirement for phosphatidylserine (PS); other phospholipids show limited activation, and several show positive or negative effects on PS binding in the presence of low calcium concentrations, and hence on enzyme activity (Takai et al., 1979a; Kaibuchi et al., 1981). Therefore in vivo the phospholipid composition of membrane may have profound effects on the requirements for activation of PKC in any particular cell type; of particular interest here are studies of lipid arrangement which found PS to be positioned at the cytosolic leaflet of the membrane bilayer (Jacobsen and Saier, 1984). It is therefore likely that the lipid composition may determine which cellular membranes can support binding of PKC. In vitro, 1,2-sn diacylglycerols stimulate PKC by increasing the affinity of the enzyme for calcium and phospholipids (Takai et al., 1979b). The enzyme exhibits specificity for the 1,2-configurations (Rando & Young, 1984). The structure of the DAG molecule is shown in figure 1.3.1.

Hannun et al. (1985) have proposed that one molecule of DAG can activate one molecule of PKC in the presence of greater than four, but less than ten, molecules of PS. Active PKC thus appears to exist as a quaternary complex comprising the enzyme, phospholipid, DAG and calcium

(see figure 1.3.2). In vivo, dissolution of the complex and inactivation would occur primarily via phosphorylation of the DAG to phosphatidic acid by DAG kinases, although degradation by DAG lipases may also be important.

The divalent calcium requirement is almost absolute. Of the divalent metal ions, only strontium will substitute for calcium and even then it is only 5% as effective in PKC activation (Takai et al., 1979a). Magnesium ions are essential for catalytic activity (5-10mM) but if manganese or cobalt replace magnesium (0.5-1mM), a half-maximal response may be attained (Takai et al., 1984).

PKC can also be activated by proteolysis with a calcium-dependent neutral protease (Inoue et al., 1977). The smaller enzymatically active component produced is totally independent of calcium, phospholipid and DAG. Membrane-associated PKC is more susceptible to this limited proteolysis (Kishimoto et al., 1983), but whether or not this proteolysis is of physiological significance is still a subject for debate (see Woodgett et al., 1987 for a discussion on this subject). Of interest here is a recent observation by Kishimoto et al. (1989) that calpain exhibits differential activity on the various PKC subspecies.

1.3.d. Diacylglycerol and tumour promoters

The tumour-promoting phorbol esters are tetracycline diterpine derivatives isolated from croton oil of plants of the family Euphorbiaceae. Although not carcinogenic themselves, repeated application of phorbol esters following a single sub-threshold dose of a carcinogenic substance causes tumour outgrowth. The active derivatives of phorbol are esterified at the 12 and 13 positions, and contain a DAG-like moiety in their structure (see figure 1.3.3). The most potent of these is 12-O-tetradecanoylphorbol-13-acetate (TPA), also called phorbol myristic acid (PMA) by an alternative nomenclature system. In

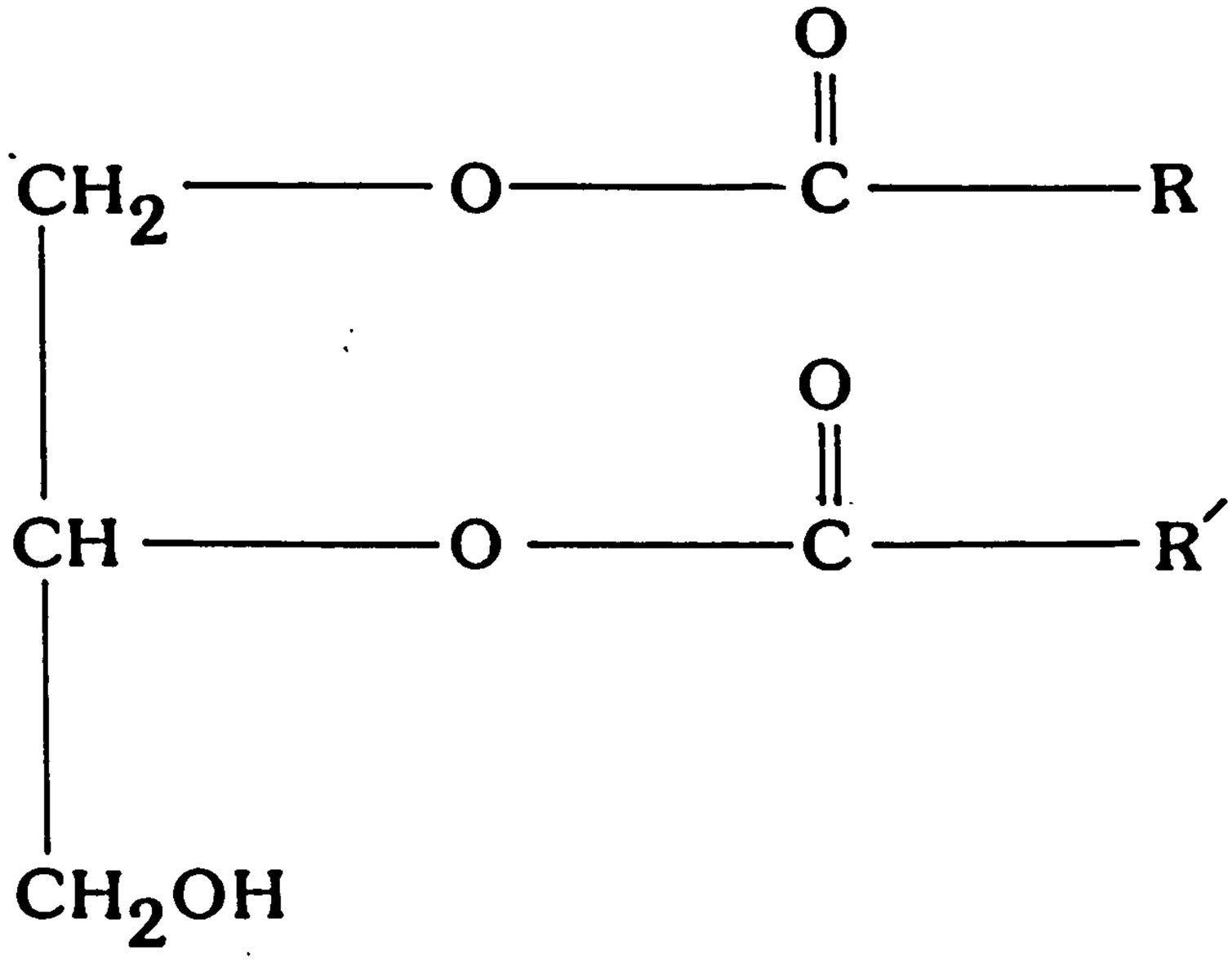
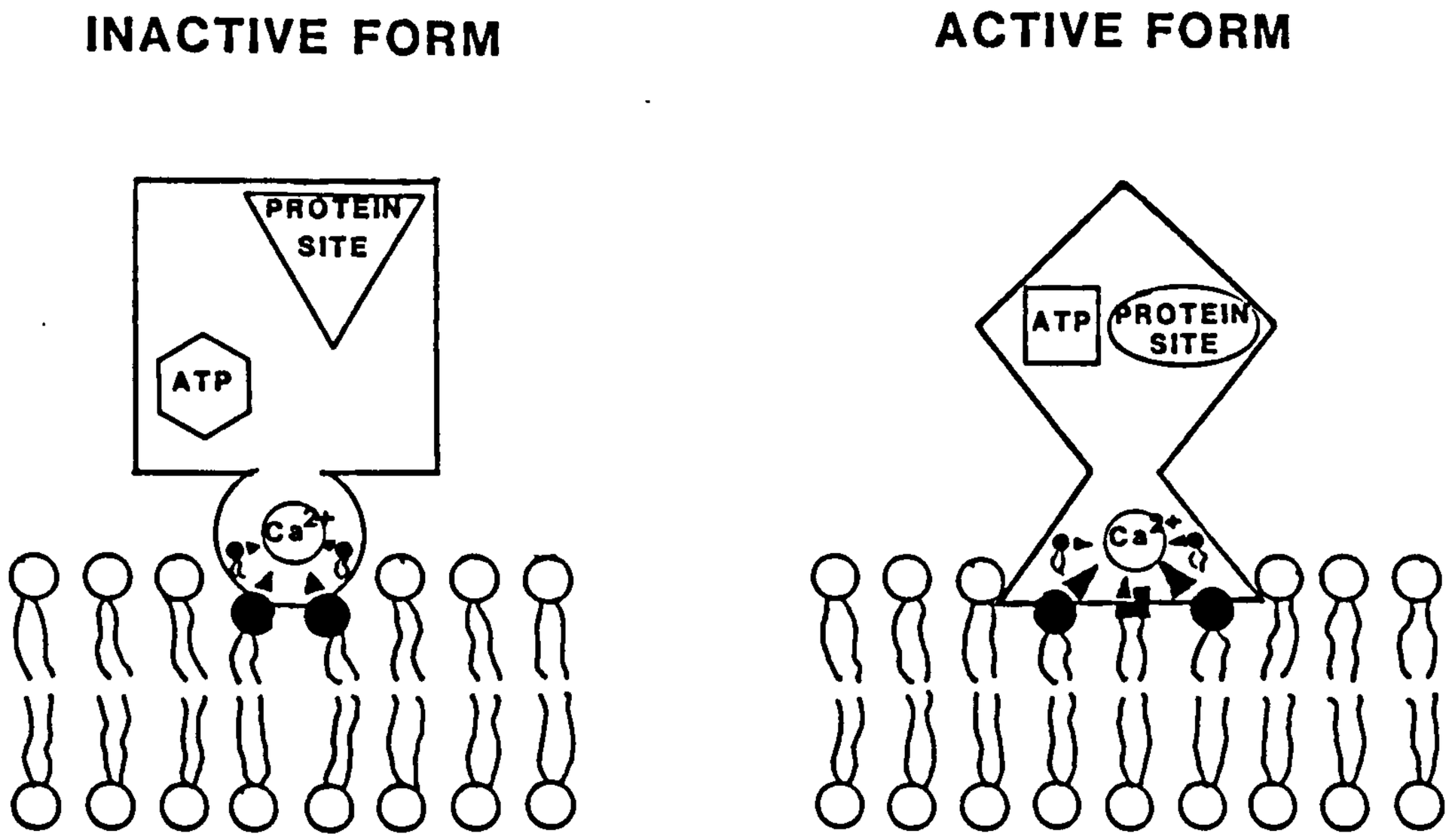


Figure 1.3.1. Structure of diacylglycerol.



(from Ganong et al., 1987)

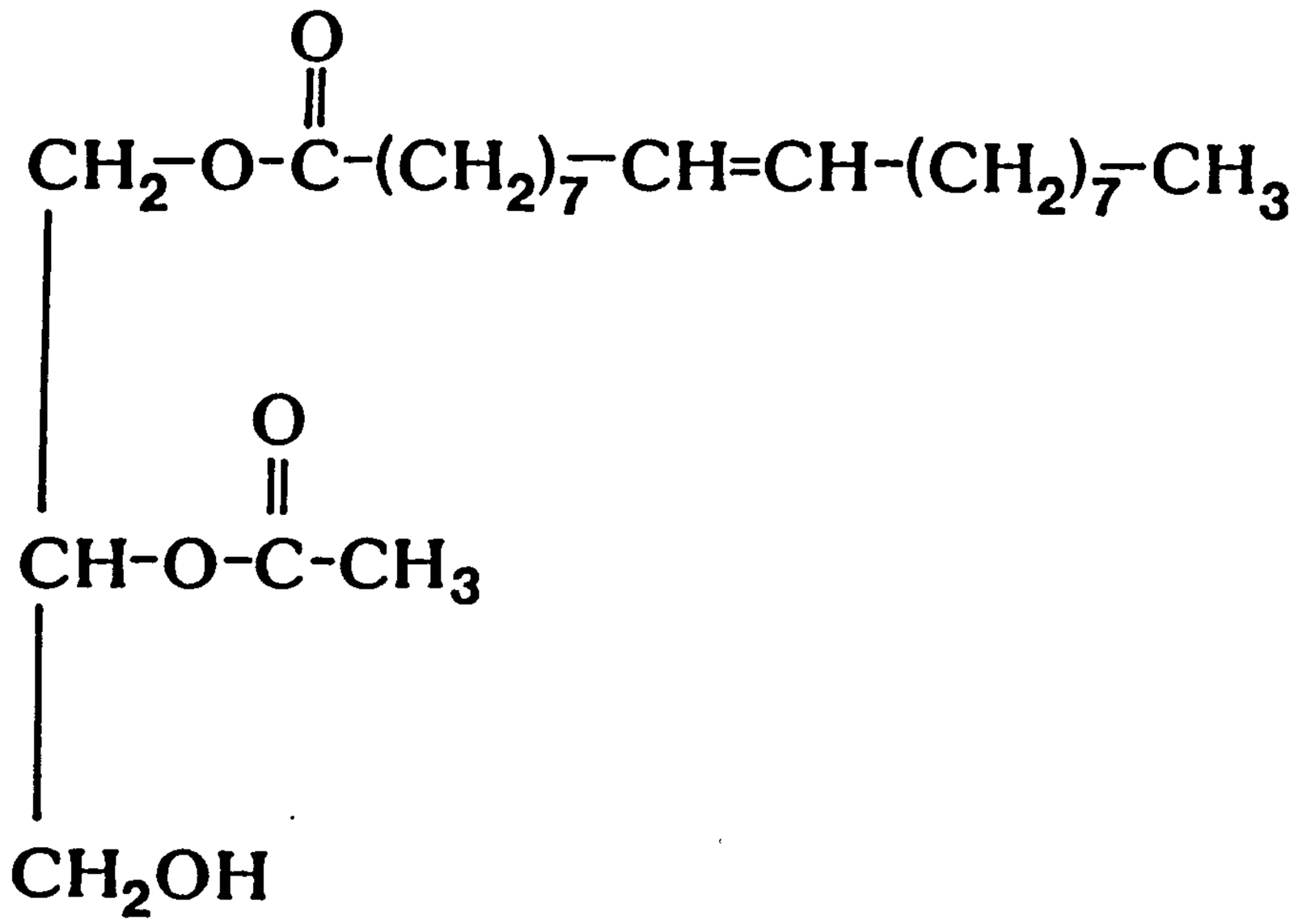
Figure 1.3.2. Activation of protein kinase C.

Left: Protein kinase C associated with a membrane surface complex of phosphatidylserine and calcium in an inactive form.
Right: Membrane-associated protein kinase C activated by diacylglycerol.

1982, Castagna et al. reported that phorbol esters could substitute for DAG in activating partially purified PKC. Moreover, the relative potencies of a series of phorbol esters in tumour promotion tended to correlate with their relative potencies in PKC activation, with the doses required for both processes being very similar. Subsequent work showed that the phorbol ester receptor copurified with PKC (Kikkawa et al., 1982; Parker et al., 1984).

Kinetic analysis of the PKC activation by phorbol esters suggests that roughly one molecule of tumour promoter can activate one molecule of the kinase (Kikkawa et al., 1983; Uratsuji et al., 1985). TPA will produce its effects at the nanogram level however, in contrast to the microgram levels required for a similar effect by DAG. In addition TPA acts on PKC directly without involving activation of the PI pathway for example. However, whilst DAG is present only transiently in the membrane, its in vivo presence being carefully regulated, no enzymes exist to regulate levels of phorbol ester. It thus tends to persist in the membrane leading to a continuous activation of PKC, and so does not necessarily give an accurate reflection of the effects induced by PKC activation via DAG production, and indeed may distort the normal sequence of events (Kikkawa & Nishizuka, 1986).

Tumour promoters structurally unrelated to phorbol esters such as teleocidin and mezerein also activate PKC (Fujiki et al., 1984; Miyake et al., 1984), thus suggesting that a DAG-like structure is not always essential. The lipophilic nature of these molecules causes perturbations of phospholipid bilayers at relatively low concentrations, analagous to that caused by DAG (Tran et al., 1983). Such changes in fluidity may affect a variety of membrane-associated proteins independently of PKC activation such as receptors, ion channels and cytoskeletal components.



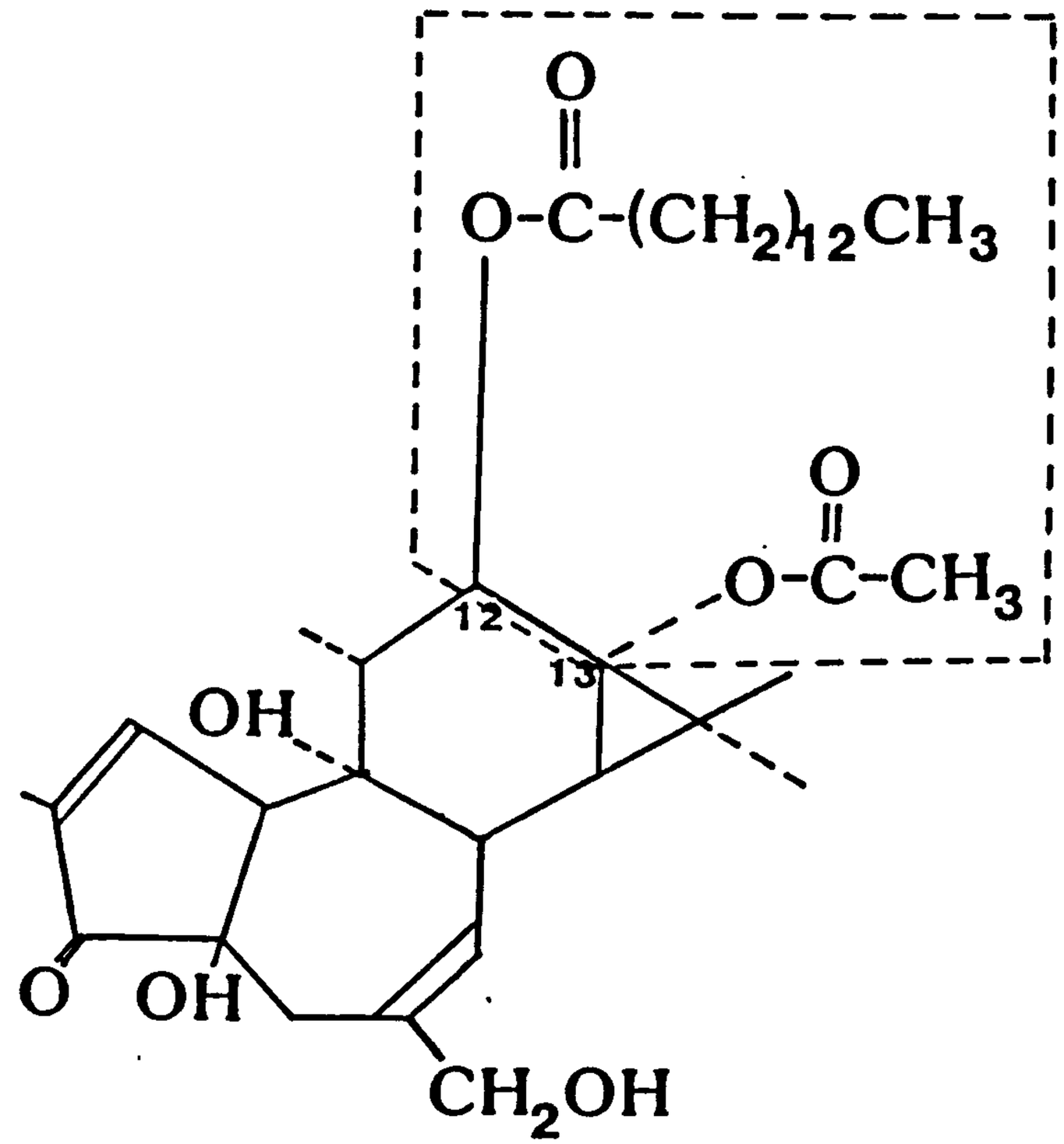


Figure 1.3.3. Structures of synthetic diacylglycerol (1-oleoyl-2-acetylglycerol; left) and tumour-promoting phorbol ester (12-o-tetradecanoylphorbol-13-acetate, TPA; right).

TPA contains a diacylglycerol-like structure in its molecule, as shown in the dotted square.

1.3.e.Inhibitors of PKC

A variety of compounds have been described that inhibit the activity of PKC. The first cited inhibitors were members of the antipsychotic drug family e.g. chlorpromazine (Mori et al., 1980). Like many other purported inhibitors of the enzyme these act by modifying the interaction between the enzyme and the phospholipid, and their action can be overcome by adding phospholipids in vitro (Uratsuji et al., 1985). In addition, these compounds will also inhibit calmodulin-dependent protein kinases.

Tamoxifen and polymyxin B, an antioestrogen and an antibiotic respectively, were also found to interact with phospholipids. Recently, sphingosine, a component of sphingomyelin phospholipids, has been shown to inhibit purified PKC competitively with respect to calcium, DAG and PS (Hannun et al., 1986). However, inhibition by sphingosine does not involve the active site of PKC (Farooqui et al., 1988); instead it appears that through competitive interactions with calcium, PS and PKC, sphingosine displaces activators such as DAG or TPA, thus preventing formation of the active lipid-enzyme complex.

All protein kinases require nucleotide trisphosphates, usually ATP, as cofactors. Hidaka et al. (1984) have described the use of isoquinolinesulphonamide compounds which are competitive inhibitors with respect to ATP, but differ in their selectivity of inhibition of various protein kinases.

The most potent inhibitor of PKC described to date is staurosporine, an antifungal microbial alkaloid, which exhibits half-maximal inhibition at 27nM (Tamaoki et al., 1986). Again, however, its action is not a specific one and it is also a potent inhibitor of PKA as well as being toxic to growing cells.

Thus it would appear that although numerous inhibitors of PKC have been cited, none are specific for the enzyme, and the use of such

compounds requires justification by corroborating results with data obtained by other means.

1.3.f. Regulation of PKC activity by various lipids

The activity of PKC is regulated according to its state of association and dissociation with membrane lipids. These interactions control not only the amount of active enzyme but also its subcellular localisation. Redistribution of PKC to specific regions of cell membranes would occur if the lipid activator accumulated in localised regions of the cell membrane. A better understanding of the modes of action and mechanisms of activation of PKC by various lipids is essential for elucidating the role of PKC in the regulation of multiple cellular functions.

PKC activity may be regulated by phospholipids, neutral lipids, glycolipids and unsaturated fatty acids. Phospholipids, sulphatides and gangliosides interact with PKC through calcium, whereas unsaturated fatty acids such as arachidonate do not require calcium for this activation (Farooqui et al., 1988).

(i) Diacylglycerol.

This has been discussed above (see section 1.3.c). It is of interest to note that besides PKC, DAG regulates other enzymes such as phospholipases A_1 and A_2 (Dawson et al., 1985), glycogen synthetase (Bouscarel et al., 1988) and ornithine decarboxylase (Kido et al., 1986). Dawson et al. (1985) believe that the stimulation of phospholipases A_1 and A_2 is caused by the ability of DAG to change the organisation or orientation of the phospholipid bilayer. Michel et al. (1976) have also indicated that high DAG concentrations can be detrimental to normal bilayer structures.

(ii) Phorbol esters.

This has been discussed above (see section 1.3.d.).

(iii) Unsaturated fatty acids.

McPhail et al. (1984) were the first to show that arachidonic acid and other unsaturated fatty acids markedly stimulated neutrophil PKC in the presence of calcium, and that stimulation was enhanced by DAG (diolein) but not by PS. Murakami & Rottenberg (1985), using a purified preparation of rat brain PKC, showed activation of the enzyme using both oleic and arachidonic acids, but that this activity was independent of calcium and phospholipid. Sekiguchi et al. (1987) have indicated that different subtypes of brain PKC respond differently to unsaturated fatty acids (see section 1.3.k). In all the above work however, non-physiological levels of the fatty acids were used; such levels of fatty acid do not occur in vivo even under pathological conditions (ischaemia and spinal cord trauma) in which unsaturated fatty acids and their metabolites have been reported to have perturbing effects on biological membranes (Williamson, 1986). According to Oishi et al. (1988), lyso-PC at concentrations of 20μm or less, possibly derived from membrane PC by the action of PLA_2, will activate PKC; concentrations above 30μM cause inhibition of the kinase. These authors consider that lyso-PC may thus play a role in signal transduction via a dual regulation of PKC.

(iv) Gangliosides.

These are complex glycosphingolipids which form complexes with calcium through their negatively charged sialic acid moieties. Both stimulatory and inhibitory actions of gangliosides on PKC have been described (Chan, 1987a,b). This class of lipid has been ascribed a potential role in regulation of phenomena such as cellular division and differentiation through PKC and other kinases (Farooqui et al., 1988).

Such postulations are supported by observations that sphingosine, a catabolic product of gangliosides, will inhibit PKC by displacement of DAG or TPA, thus preventing formation of the active lipid-enzyme complex (Hannun et al, 1986).

(v) Lipoxin A and other eicosanoids.

Oxygenated products of the arachidonic acid cascade are known to have profound effects on cellular metabolism (Irvine, 1982). Lipoxin A (5, 6, 15L-trihydroxy-7, 9, 11, 13-eicosatetraenoic acid), a recently discovered metabolite of arachidonic acid, activates human placental PKC at a 30-fold lower concentration than does arachidonic acid or DAG (Hansson et al., 1986). Other eicosanoids had either decreased (e.g. linolenic acid) or no effects (e.g. leukotriene B_4) on PKC activity.

(vi) Sulphatides.

Sulphatides, sulphate esters of cerebrosides, will stimulate PKC to the same extent as PS in the presence of TPA (Fujiki et al, 1986). Other sphingolipids were found to be without effect. In addition, substitution of TPA by DAG led to a fourfold lower activity in the presence of sulphatide than in the presence of PS.

(vii) Others.

The active lipid moiety of lipopolysaccharides of Gram-negative bacteria, diacylglucosamine-1-phosphate, markedly stimulates PKC activity (Wightman et al., 1975), possibly due to the structural similarity of this active lipid moiety to PS. Tumour-promoting organic solvents such as benzene and toluene also activate PKC (Roghani et al., 1987) but without competing with TPA for its binding site, indicating that the mechanism of action of tumour promoter solvents is different from the phorbol esters.

1.3.g. Role of PKC in response to cellular activation

PKC activation and calcium mobilisation can be induced selectively and independently by the application of a permeable DAG or phorbol ester for the former and a calcium ionophore such as A23187 for the latter. Use of such a system demonstrates that both limbs of the signal pathway are essential in order to elicit full cellular responses. Nishizuka's group first demonstrated this role for PKC in stimulus-response coupling (Kaibuchi et al., 1983). The system was subsequently employed in a number of cell types, and some of the postulated roles of PKC which have arisen as a result of this type of experiment are listed in table 1.3.1., together with examples of workers who have undertaken such studies. The potential role of PKC in signal transduction at the cell surface has also been extrapolated to neural tissues, particularly in relation to neuropeptide and transmitter release in the CNS and PNS. The activation of cellular responses by PKC appears to be separate from, but often synergistic with, activation via an increase in intracellular calcium concentration (Kaibuchi et al., 1983, for example).

While IP_3 and DAG play a role in short-term responses, these second messengers are likely also to be of importance in long-term responses such as gene expression and cell proliferation. Both limbs of the pathway are essential and act synergistically to promote DNA synthesis, although an additional pathway is required to elicit maximal activation of cell proliferation as some growth factor must be added (Kaibuchi et al., 1985). Growth factors which operate through the DAG and IP_3 signal pathways include PDGF (Habenicht et al., 1981), insulin (Rozengurt et al., 1984) and interleukin-1 (Truneh et al., 1985). Expression of a number of genes has been reported to be induced by TPA and DAG including ornithine decarboxylase (Otani et al., 1985) and prolactin (Murdoch et al., 1985).

The relationship between PKC and oncogenes has received much attention. Nerve growth factor has been shown to induce the expression of Harvey and Kirsten cellular ras oncogenes (cH-ras and cKi-ras respectively) (Hagag et al., 1986) and to stimulate a PLC with the consequent activation of PKC (Cremins et al., 1986). Activated ras appears to give an overproduction of DAG, not the equimolar quantities of DAG and IP_3 that would be obtained through the PI pathway, similar to an insulin-like effect on de novo synthesis of phosphatidic acid which is rapidly converted to DAG (Wolfman & Macara, 1987). Evidence is also available that the src oncogene may utilise the DAG synthesis pathway in addition to PLC activation (Chiarugi et al., 1987). c-fos, c-myc and cKi-ras oncogenes are also potential targets of PKC action (Greenberg et al., 1985; Ballester et al., 1987).

1.3.h. Regulation of PKC by feedback control

In biological systems activation responses are normally followed by rapid deactivation responses to prevent an overshoot and so allow subsequent responses to signals received. The activity of PKC is no different in this respect; its function appears to be sensitive to feedback control on cell surface receptors. This is often termed downregulation. Feedback control involving receptors coupled to inositol phospholipid breakdown has been reported in astrocytoma cells (Orellana et al., 1985), hippocampal slices (Labarca et al., 1984) and platelets (Rittenhouse & Sasson, 1985) to name but a few systems. Not only does PKC regulate its own receptors but it also extends its action to receptors of other signalling systems. The epidermal growth factor (EGF) receptor is phosphorylated by PKC, resulting in a decrease in both its tyrosine-specific protein kinase and growth factor binding activities (Cochet et al., 1984). The receptors for insulin (Jacobs et al., 1983), transferrin (May et al., 1984) and interleukin-2

TABLE 1.3.1. POTENTIAL ROLES OF PKC IN CELLULAR RESPONSES

TISSUE OR CELL TYPE	RESPONSES	REFERENCE
	Blood Cell Systems	
Platelets	Serotonin release	Kaibuchi et al., 1983
	Arachidonate release	Halenda et al., 1985
	Thromboxane synthesis	Mobley & Tai, 1985
Neutrophils	Superoxide generation	Serhan et al., 1983
Mast cell	Histamine release	Katakami et al., 1984
	Endocrine Systems	
Adrenal medulla	Catecholamine secretion	Knight & Baker, 1983
Adrenal cortex	Aldosterone secretion	Kojima et al., 1983
Pancreatic islets	Insulin release	Zawalich et al., 1983
Pituitary cells	Growth hormone release	Ohmura & Friesen, 1985
	Luteinising hormone release	Conn et al., 1985
	Prolactin release	Delbeke et al., 1984
	Thyrotropin release	Martin & Kowalchyk, 1984
Parathyroid cells	Parathyroid hormone release	Brown et al., 1984a
	Exocrine Systems	
Pancreas	Amylase secretion	du Pont & Fleuren-Jakobs, 1984
Gastric gland	Pepsinogen secretion	Sakamoto et al., 1985
	Nervous Systems	
Ileal nerve endings;caudate nucleus	Acetylcholine release	Tanaka et al., 1984, 1986
PC12 cells; neurones	Dopamine release	Pozzan et al., 1984; Zurgil & Zisapel, 1985
	Muscular Systems	
Smooth muscle	Contraction	Rasmussen et al., 1984
	Other Systems	
Adipocytes	Lipogenesis	van de Werve et al., 1985
	Glucose transport	Kirsch et al., 1985
Hepatocytes	Glycogenolysis	Roach & Goldman, 1983

(Shackelford & Trowbridge, 1984) may also be targets for PKC action.

In short-term cellular responses, PKC may inhibit calcium mobilisation by blocking the receptor-mediated hydrolysis of inositol phospholipids, and hence production of IP_3 and DAG (Nishizuka, 1988). An alternative regulatory method would be stimulation of IP_3 hydrolysis by activation of an IP_3 phosphatase (Connolly et al., 1986). A third possible route is the stimulation of removal of intracellular calcium by activation of the calcium-transport ATPase and the sodium/calcium exchange protein (Nishizuka, 1986).

Feedback control may also apply to long-term responses such as cell proliferation, an example being phosphorylation of the EGF receptor mentioned above. An analagous situation appears to exist with the T-cell receptor present on T-lymphocytes. Stimulation of these cells with TPA and calcium ionophore results in the phosphorylation and subsequent downregulation of the receptor, preventing a further proliferative response to the antigen (Cantrell et al., 1985).

1.3.i. Substrates for PKC phosphorylation

The first step in identifying the mechanism of action of agonists which lead to PKC activation is to identify the proteins that are phosphorylated by this kinase. Such a task is far from simple as PKC will phosphorylate a huge number of proteins in vitro of seemingly every function, subcellular localisation and structure. This has tended to complicate attempts to rationalise observations of effects of agonists in vivo with changes in the phosphorylation state of proteins phosphorylated by PKC in vitro. Some workers have tried to get around this problem by proposing certain criteria which must be satisfied before target proteins for PKC may be 'identified' (see Woodgett et al., 1987). Table 1.3.2 lists a number of postulated substrates of PKC,

together with some of the workers who have undertaken these studies.

Activation of PKC in platelets by exposure to agonist leads to increased phosphorylation of a 40kDa component identified as IP_3 phosphatase (Connolly et al., 1986). Phosphorylation of this protein in response to agonists such as phorbol ester, synthetic diacylglycerols and platelet-activating factor parallels the release of various constituents of platelet granules. Another useful marker of PKC activation in intact cells appears to be an agonist-based change in an acidic phosphoprotein of approximately 80kDa that has been identified in a number of systems (for example, Rozengurt et al., 1984; Blackshear et al., 1986; Rumsby et al., 1988). The phosphorylation of this protein increases dramatically in response to phorbol ester and a variety of agonists believed by other criteria to act in part through PKC activation. The 80kDa protein appears not to be significantly phosphorylated by any other kinase and is present in all cells and species examined to date with the richest sources being the CNS and retina (Witters & Blackshear, 1987). The identity and function of this protein however are at present unknown.

Phosphorylation of target proteins occurs at seryl and threonyl but not tyrosyl residues. In this respect PKC is similar to PKA, and indeed the two kinases share common substrates e.g. calf thymus histone (histone H1) and myelin basic protein (MBP). Closer analysis however reveals residue specificity; PKA phosphorylates the serine-38 residue located close to the N-terminal end of histone H1 for example, whereas PKC rapidly phosphorylates seryl and threonyl residues close to the C-terminus. Like a number of other protein kinases, PKC will autophosphorylate; this occurs in the presence of calcium, phospholipid and DAG, but the significance of this phosphorylation

TABLE 1.3.2. PROTEINS PHOSPHORYLATED BY PKC *in vitro* AND WHOSE PHOSPHORYLATION IS STIMULATED BY TPA *in vivo*

Actin-binding protein	Carroll *et al.*, 1982
Myosin light chain	Castagna *et al.*, 1982
47kDa (IP_3 phosphatase)	Castagna *et al.*, 1982; Connolly *et al.*, 1986
Ribosomol protein S6	LePeuch *et al.*, 1983
Glycogen synthase	Roach & Goldman, 1983
Vinculin	Werth *et al.*, 1983
Tyrosine hydroxylase	Albert *et al.*, 1984
EGF receptor	Cochet *et al.*, 1984
IL-2 receptor	Shackelford & Trowbridge, 1984
80kDa	Rozengurt *et al.*, 1984
$pp60^{src}$	Tamura *et al.*, 1984
Class 1 HLA antigens	Feuerstein *et al.*, 1985
NADPH oxidase	Papini *et al.*, 1985
Glucose transporter	Witters *et al.*, 1985
Transferin receptor	Davis *et al.*, 1986
p36/p35	Gould *et al.*, 1986
MBP	Vartanian *et al.*, 1986

remains to be determined. In many systems PKC-dependent phosphorylation is believed to be relatively resistant to phosphatase action (Chiarugi et al., 1989), so allowing substrate modification induced by PKC to persist.

1.3.j. Interaction of PKC with other signalling systems

(i) Interaction with the cAMP pathway

The transduction mechanism involving the generation of the second messenger 3', 5'-cAMP was the first to be extensively characterised (see Krebs and Beavo, 1979). The cAMP system operates by activating a serine/threonine-specific protein kinase, PKA. The two kinases PKA and PKC often interact with each other; Nishizuka (1986) has divided the cellular responses into two modes (see figure 1.3.4). In bidirectional control systems the two classes of receptor appear to counteract each other, whereas in monodirectional control systems one receptor class may potentiate the other. For example, in platelets and lymphocytes there is evidence that cGMP is elevated upon TPA treatment leading to cAMP inhibition (Coffey & Hadden, 1983; Nishizuka, 1983), a bidirectional control system. In cerebral cortex, in contrast to this, agonists of PKC stimulate the cAMP pathway (Hollingsworth et al., 1986). The molecular mechanisms responsible for this interaction between the two pathways are unknown but the complex cell-specific interrelationships displayed reflect the adaptability of control pathways (Nishizuka, 1986).

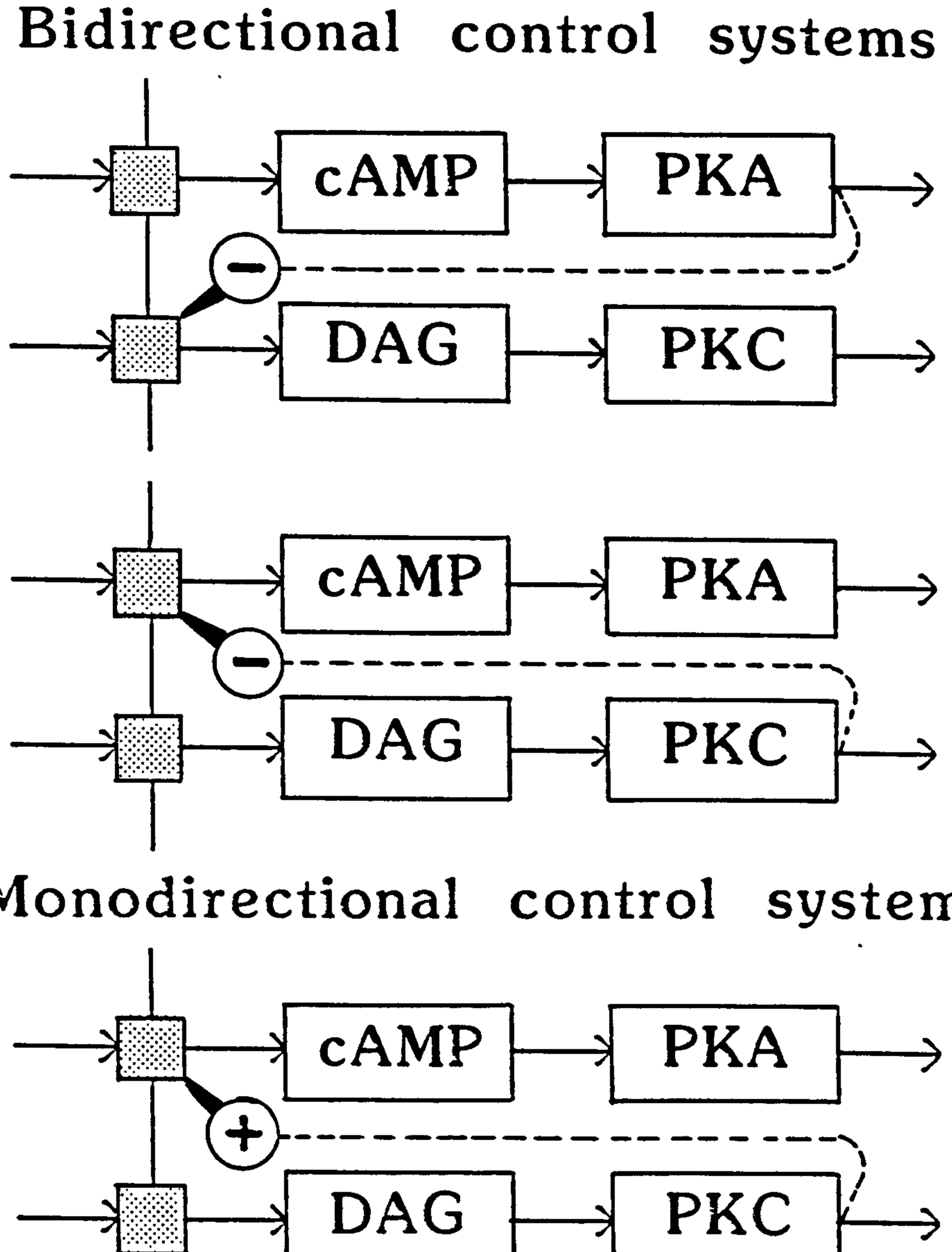


Figure 1.3.4. Modes of interaction of two major signal - transducing systems.

(ii) Interaction between PKC and tyrosine kinases

Although little is known about how these two signalling systems are linked, a number of observations point to such a relationship. The two kinases have a number of substrates in common such as the EGF receptor (Cochet et al., 1984) and p36 (Gould et al., 1986); the latter is both a major cellular substrate for protein-tyrosine kinases and a physiological substrate for PKC. In addition, a 42kDa protein is phosphorylated on tyrosine when certain cell lines are treated with phorbol esters (Bishop et al., 1983; Gilmore & Martin, 1983) indicating that PKC must either activate a protein-tyrosine kinase or inhibit a protein-phosphotyrosine phosphatase (Woodgett et al., 1987). Finally, TPA will act as a partial mitogen for certain cell lines in a manner analagous to some of the polypeptide mitogens that act through protein-tyrosine kinase receptors (for example, Dicker & Rozengurt, 1978).

1.3.k. Multiple forms of PKC

Until recently it was believed that the PKC detected throughout the animal kingdom was a single protein. It is now apparent, however, that far from being a single entity, there are at least seven subspecies of the enzyme. This is not a previously unknown phenemenon with kinases; PKA has two forms which differ in their mechanisms of regulation and tissue distribution (Nairn, 1985).

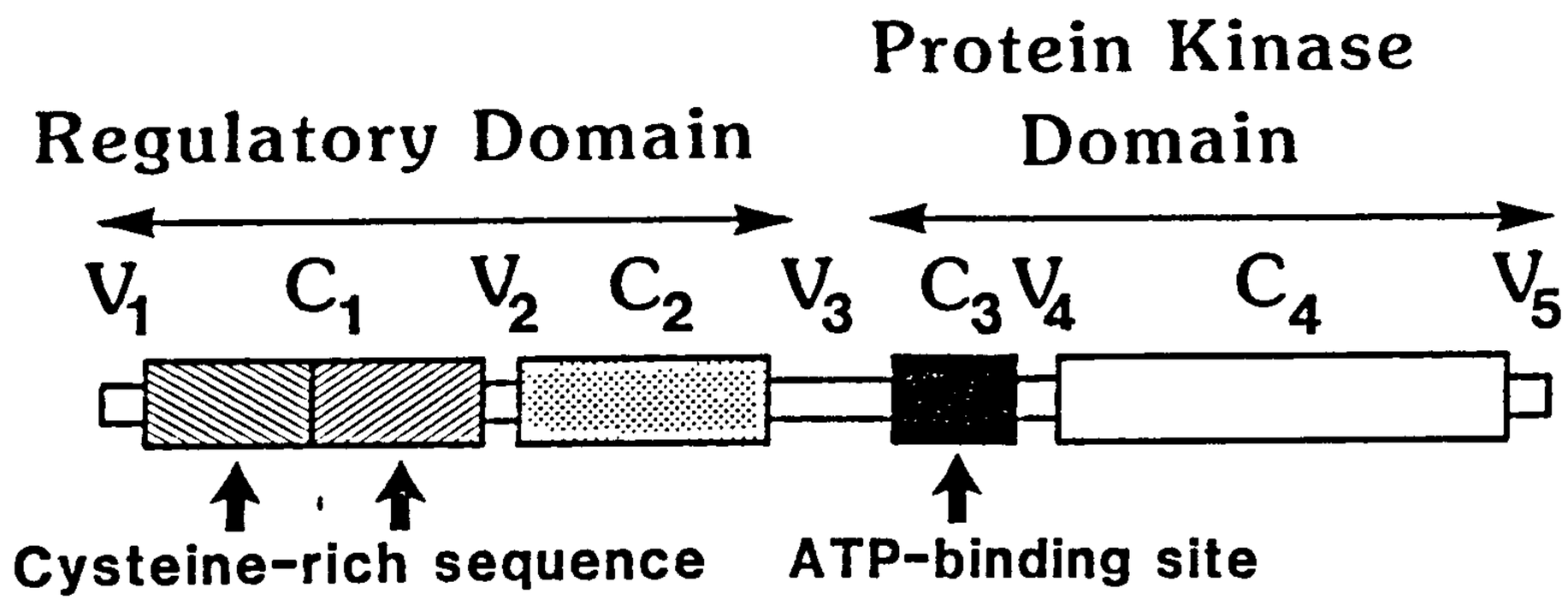
The existence of multiple forms of PKC was reported at about the same time by isolating four distinct cDNA clones of PKC (Coussens et al., 1986; Knopf et al., 1986; Ono et al., 1986; Parker et al., 1986). Two methods of nomenclature exist to describe the subspecies. The lettered variety now favoured by

most groups and originally proposed by Coussens et al. (1986) is used in this study. In this the four subspecies are termed α, β_1, β_{11} and γ (corresponding to numerical types III, IIA, IIB and I respectively). These subspecies are products of three distinct genes, with β_1 and β_{11} being formed by alternative splicing of a single gene (Ono et al., 1986). These subspecies can be separated into three distinct fractions by hydroxylapatite column chromatography (Ono et al., 1987).

More recently, at least three further subspecies, delta (δ), epsilon (ε) and zeta (z) have been isolated from a rat brain cDNA library (Ono et al., 1988), but these cannot yet be isolated using chromatographic means. The newly-discovered subspecies have a common structure closely related to, but clearly distinct, from the four subspecies initially described. See figure 1.3.5. The main difference between the α, β, γ and δ, ε, z groups is that the latter subspecies lack the second conserved region, C_2. β_1 and β_{11} differ from each other only in approximately fifty amino acid residues at their carboxy-terminal end regions V_5, and even in this area they possess a high degree of sequence homology (Ono et al., 1987).

(i) Distribution

Biochemical and immunocytochemical approaches to investigating PKC subspecies have been undertaken in a number of laboratories with the result that much is now known about the distribution of the α, β and γ forms but rather less about the remaining three. The most striking example of subspecies specificity in localisation is that the γ-subspecies appears to be expressed solely in the brain and spinal cord, with particularly high concentrations in the hippocampus and cerebral



Clone	Amino Acids	Molecular Weight
α	672	76,799
βI	671	76,790
βII	673	76,933
γ	697	78,366
δ	673	77,517
ε	737	83,474
ζ	492	——

(from Kikkawa et al., 1987)

Figure 1.3.5. Structures, amino acid residues and deduced molecular weights of PKC subspecies. Conserved regions (C_1 - C_4) and variable regions (V_1 - V_5) are shown by thick and thin boxes respectively.

cortex (Nishizuka, 1988). Because of its localisation, γ-PKC has been proposed to play a role in long-term potentiation (LTP) (Nishizuka, 1988). Levels of the γ-subspecies appear to increase postnatally, reaching a maximum in the rat approximately three weeks after birth.

β_1 and β_{11} are found in both the CNS and PNS, with β_{11} being far more prevalent. Each subspecies has a distinct pattern of cellular localisation, however. For example, in the rat cerebellar cortex β_1 is found in the granule cell body and β_{11} in the molecular layer (Huang et al., 1987a). Many tissues contain β_1 and β_{11} subspecies in varying amounts, but α appears to be the most widely distributed of the subspecies isolated to date (Shearman et al., 1987). In addition, most tissues contain more than one subspecies of PKC.

(ii) Individual characteristics

The presence of different subspecies of PKC suggests different biological roles according to difference in tissue distribution. This suggestion is strengthened if the subspecies exhibit differences in enzymatic properties; this indeed is the case. β_1 and β_{11} subspecies are surprisingly active when stimulated by DAG and PS in the absence of calcium, and reach 60% of maximal possible activity. This contrasts with the 25% of maximal activity obtained with the α and γ forms under the same conditions (Nishizuka, 1988). γ-PKC can be significantly activated by arachidonic acid (AA) (Sekiguchi et al., 1988), activation being nearly 80% of maximal with 30μM arachidonic acid. Activation does not require Ca^{2+}, nor does it depend upon phospholipid and DAG. β_1 and β_{11}-PKC's show substantial activity without added Ca^{2+} in the presence of DAG and phospholipid, but respond much less to AA. α-PKC is similar to the γ-subspecies in properties but responds to high levels of AA only

when Ca^{2+} levels are increased. The activation properties of the α-, β- and γ-subspecies are summarised in table 1.3.3. It is possible that some PKC subspecies may be activated at different stages of cellular responses by a series of phospholipid metabolites such as DAG, which may arise from various phospholipid sources, and AA and its metabolites which are formed subsequent to stimulation of the receptor.

TABLE 1.3.3. ACTIVATION PROPERTIES OF SUBSPECIES OF PROTEIN KINASE C FROM MAMMALIAN TISSUES

SUBSPECIES	ACTIVATORS
α	PS + DAG + Ca^{2+} AA + Ca^{2+}
β_1	PS + DAG + Ca^{2+}
β_{11}	PS + DAG + Ca^{2+}
γ	PS + DAG + Ca^{2+} AA

(iii) PKC and the arachidonate cascade

Arachidonic acid may be derived via PI turnover through two consecutive reactions catalysed by PI-PLC and DAG lipase (see figure 1.2.2) (Bell et al., 1979). However, although this pathway produces arachidonate specifically, PI appears to be a relatively minor production source (Takai et al., 1984). A second pathway is a PLA_2-mediated breakdown of phosphatidylcholine, phosphatidylethanolamine, and phosphalidylinositol (Sekar & Hokin, 1986). This enzyme has a lower affinity for Ca^{2+} than PLC, suggesting that PLA_2 action on phospholipids is likely to be stimulated by agonist-evoked rises in intracellular Ca^{2+} concentrations such as those seen on IP_3 formation from PIP_2 (Billah et al., 1980). These same authors proposed a third pathway of AA formation via a PA-specific PLA_2, the PA being derived from DAG during receptor-linked PI turnover by a DAG kinase. These pathways are summarised diagrammatically in figure 1.3.6. It is probable that pathways of arachidonate liberation employed vary widely according to tissue and cell type, with much depending upon the messenger invoking phospholipid (especially PI) turnover.

Arachidonate liberated by any of these pathways is rapidly converted to various biologically active metabolites (prostaglandins, thromboxanes and leukotrienes) which mediate or modulate numerous physiological functions. Thromboxane A_2 in platelets may induce PI turnover and initiate another cascade of the activation of cellular functions while prostaglandins such as prostacyclin will interact with receptors to cause formation of cAMP and so lead to feedback control of cellular functions (Sekar & Hokin, 1986). Arachidonate has also been implicated in calcium mobilisation in a number of cell types (for example, Whiting & Baritt, 1982; Kolesnick & Gershengorn, 1985), and

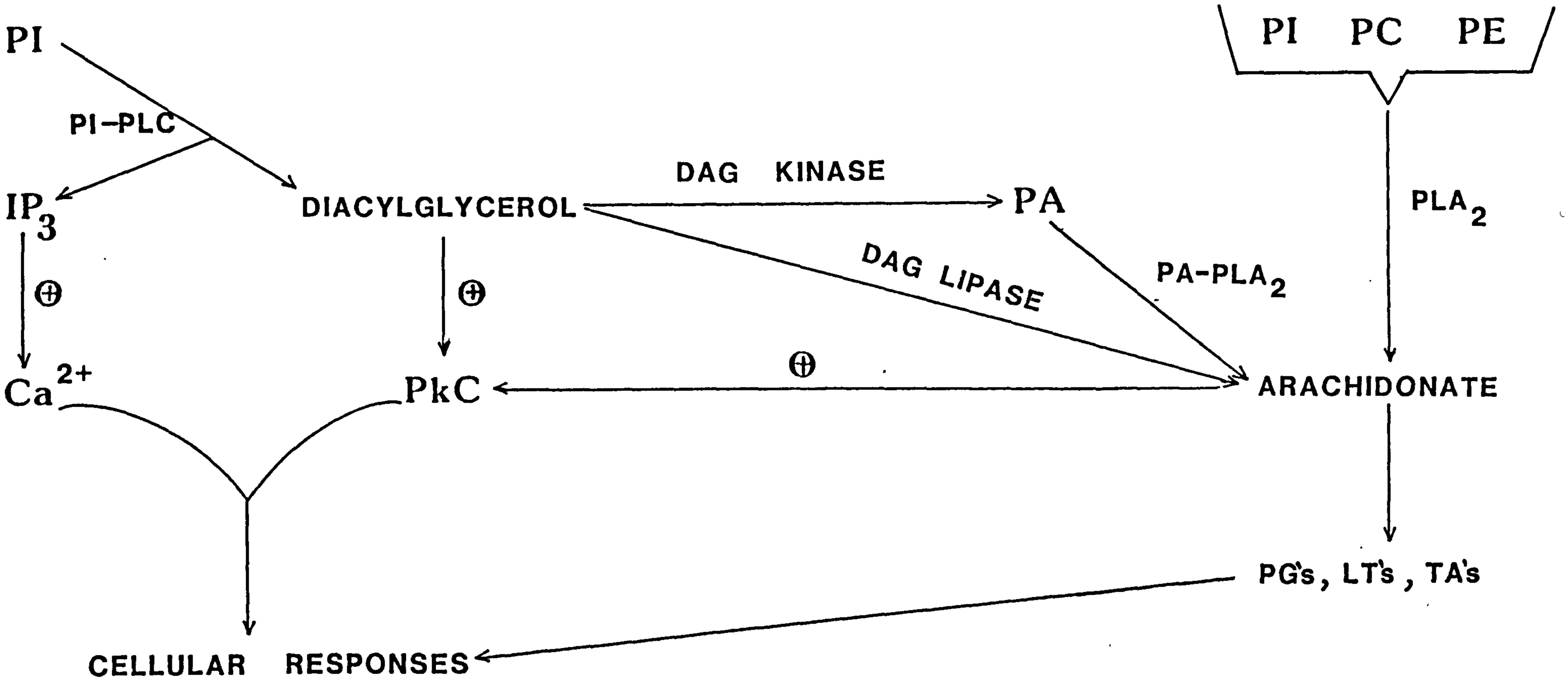


Figure 1.3.6. Pathways for formation of arachidonic acid.

(PA = phosphatidic acid, PC = phosphatidylcholine, PE = phosphatidylethanolamine
PI = phosphatidylinositol, PLA_2 = phospholipase A_2, PLC = phospholipase C,
PG's = prostaglandins, LT's = leukotrienes, TA's = thromboxanes.)

PKC may be involved in the regulation of arachidonic acid metabolism via phosphorylation of the lipocortins, proteins which inhibit the rate limiting step in prostaglandin synthesis (Khanna et al., 1986).

1.4 PKC AND GLIA

Much work has been carried out investigating the role of PKC in neurones (for example, Kikkawa et al., 1986; Miller, 1986), but there have been few investigations of PKC activity in glial cells. This is despite a number of observations linking the enzyme to this cell type:

(i) immunocytochemical observations by Girard et al. (1985) identified PKC activity in cells resembling oligodendrocytes in rat brain white matter;

(ii) myelin basic protein, a major constituent of the myelin sheath and synthesised by oligodendrocytes, is a good substrate for PKC (Turner et al., 1982);

(iii) tumour-promoting phorbol esters enhanced the differentiation of astrocytes in cultures derived from foetal rat brain (Honegger, 1986);

(iv) PKC has been identified in primary astrocyte cultures (Neary et al., 1986b);

(v) receptor-linked PI turnover has been demonstrated in astrocytes (Pearce et al., 1986) and

(vi) proliferation of glial cells in a primary neuronal cell culture led to significantly increased levels of phorbol ester receptor binding (Burgess et al., 1986).

There is thus substantial evidence that PKC is associated with glia which would be in keeping with the role of the enzyme in controlling regulatory processes in these active cells.

1.5 PLATELET-DERIVED GROWTH FACTOR

A polypeptide growth factor recently implicated in the regulation of glial cell development in rat optic nerve has been identified as PDGF (Noble et al., 1988; Raff et al., 1988; Richardson et al., 1988). This work has been mentioned in an earlier section (see 1.1.b) but is discussed in more detail here. Information concerning the PDGF molecule and its role in signal transduction is also presented.

1.5.a. PDGF and glial cell development

The differentiation of an O-2A progenitor cell in vitro is dependent upon the culture conditions employed. In the presence of concentrations of foetal calf serum of 10% or more the cell, when cultured in the absence of other cell types, will differentiate into a type-2 astrocyte (Raff et al., 1983). At concentrations of FCS below 1%, however, an oligodendrocyte is produced. Thus it was proposed that O-2A progenitor cells would differentiate to become oligodendrocytes unless an inducing factor, mimicked in cell culture by FCS, were present (Raff et al., 1985). In vitro experiments by Noble and Murray (1984) indicated that type-1 astrocytes secreted one or more factors to keep O-2A progenitor cells proliferating and prevent their premature differentiation. However, at a given point in time (i.e. day of birth = E21 in rats), the cells became unresponsive to this stimulus and developed into oligodendrocytes, a situation which can be reflected in vitro by culturing O-2A progenitor cells from embryonic rat nerve with type-1 astrocytes, or in type-1 astrocyte-conditioned medium (Raff et al., 1985).

Evidence to date suggests that PDGF is the growth factor secreted by type-1 astrocytes which stimulates O-2A cells to proliferate. Noble et al. (1988) investigated a wide range of growth factors for their

ability to modulate DNA synthesis and differentiation of O-2A progenitor cells in cultures derived from optic nerves of 7-day old rats. Only PDGF was found to mimic the effects of type-1 astrocytes. PDGF also promoted DNA synthesis in O-2A progenitor cells as effectively as type-1 astrocytes or type-1 astrocyte-conditioned medium, and inhibited the rapid differentiation of progenitor cells into oligodendrocytes that is otherwise seen when these cells are grown in the absence of type-1 astrocytes.

Richardson et al. (1988) made a number of observations supporting this proposed role for PDGF. Analysis of cultures of type-1 astrocytes showed that they secrete PDGF and contain messenger RNA which encodes for the PDGF-A chain (the structure of PDGF is discussed in section 1.5.b). Purification of type-1 astrocyte conditioned medium by gel filtration led to mitogenic activity comigrating with PDGF. This mitogenic activity competed with PDGF for receptors and was neutralised by antibodies to PDGF.

PDGF has also been identified in extracts of developing optic nerve (Raff et al., 1988). The same authors have shown that the polypeptide can replace exogenous type-1 astrocytes or type-1 astrocyte-conditioned medium in reconstituting the normal timing of oligodendrocyte development in cultures of embryonic rat nerve cells, and that antibodies to PDGF neutralise the ability of type-1 astrocyte-conditioned medium to reconstitute this normal timing.

At a specific time the O-2A cells become unresponsive to PDGF and drop out of division to become oligodendrocytes. Theories proposing how this might occur have been discussed earlier, as has the role of type-1 astrocytes in development of type-2 astrocytes from O-2A progenitor cells (see section 1.1.b).

1.5.b.PDGF: structure and properties

PDGF is an ubiquitous peptide regulatory factor which was first identified when it was realised that whole blood serum was an essential component of culture media for the successful growth of mesenchyma connective tissue-forming cells (Ross et al., 1974). Cell-free, plasma-derived serum, lacking any components derived from circulating blood, lacks growth-promoting activity. Only the platelet can restore this activity, hence the introduction of the term platelet-derived growth factor. Despite this terminology, the molecule is synthesised and secreted by a number of cell types such as tumour cells and activated macrophages (Ross et al., 1986). Various roles have been attributed to PDGF including healing and repair of wounds and inflammation (Shimokado et al., 1985). More recently, roles in development have been proposed as a result of finding PDGF in the placenta (Goustin et al., 1985), the early mouse embryo (Rappolee et al., 1988) and the Xenopus embryo (Mercola et al., 1988). In addition PDGF has now been shown to play a major role in the differentiation and development of glial cells, as discussed in the previous section.

PDGF from human platelets is a cationic glycoprotein of approximately 30kDa (Antoniades, 1981). Reduction of disulphide bonds destroys its mitogenic activity and generates multiple protein species of 14-17kDa. Sequence analysis revealed two distinct but related sequences in these multiple species suggesting that PDGF from human platelets is a heterodimer of two chains termed A and B (Johnsson et al., 1984), both of which can be mitogenic. This contrasts with porcine PDGF which consists of B-B homodimers (Stroobant and Waterfield, 1984) and the A-A homodimers of osteosarcoma cells (Heldin et al., 1986). In fact purified PDGF from human platelets contains about 70% PDGF-AB and 30% PDGF-BB (Hammacher et al., 1988).

The PDGF-AB chain has been found to have a stimulatory effect in

assays of chemotoxins and actin reorganisation of human fibroblasts (Nister et al., 1988). PDGF-AA is a potent mitogen for Swiss 3T3 cells (Kaslauskas et al., 1988). In addition, it is mRNA encoding the AA homodimer that is found in cultures of type-1 astrocytes (Richardson et al., 1988) and thus it is this homodimer that is mitogenic for the O-2A progenitor cell (Anderson et al., 1989).

1.5.c. Signal transduction by PDGF

Analysis of binding of the various PDGF dimers to cultured fibroblasts led to the introduction of two distinct PDGF receptor types (Hart et al., 1988), denoted A and B. The A-type receptor binds all three forms of PDGF (homodimers AA and BB, and heterodimer AB) whereas the B-type receptor binds PDGF-BB with high affinity, PDGF-AB with lower affinity, and doesn't appear to bind PDGF-AA at all (Heldin and Westermark, 1989). The difficulty in investigating signal transduction involving PDGF is its ability to act via two signal pathways, PKC and tyrosine kinase (Kaplan et al., 1987; Ek et al., 1982). Our knowledge of signal transduction involving the first pathway is growing all the time, but the second-named pathway is rather less well detailed. Work by Williams (see Williams et al., 1988; Williams, 1989) has attempted to remedy this latter fact and, in detailed studies, he has made a number of important observations:

(i) the kinase-insert region of the receptor (Yarden et al., 1986) plays an important part in mitogenesis, but is not essential for many of the early responses to PDGF including tyrosine kinase activation, PI turnover, increased intracellular calcium, receptor internalisation and change in intracellular pH;

(ii) the tyrosine kinase activity of the receptor is essential for mitogenesis and PI hydrolysis, but is not required for ligand-induced receptor downregulation;

(iii) the transmembrane region of the receptor serves an important and specific role in signal transduction other than simply providing a membrane anchor for the receptor;

(iv) when activated, the wild-type receptor associates with PDGF-sensitive PI kinase, but non-mitogenic mutants of the receptor failed to do this;

(v) the receptor undergoes a nonconformational change when activated by PDGF.

In investigating the PDGF receptor, all this above work was carried out using the PDGF-B receptor; the less well characterised PDGF-A receptor is structurally related however (Heldin and Westermark, 1989), and so similar properties might be expected.

Recent evidence suggests that the PDGF-B receptor is not expressed, or is expressed only at very low levels, on normal connective tissue cells _in vivo_ and that the receptor is induced in conjunction with inflammation _in vivo_ (Rubin _et al._, 1988) or when cells are explanted into tissue culture _in vitro_ (Terracio _et al._, 1987). Thus the _in vivo_ response appears to be largely dependant upon type-B receptor induction and not merely the levels of available ligand. How the type-A receptor functions is not yet known.

1.6. AIMS OF THE PROJECTS

The aims of this study were to investigate CNS glia for the presence of PKC activity and to define the characteristics of the enzyme present. A further aim was to examine oligodendrocytes and astrocytes separately for evidence of PKC activity. Because of the complexity of separating glia from CNS tissue, primary culture systems derived from neonatal rat brain (Walker et al., 1985) were used as the basis of this work.

Initially, an assay to measure PKC in this system had to be developed prior to investigations concerning levels of PKC activity, localisation of this activity and enzyme characterisation could take place. Finally, this study aimed to postulate a role for PKC in glial cells.

2. METHODS

2.1. CELL CULTURE

2.1.a. Preparation of mixed glial cell primary cultures

This method was performed essentially as described by Walker et al. (1985); the entire preparation was carried out under aseptic conditions.

1-2 day old Wistar rat pups were decapitated and the cerebral hemispheres removed into culture medium consisting of Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% foetal calf serum (FCS), 2mM L-glutamine and 50 IU/ml penicillin and 0.05mg/ml streptomycin in a 10cm petri dish. The cerebra were dissected along the longitudinal fissure and the meninges removed by gently rolling the hemispheres across sterile filter paper. This technique significantly reduces contamination of cultures with fibroblasts. Cleaned hemispheres were placed inside a nylon mesh bag (212µm mesh; Swiss Silk Bolting Cloth Mfg. Co. Ltd., Zurich) which was resting in a petri dish containing medium. The surface of the bag was stroked gently with a glass rod to allow the dissociated tissue to pass out into solution. The suspension of tissue fragments and one wash of the bag were passed through two stainless steel sieves (230µm and 140µm mesh; Cellector E-C Apparatus Corp., USA) and the cells collected by centrifugation at 1000g for 5 min. on a Sorvall RT6000 bench centrifuge. The cell pellet was resuspended in culture medium and cell viability determined by nigrosin dye exclusion using phase contrast microscopy.

$25cm^2$ tissue culture flasks (Nunc) were seeded with 1.75×10^6 viable cells per flask and cultures left undisturbed in a humidified incubator in an atmosphere of 5% CO_2 at 37°C for 5 days. The culture medium was then changed, and subsequent changes took place every 3-4

days. Figure 2.1.1.A shows the appearance of primary glial cells at four weeks in culture.

2.1.b. Preparation of glial subcultures

Oligodendrocyte-enriched cultures were obtained by following a procedure performed essentially as detailed by McCarthy and de Vellis (1980).

Primary mixed glial cell cultures were grown for 9 days in 25cm^2 flasks as detailed above. The flasks were then placed on an orbital shaker (Gyrotory G2, New Brunswick Sci., Canada) positioned within a 37°C incubator, the caps of each flask tightened, and the flasks shaken for 1h. at 190rpm. Flasks were then removed, the culture medium (containing mainly macrophages) discarded, and 4ml of fresh medium added. Shaking was then continued for a further 16h.

At the end of this time cell-containing supernatant was removed, a viable cell count performed, and the subcultures seeded at 1.75 x 10^6 viable cells per 25cm^2 flask. Flasks were returned to the incubator and left for 5 days prior to medium change. Figure 2.1.1.B shows the appearance of glial subcultures at three weeks in subculture.

2.1.c. Use of oligodendrocyte-defined medium

The medium used was as described by Espinosa de los Monteros et al. (1988) and termed OLDEM. The basal media were DMEM and Ham's F-12 medium (HAM). OLDEM contained the following constituents (all given as final concentrations):

DMEM : HAM	1 : 1 (v/v)
Sodium bicarbonate	3037 mg/litre
D(+) - galactose	2500 mg/litre
D(+) - glucose	1300 mg/litre
L-glutamine	200 mg/litre

Streptomycin	50 mg/litre
Penicillin	50000 IU/litre
Putrescine dihydrochloride	16.1 mg/litre
Bovine insulin	5 mg/litre
Sodium selenite	0.008 mg/litre

Approximate pH was 7.2 - 7.4 at 37°C.

2.1.d. Preparation of type-1 astrocyte cultures

Cultures were prepared according to Noble and Murray (1984). Cells remaining following subculturing were essentially a population of type-1 astrocytes with a small number of contaminating O-2A progenitor cells. The flasks were tapped against a solid surface 3-4 times and then washed with medium to remove any cells not tightly attached to the substratum; this further reduced the number of contaminant cells. Cultures then had 4ml medium added and were grown for a further 24h. at 37°C. 10μM cytosine arabinoside was added for a further 24h. in order to preferentially kill any rapidly dividing cells. Cells were passaged either 1 to 2 or 1 to 4 using trypsin and grown to near confluency (3-4 days for 1 to 2 passage, about 1 week for 1 to 4 passage). At this stage the cultures consisted of greater than 95% type-1 astrocytes as determined by phase contrast and immunofluorescent microscopy. Figure 2.1.1.C shows the the appearance of an astrocyte culture following treatment with cytosine arabinoside and prior to passage.

2.1.e. Use of astrocyte-conditioned medium

Medium was prepared as described by Richardson et al. (1988). Semi-confluent monolayers of astrocytes were washed three times with serum-free DMEM, and then incubated at 37°C in an atmosphere of 5% CO_2 for 48h. in the same medium. The conditioned medium was then removed and stored at -20°C.

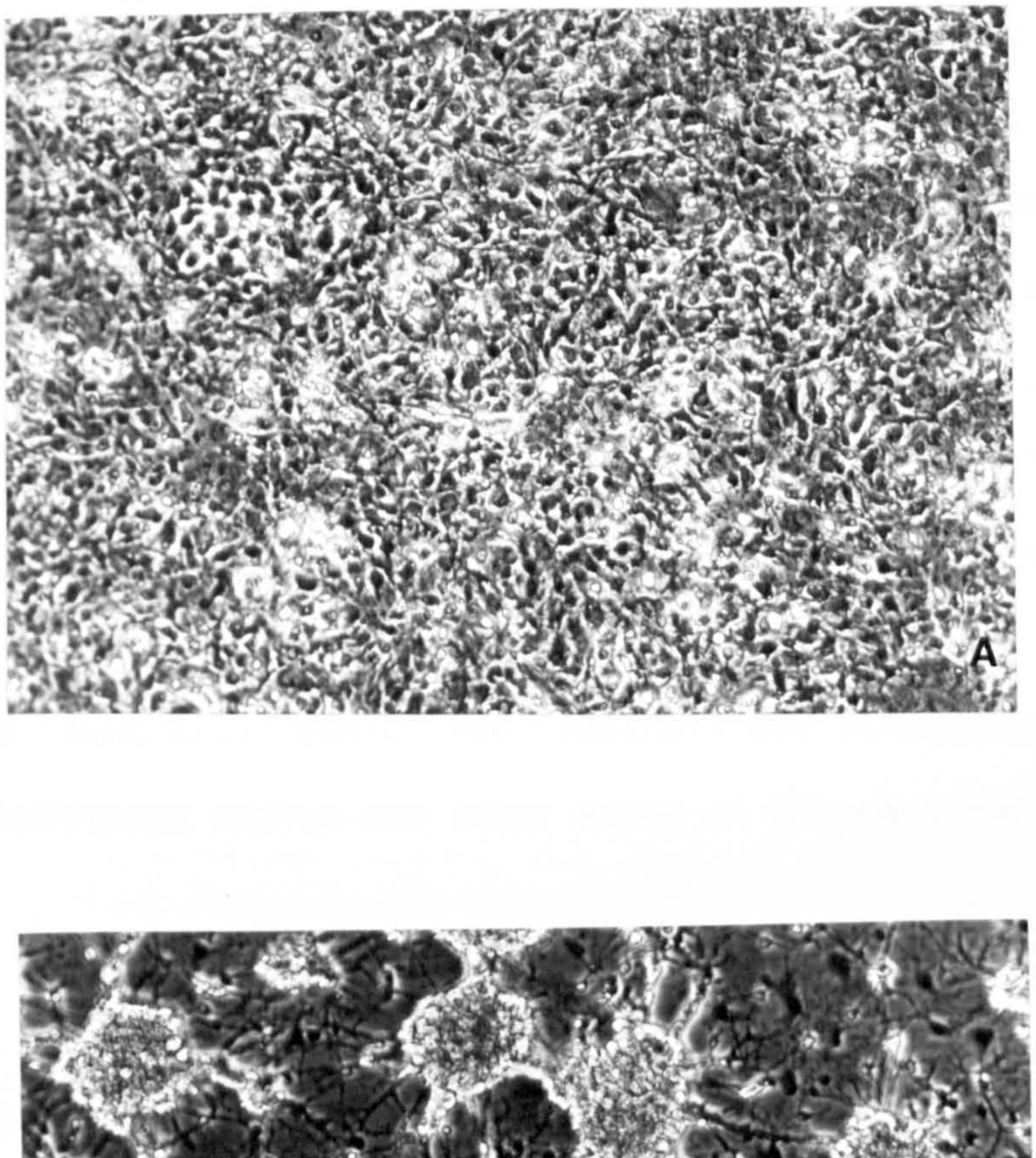
Figure 2.1.1. Glial cell cultures at different stages of development.

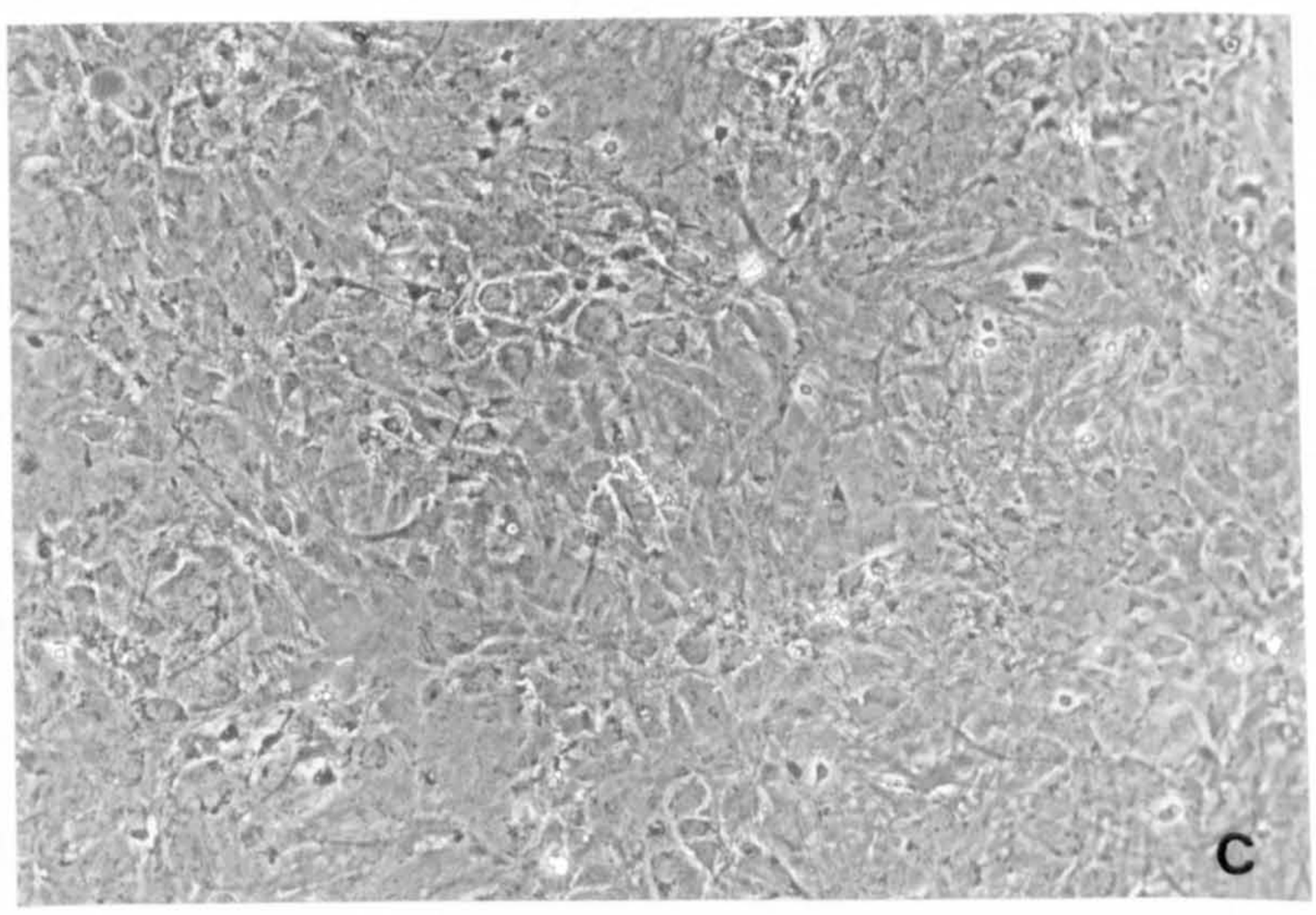
A: 21-day old primary glial cell culture

B: 21-day old glial subculture

C: 12-day old primary astrocyte culture prior to passage

(Magnification x 200)


A
B


C

For use of the astrocyte-conditioned medium, the method of Richardson et al. (1988) was followed. 24h. prior to use of the conditioned medium, cells were rinsed twice with serum-free DMEM and incubated in a modified Bottenstein and Sato (1980) medium. This consisted of DMEM with the following supplements; D-glucose (5.7 mg/ml), bovine insulin (50ng/ml), bovine serum albumin (0.1mg/ml), human transferrin (10.1mg/ml), progesterone (62ng/ml), putrescine chloride (1.6µg/ml), sodium selenite (40ng/ml), L-thyroxine (40ng/ml), triiodothyronine (30ng/ml), penicillen (100U/ml) and streptomycin (100µg/ml). Conditioned medium was later added at the required dilution.

2.2. IMMUNOFLUORESCENT LABELLING OF GLIAL CELLS

Cell-specific markers for identification of glial cells have been discussed earlier (see section 1.1.c.).

2.2.a. Cell specific markers

The antibodies that were used routinely were galactocerebroside (GC), a marker for oligodendrocytes (Raff et al., 1978), the astrocyte-specific marker glial fibrillary acidic protein (GFAP; Eng et al., 1971) and the progenitor cell and type-2 astrocyte marker A2B5 (Raff et al., 1983).

Monoclonal anti-GC antibody (culture supernatant) was a gift from Dr. M. Noble (Institute of Neurology, London) and was used at a 1+19 dilution. Monoclonal anti-GFAP antibody (ascites fluid) was a gift from Dr. N. Groome (Oxford Polytechnic) and used at a 1+99 dilution. Monoclonal anti-A2B5 antibody (ascites fluid) was a gift from Dr. F. Walsh (Institute of Neurology, London) and was used at a 1+159 dilution.

Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (affinity purified) was purchased from Sigma and used at a 1+19 dilution. Thimerosal (Sigma) was added to all antibody solutions to give a final concentration of 0.01% (w/v) in order to retard microbial growth.

2.2.b. Procedure

Cells were identified using cell-specific markers according to the method of Walker et al. (1985).

Culture medium was removed from the 25cm^2 flasks and the cells washed three times with Hank's balanced salt solution (HBSS), Ca^{2+} and Mg^{2+} free (Gibco), containing 20mM Hepes, pH 7.3, to remove all traces of media. 4mm diameter discs were cut from the base of each flask using a heated cork burner and taking care to prevent excessive drying of the cells during this process. An aliquot of HBSS-Hepes was added immediately after cutting each disc. The discs were then rinsed a further three times in HBSS-Hepes before fixing with 3.8% (v/v) formaldehyde in HBSS-Hepes for 5-10 min. at room temperature. Cells were then washed extensively in HBSS-Hepes prior to staining.

Protocols varied according to whether the antigens under investigation were situated on the cell surface or were intracellular. For labelling with A2B5 or GC, cells were initially incubated with 3% (v/v) goat serum in HBSS-Hepes for 30 min. to block non-specific binding of antibody; cells to be labelled with GFAP underwent a second fixation step (10 min. in 5% glacial ethanoic acid/95% ethanol at -20°C) followed by washing in HBSS-Hepes prior to incubation with primary antiserum. Cells were then incubated with the appropriate monoclonal antibody for 45 min.. At the end of the term of incubation the plastic discs were rinsed three times in HBSS-Hepes before a second 30 min. incubation in 3% (v/v) goat serum in HBSS-Hepes. Incubation with goat anti-mouse IgG-FITC for 30 min. followed. Cells were then washed in HBSS-Hepes and

mounted with glass coverslips using a solution of glycerol/PBS (9:1 v/v), pH 8.6 containing 2.5% (w/v) DABCO (1,4 diazobicyclo [2,2,2] octane; Aldrich).

Cells were viewed with a Nikon Labophot microscope equipped with phase contrast and epifluorescence optics providing an excitation wavelength of 460nm. Figure 2.2.1. shows the appearance of cells stained with anti-A2B5, anti-GC and anti-GFAP antibodies.

2.2.c. Photography

Cells were photographed using Kodak Technical Pan 2415 film at 400 ASA. A stock solution of Kodak HC110 developer diluted 1+3 in water and kept at 4°C was further diluted 1+9 in 20°C water. Developing of the film in a Paterson developing canister was carried out for 24 min. with inversions every 30 seconds. After rinsing twice in water the film was fixed in Kodafix (1+3 in water) for 5 min. at 20°C. A 30 min. rinse in water followed prior to drying the film in a heated cabinet.

2.3 COMPLEMENT-MEDIATED CYTOTOXICITY

Normal guinea pig serum was used as a source of complement; blood obtained by cardiac puncture from anaesthetised Strain-13 guinea pigs was left to clot at room temperature for 20 min. and then centrifuged (1000g for 30 min.). Alioquots of serum were stored at -80°C for up to four weeks without any significant loss of activity.

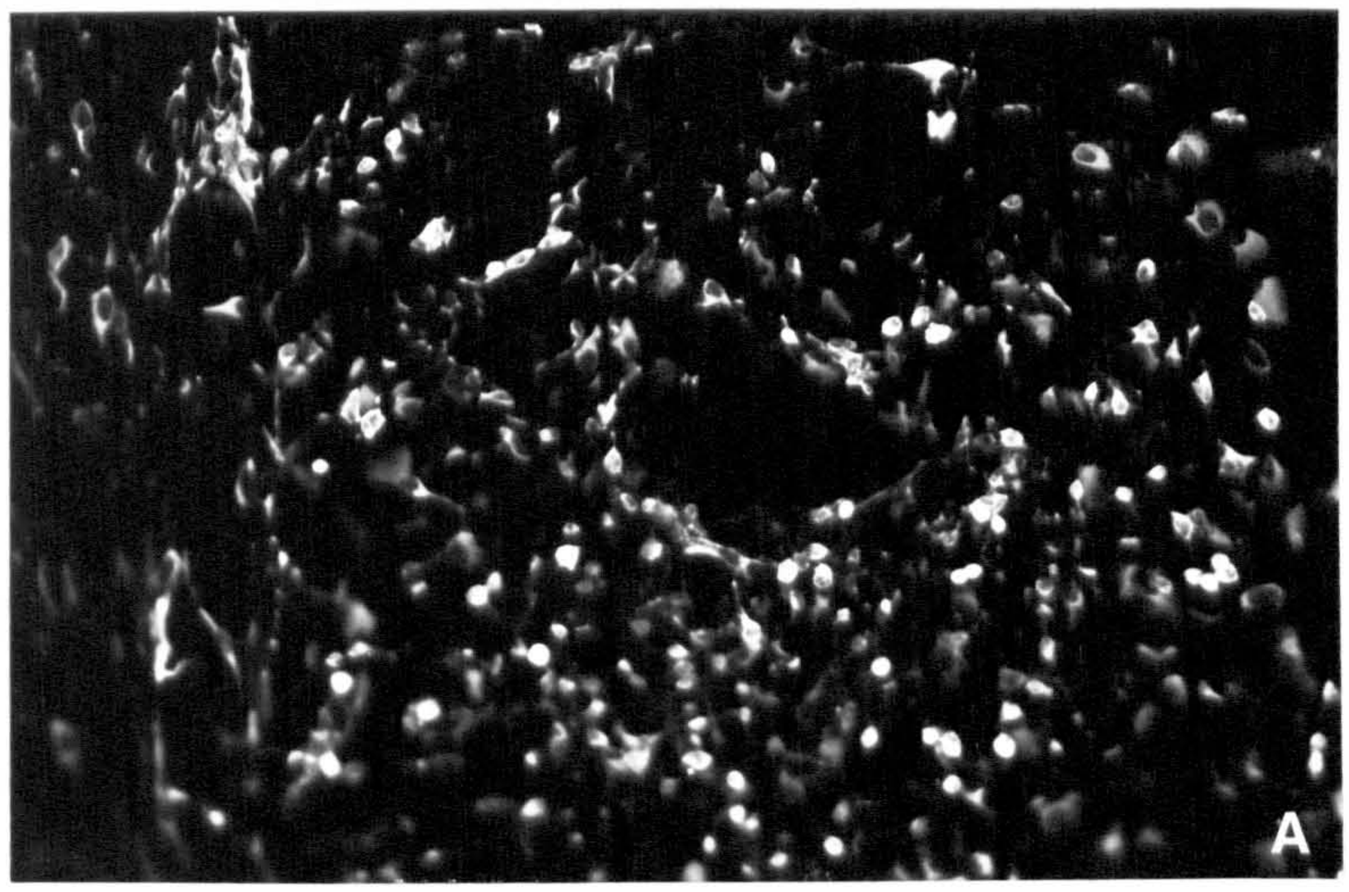
Flasks were rinsed with DMEM to get rid of any floating cells or debris and incubated with anti-A2B5 antibody (1+639 dilution) for 3h. at 37°C. Following two rinses with DMEM, a 20% (v/v) dilution of guinea pig serum was added and the flasks incubated for a further 30 min. at 37°C. Control cells were treated with guinea pig serum only in order to determine the level of non antibody-mediated complement cytotoxicity.

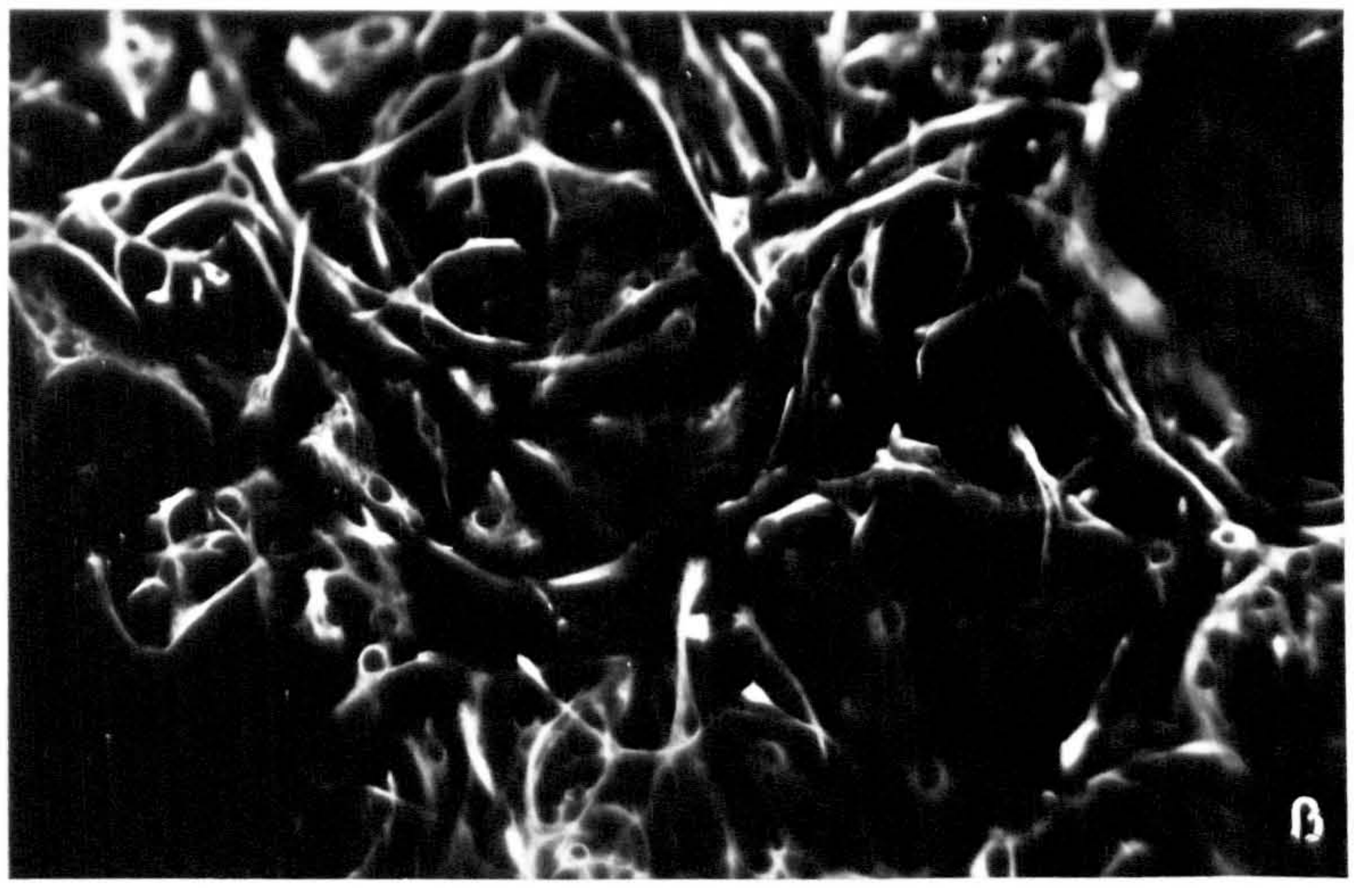
Figure 2.2.1. FITC-fluorescent labelling of 10-day old (A,B) and 21-day old (C) primary glial cell cultures.

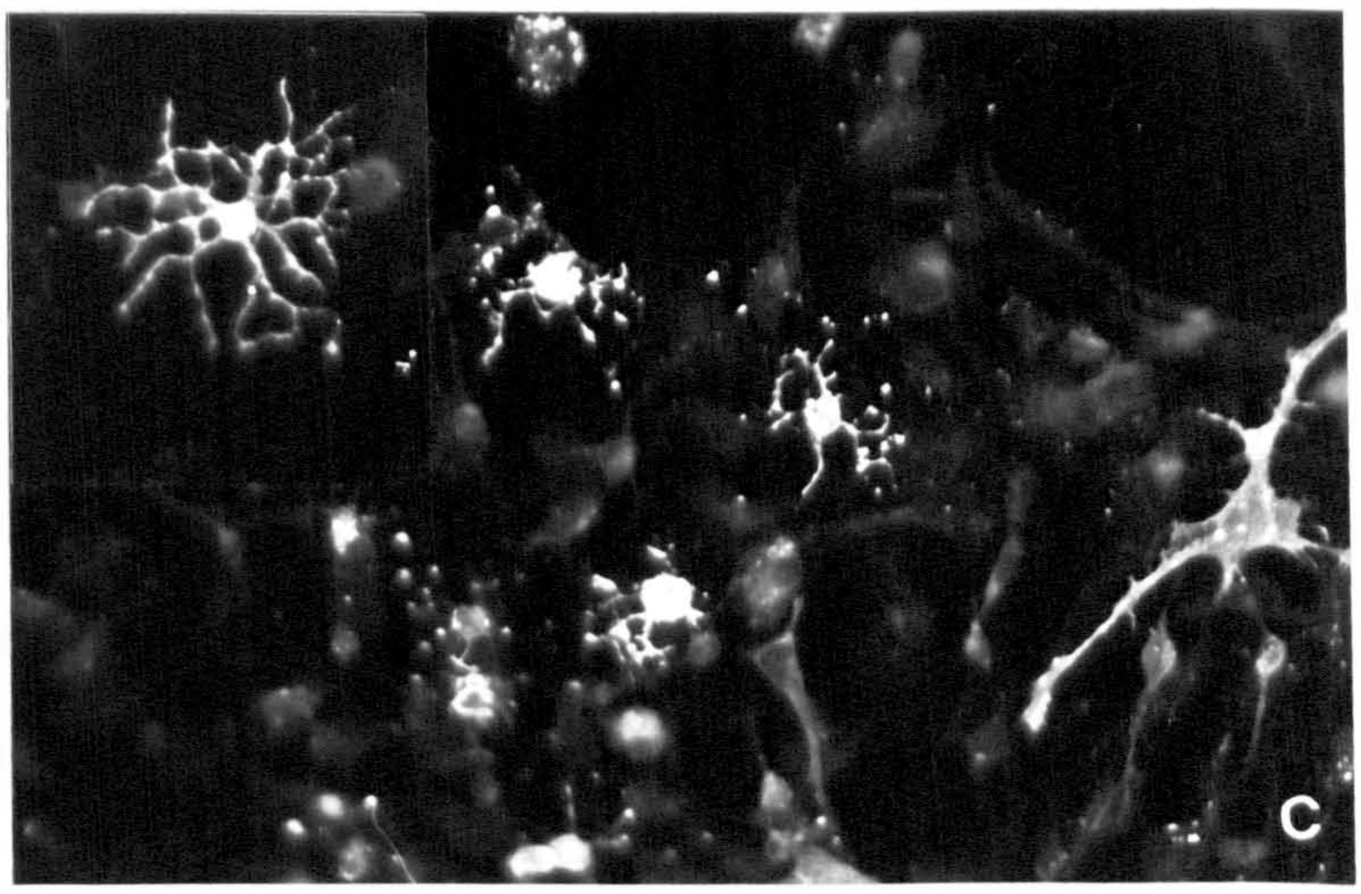
A: using anti-A2B5 antibody (magnification x 200)

B: using anti-GFAP antibody (x 200)

C: using anti-GC antibody (x 400)


A


B


C

Serum from three guinea pigs was tested at dilutions ranging from 1 to 50%; in all cases a 20% dilution was found to be appropriate. Cell damage was assessed using nigrosin dye exclusion and phase contrast microscopy.

2.4 PROTEIN KINASE C ASSAY

This method was adapted from that of Kikkawa et al. (1983).

2.4.a. Principle

PKC catalyses the transfer of the γ-phosphate of ATP to the seryl or threonyl residues of various protein substrates. The enzyme was routinely assayed by measuring the incorporation of ^{32}P from γ-^{32}P-ATP into calf thymus histone as a phosphate acceptor in the presence of Ca^{2+}, phospholipid and unsaturated DAG. After incubation, the radioactive histone was separated from the reaction mixture by precipitation with 25% (w/v) TCA, followed by vacuum filtration on a glass fibre membrane.

Basal activity was measured in the presence of ethylene glycol bis (β-aminoethylether) N, N, N', N'-tetraacetic acid (EGTA) instead of Ca^{2+}, phospholipid and DAG; activity was also measured omitting phospholipid and DAG. In this way the assay measured three types of kinase activity; calcium-independent (in the presence of EGTA), calcium-dependent (in the presence of Ca^{2+}) and calcium and phospholipid-dependent (in the presence of Ca^{2+}, phospholipid and DAG) kinase activity. Because the histone is not a specific substrate for PKC, indeed to date none have been identified which might be suitable for such an assay, one must depend upon the demonstration of Ca^{2+}- and phospholipid-dependence to identify PKC activity.

2.4.b. Procedure

The reaction mixture (250µl) contained 50mM Tris/HCl pH7.7, 50µg type IIIS histone (Sigma), 10µg phosphatidylserine (Lipid Products), 0.2µg diolein (Sigma), 100µM calcium chloride, 50µM ATP, 2µCi γ-^{32}P-ATP (3000 Ci/mmol, Amersham International) plus the enzyme fraction.

Before being added, the PS and diolein in chloroform/methanol (2:1 v/v) were mixed and the solvent removed under nitrogen. The residue was then resuspended in a small volume of 50mM Tris/HCl at pH 7.5 by sonication with a Decon sonifier FS100 for 10 min. at 4°C. The reaction was started by addition of enzyme to reaction mixture preequilibrated to the requisite temperature and the reaction continued for 10 min. at 30°C in a shaking water bath. All assays were in quadruplicate and addition of 2ml of cold 25% (w/v) TCA to each sample terminated the reaction. Samples were left on ice for at least 1h. when acid-precipitable material was collected on a glass fibre membrane filter (Whatman GF/F, pore size 0.7µm) by vacuum filtration. The tubes were washed out three times, each time with 3ml of 25% (w/v) TCA, and the membrane filter was successively washed with 25% (w/v) TCA, 8% (w/v) TCA and 70% (v/v) ethanol. Filters were then dried in an oven set at 37°C for one hour and placed in plastic minivials. 3ml of Optiphase liquid scintillant (LKB) was added to each minivial and the radioactivity determined with an LKB Minibeta 1212 liquid scintillation counter using the ^{32}P window for 6 min. or until an error of 1% was reached. PKC activity was determined by subtracting the level of ^{32}P incorporation into histone noted in the presence of EGTA alone from that noted in the presence of Ca^{2+}, phospholipid and DAG. Results were corrected for protein by a modification of the method of Lowry et al. (1951).

2.5. PREPARATION OF GLIAL CELL EXTRACT

Flasks of cells were washed three times with 1ml of homogenisation buffer (50mM Tris/HCl pH 7.5, 5mM dithiothreitol, 2mM EGTA, 100µg/ml leupeptin and 2mM phenylmethylsulfonyl fluoride [PMSF]) to remove all traces of culture medium, and the cells harvested by scraping them into 0.5 - 1ml homogenisation buffer using a rubber policeman. The cell suspension was disrupted by 20 strokes in a Potter-Elvehjem teflon-glass homogeniser at 700rpm, and the homogenate spun at 100,000g for 60 min. at 4°C using a Beckman SW60Ti rotor in an L2-65B ultracentrifuge (Beckman).

The supernatant recovered was employed as the crude cytosolic extract. To solubilise any PKC activity associated with the membrane, the particulate pellet remaining after the above spin was resuspended in homogenisation buffer containing 1% (v/v) Triton-X-100. The pellet was homogenised and left mixing for 30 min. at 4°C on a roller. The mixture was then centrifuged as before and the supernatant recovered employed as the crude source of membrane-bound PKC.

2.6 DE-52 COLUMN PURIFICATION OF CRUDE ENZYME EXTRACT

This method was previously described by Anderson et al. (1985). The entire procedure was carried out at 4°C.

Glass columns were manufactured in the departmental workshops and were 12cm x 1cm i.d. A glass wool plug was located at the tapered end of the column and a 2cm length of autoclavable plastic tubing sealed with a jubilee clip attached to that end.

DE-52 cellulose (Whatman) was equilibrated with 20mM Tris/HCl pH 7.5, and 2ml of a 1:1 (v/v) slurry of equilibrated DE-52 cellulose was poured into the columns to give a 1ml packed-bed volume. The packed

column was then washed with 10ml of column buffer (20mM Tris/HCl pH 7.5, 2mM ethylenediaminetetraacetic acid (EDTA), 0.5mM EGTA, 50mM β-mercaptoethanol) and left for at least 12h. prior to use.

Both cytosolic and particulate fractions (2-4ml) were applied to the DE-52 columns which had previously been washed with 15ml each of column buffer. Following sample addition, the columns were washed with 10ml of column buffer prior to elution of bound PKC with 3ml of column buffer containing 120mM NaCl. An alternate method of Kikkawa et al.(1986), eluting with a 10ml 0-0.3M gradient and collecting 0.5ml fractions was used at first, but later replaced with this method of Anderson et al. (1985). Fractions collected were assayed for PKC activity on the same day.

2.7 TREATMENT OF CELL CULTURES WITH PHORBOL ESTER AND CALCIUM IONOPHORE

12-O-tetradecanoylphorbol-13-acetate (TPA), 4-α-phorbol and dimethylsulphoxide (DMSO) were all obtained from Sigma. TPA and 4-α-phorbol were diluted in DMSO to give a 1mg/ml stock solution. 100µl aliquots of the stock solutions were frozen at -80°C, and stocks replenished monthly.

Prior to use, aliquots of stock solution were diluted appropriately in tris saline and a 10-50µl aliquot added to the cell cultures. These dilution factors meant that the final concentration of DMSO added to the cultures was less than 0.001% (v/v).

Calcium ionophore (A23187) was obtained from Sigma and dissolved in DMSO to give a stock solution of 5mg/ml. The final concentration of DMSO added to the cultures was less than 0.01% (v/v).

Extreme caution was necessary when using the phorbol ester and calcium ionophore. Suitable protective clothing was worn at all times, including two pairs of rubber gloves. All solutions containing phorbol

ester were brought to pH≥11 after use to destroy their potential tumour-promoting activity (Wooge and Conn, 1987) and all contaminated material was left to soak in 1M sodium hydroxide for 48h. prior to disposal.

2.8 USE OF HEAVY METALS

The following compounds were used as sources of divalent heavy metal ions. All were of the purest grade commercially available: arsenous oxide (As_2O_3); barium chloride ($BaCl_2.2H_2O$); cadmium chloride ($2CdCl_2.5H_2O$); copper chloride ($CuCl_2.2H_2O$); lead chloride ($PbCl_2$); manganese chloride ($MnCl_2.4H_2O$); mercuric chloride ($HgCl_2.2H_2O$); nickel chloride ($NiCl_2.6H_2O$); zinc oxide (ZnO).

Due to the extreme toxicity of some of the above compounds great care had to be exercised in the handling of the solids and the solutions made up, and in the disposal of contaminated glassware and pipette tips. Suitable protection, including the use of two pairs of rubber gloves, was worn at all times.

2.9 CELL PROLIFERATION ASSAY

2.9.a. Labelling of cells

Methyl-^{3}H-thymidine (5Ci/mmol; Amersham) was added to newly-fed cultures at 1µCi/ml for the appropriate time period. Cultures were then rinsed twice with tris saline to remove excess label and the cells scraped into a known volume of double distilled water using a rubber policeman.

2.9.b. Determination of levels of incorporated radioactivity

The cell extracts were disrupted by 10 strokes in a Potter-

Elvehjem teflon-glass homogeniser at 700rpm. Triplicate 30µl aliquots were added to 2ml of ice-cold 8% (w/v) TCA and the tubes left on ice for at least 1h. Triplicate 10µl aliquots were taken for protein estimation as detailed in section 2.11.

TCA-precipitable material was collected on a glass fibre membrane filter (Whatman GF/A, pore size 1.2µm) by vacuum filtration. Tubes were rinsed twice with 3ml of 8% (w/v) TCA and the membrane filter washed with 8% (w/v) TCA and 70% (v/v) ethanol. Filters were then dried in an oven set at 37°C for one hour and placed in plastic minivials. 3ml of Optiphase liquid scintillant (LKB) was added to each minivial and the radioactivity determined with an LKB Minibeta 1212 liquid scintillation counter using the ^{3}H window for 6min. or until an error of 1% was reached. All results were corrected for protein .

2.9.c. Photography

Cells were routinely examined using a Nikon inverted microscope, and photographs taken using Kodak Technical Pan 2415 film exposed at 70 ASA and developed at 50 ASA using Kodak HC110 developer (1+79 dilution with water for 8 min. at 18°C) with fixing as described earlier (section 2.2.c.).

2.10 HYDROXYLAPATITE COLUMN CHROMATOGRAPHY

2.10.a. Initial Method

DE-52 purified fractions obtained from 100,000g whole glial cell extract or rat cerebral extract were applied directly to a packed Biogel hydroxylapatite column (Biorad, 0.78x10cm with 0.4x5cm guard column) connected to a Pharmacia FPLC system as advised by M. Shearman (personal communication) and detailed by Kosaka et al. (1988). The column was previously equilibrated with 20mM potassium phosphate buffer pH 7.5

containing 0.5mM EGTA, 0.5mM EDTA, 10% (v/v) glycerol and 10mM β-mercaptoethanol. At least 10^7 cells were required for the DE-52 purification and resolution of the enzymes (M. Shearman, personal communication), representing a protein level in the region of 2-3mg. The column was washed with two column volumes of the buffer and the enzyme eluted by application of a linear 20-215mM potassium phosphate buffer gradient in 84ml at a flow rate of 0.4ml/min. collecting 1ml fractions. All procedures were carried out at 0-4°C.

Aliquots of the collected fractions were then tested for PKC activity as detailed previously (see section 2.4).

2.10.b.Refined Method

(i) Sample preparation

The 3ml DE-52-purified fractions obtained from glial cell extract or rat cerebral extract were dialysed overnight against 500ml of 20mM potassium phosphate buffer pH 7.5 at 4°C.

(ii) Separation of multiple subspecies

Sample (1-2mg protein) was loaded onto a packed ceramic hydroxylapatite column (Tonen, Tokyo, 0.78x10cm with no guard column) connected to a Pharmacia FPLC system. The previously equilibrated column was washed with two column volumes of the same 20mM potassium phosphate buffer and the PKC eluted by application of a linear 20-300mM potassium phosphate buffer gradient in 84ml, at a flow rate of 0.4ml/min. collecting 1ml fractions. Aliquots from collected fractions were tested as before. The whole process from homogenisation of glial cells or rat brain through to assessment of PKC activity by transfer of γ-^{32}P from ATP to histone, and including overnight dialysis, took approximately twenty-four hours.

A list of important points to consider when using an HA column is included in the Appendix.

2.11 PROTEIN ESTIMATION

The protein content of all samples was estimated using a modification of the method of Lowry et al. (1951). Four reagents were prepared:

A. 2% (w/v) Na_2CO_3 in 0.1M NaOH

B. 0.5% (w/v) $CuSO_4.5H_2O$ in 1% (w/v) sodium citrate

C. 1ml of B mixed with 49ml of A just prior to use

D. Folin-Ciocalteau reagent (Sigma) diluted 1:1 with water to give a 1M solution.

An aliquot of the sample to be assayed containing 5-50µg protein was made up to 100µl with distilled water, and 1ml of reagent C was added. The solution was allowed to stand at room temperature for 10 min. when 100µl of solution D was added and mixed immediately using a vortex mixer. The colour was allowed to develop for 60-120 min. at room temperature when its absorbance was measured at 750nm using a CE-292 UV-spectrophotometer (Cecil Instruments) fitted with a microsipette attachment.

Because the absorbance varies non-linearly with protein concentration using this method, a standard calibration curve was constructed each time using bovine serum albumin at concentrations ranging from 1-50mg/ml in distilled water. All estimates were conducted in triplicate. A typical standard curve is shown in figure 2.11.1.

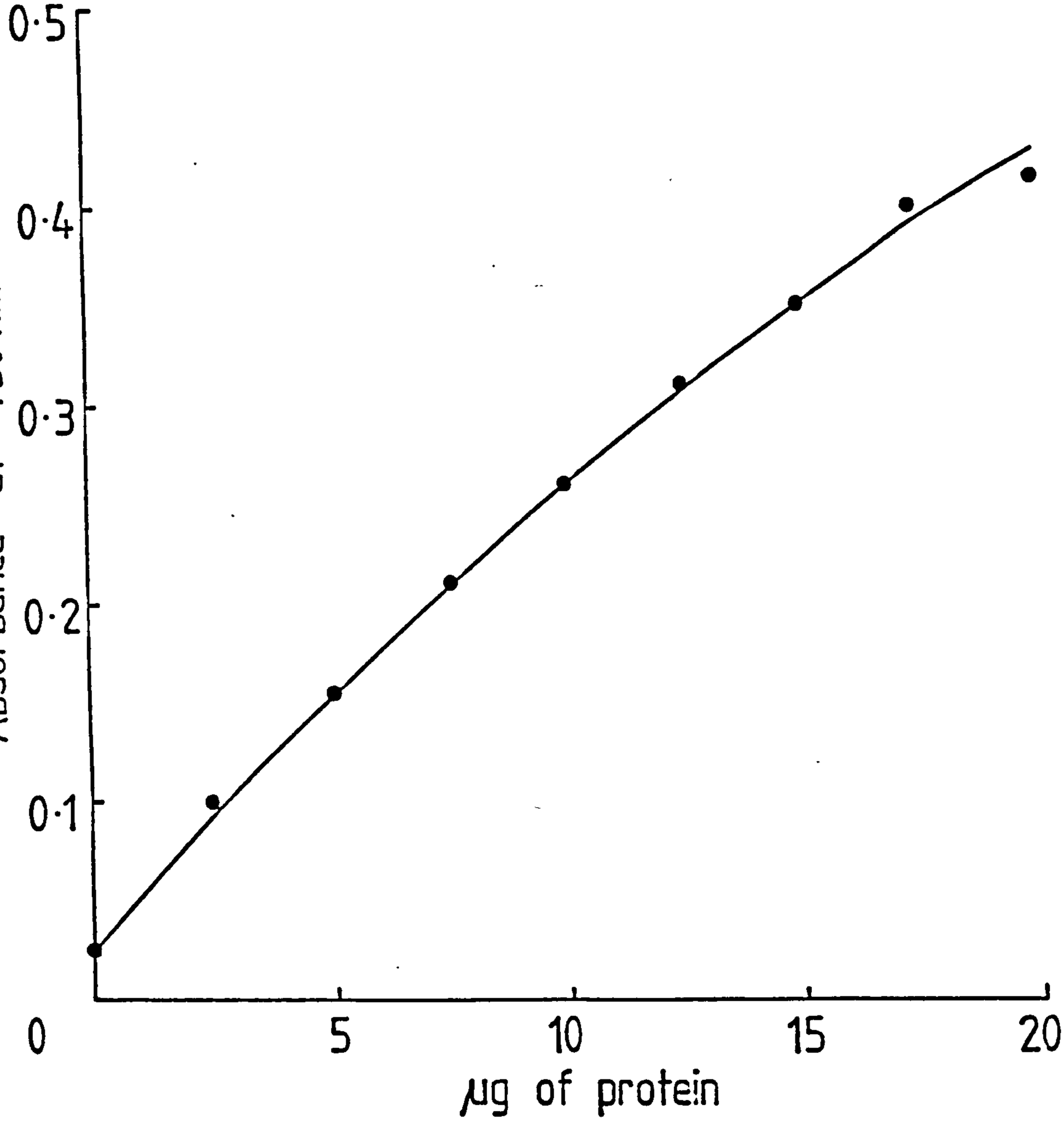


Figure 211.1. A typical protein standard curve produced using the method of Lowry et al. (1951).

Each point represents the mean of triplicate samples.

2.12 SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Proteins were separated using SDS-PAGE with a discontinuous buffer system as described by Laemmli (1970).

2.12.a. Reagents

(i) 60% (w/v) acrylamide : 1.6% (w/v) N'N'-bis-methylene acrylamide in double distilled water. 0.1g of activated charcoal was added, stirred for 15 min. to absorb impurities, and then removed by filtering twice through Whatman 541 filter paper. The solution was stored at 4°C.

(ii) 10% (w/v) sodium dodecyl sulphate

(iii) Resolving gel buffer : 1.5M Tris/HCl, pH 8.8

(iv) Stacking gel buffer : 0.5M Tris/HCl, pH 6.8

(v) Running buffer : 25mM Tris, 192mM glycine, 0.05% (w/v) SDS, adjusted to pH 8.3 with HCl

(vi) NNN'N'-tetramethylethylenediamine (TEMED)

(vii) 10% (w/v) ammonium persulphate

(viii) Sample buffer : 65mM Tris/HCl, pH 6.8 containing 2% (w/v) SDS, 5%(w/v) β-mercaptoethanol, 10% (v/v) glycerol and 0.002% (w/v) malachite green

(ix) Staining solution : 0.1% (w/v) Coomassie blue, 50% (v/v) methanol, 7.5% (v/v) glacial ethanoic acid

(x) Destaining solution : 5% (v/v) methanol, 7.5% (v/v) glacial ethanoic acid

2.12.b. Procedure

Resolving gels were made with a gradient of 6-30% (w/v) acrylamide, with 6% (w/v) acrylamide stacking gels. Gradients were formed using a two chamber gradient maker attached to a peristaltic pump. Gels were of 0.75mm thickness and volumes sufficient for two such gels were mixed as follows:

Reagent	Volume Added	
	6% acrylamide	30% acrylamide
Acrylamide	1.50ml	7.50ml
Gel buffer	3.75ml	3.75ml
SDS	0.15ml	0.15ml
Water	9.60ml	3.60ml
Ammonium persulphate	30µl	30µl
TEMED	15µl	15µl

The ammonium persulphate was made freshly on each occasion a gel was run, and it and the TEMED were added to the gel mixtures just prior to casting the gel. An overlayer of butan-1-ol was added once the gels were cast; this was removed by rinsing four times with running buffer once the gel had set. 15-place combs were then inserted between the gel plates, and a 6% stacking gel solution (prepared as for the 6% acrylamide gel solution tabled above but with the replacement of resolving gel buffer by stacking gel buffer) added. Following full polymerisation of the stacking gel the comb was removed and the wells filled with running buffer prior to the loading of samples.

2.12.c. Loading of samples and electrophoresis

Sample protein concentration (estimated by the Lowry method, see section 2.11) was adjusted to between 1 and 5mg/ml. Samples were boiled for 2-3 min. and then sonicated for 10 min. in a Decon ultrasonic

bath to achieve complete solubilisation. 10-40µl aliquots were applied to each well of the gel using a microsyringe and the gels assembled into the electrophoretic apparatus according to the manufacturer's instructions. Top and bottom reservoirs were filled to the appropriate levels with running buffer and the apparatus connected to a Shandon-Southern Vokam power pack. Gels were run at a constant current of 4mA per gel for 15-20h.

2.12.d.Recovery and staining of gels

Gels were recovered by removing the side spacers of the gel former and gently levering the glass plates apart using a spacer. Gels were then transferred to a plastic tray containing staining solution and shaken for 1h. At the end of this time the staining solution was replaced with destaining solution and shaking continued, with periodic renewal of destain, until excess stain has been removed.

2.12.e.Detection of radioactivity in polyacrylamide gels

Gels to be autoradiographed were dried under vacuum onto 4MM chromatography paper. Radioactive ink was spotted onto the paper to act as a marker for later alignment. Under safelight (Ilford F904) a sheet of Hyperfilm β-max (Amersham) was placed against the gel in an autoradiographic casette, and the autoradiograph left exposed to the gel for up to two weeks. Films were developed under safelight for 5 min. in D19 developer (Kodak) with constant agitation followed by a 1 min. rinse in running tap water, and then fixed for 5 min. in Kodafix (1+3 in water). Films were then thoroughly rinsed in running tap water and dried in a heated cabinet.

2.12.f. Photography

Gels and autoradiographs were photographed using Kodak Technical Pan 2415 film at 100 ASA. A stock solution of Kodak HC 110 developer

diluted 1+3 in water was kept at 4°C. This was diluted 1+9 with water and the film developed in a Paterson developing canister at 20°C for 8 min. with 10 inversions per min. The film was then rinsed twice with water (20°C) and fixed in Kodafix (1+3 in water) for 5 min., again at 20°C. A 30 min. rinse in water followed prior to drying the film in a heated cabinet.

3. RESULTS AND DISCUSSION

3.1. DEVELOPMENT OF AN ASSAY TO MEASURE PROTEIN KINASE C IN GLIA

INTRODUCTION

Despite many suggestions concerning the likelihood that CNS glia contained PKC activity no biochemical investigations into such a possibility had taken place. Preliminary experiments (Rumsby and Murphy, unpublished) showed that nanomolar concentrations of phorbol ester caused a stimulation in ^{32}P-orthophosphate uptake into protein and lipid in mixed glial cell cultures, inferring a PKC-mediated response. However, such a response required quantitating, and an assay for PKC was needed. Hence, the initial aim of the work was to develop a satisfactory and reproducible assay for measurement of PKC activity in glial cells to answer the question "Is there any detectable PKC activity in glia?". Although a number of methods existed for assaying PKC in other cell types (e.g. Donnelly et al., 1985; Neary et al., 1986b; Kikkawa et al., 1986), there was considerable variability in technique so the assay had to be adapted and developed for use in glia. With a standardised procedure further investigations could take place and measurements be made.

RESULTS AND DISCUSSION

3.1.a. First Attempts

Initially, for the sake of simplicity, the method of Neary et al. (1986b) was followed; a 23,000g cytosolic extract was used as the source of glial PKC. This method purported to allow measurement of cellular PKC levels quickly using only a small number of cells. Levels of PKC were assayed in both rat cerebrum and in cytosolic extracts from

TABLE 3.1.1. KINASE ACTIVITY IN 23,000g SUPERNATANT OF 4 WEEK OLD GLIAL SUBCULTURES USING HISTONE TYPE IIAS AS SUBSTRATE

cpm/µg protein		
EGTA[a]	Ca^{2+} [b]	Ca^{2+}/PS[c]
54.0±14.3	44.4±14.1	50.6± 7.6

[a] Ca^{2+}-independent kinase activity

[b] Ca^{2+}-dependent kinase activity

[c] Ca^{2+}-and phosphatidylserine-dependent kinase activity

Results are expressed as mean ± standard deviation (S.D.) from three separate sets of flasks.

TABLE 3.1.2. KINASE ACTIVITY IN 23,000g SUPERNATANT OF RAT CEREBRUM USING HISTONE TYPE IIAS AS SUBSTRATE

cpm/µg protein		
EGTA[a]	Ca^{2+} [b]	Ca^{2+}/PS[c]
38.5±3.0	168.5±9.1	174.5±12.0

[a] Ca^{2+}-independent kinase activity

[b] Ca^{2+}-dependent kinase activity

[c] Ca^{2+}-and phosphatidylserine-dependent kinase activity

Results are expressed as mean ± S.D. from three separate sets of determinations using the same enzyme source.

TABLE 3.1.3. KINASE ACTIVITY IN 23,000g SUPERNATANTS OF MIXED GLIAL CELL CULTURES OF DIFFERENT AGES USING HISTONE TYPE IIAS AS SUBSTRATE

CULTURE TYPE AND AGE	cpm/µg protein EGTA[a]	Ca^{2+} [b]	Ca^{2+}/PS[c]
PRIMARY CULTURES			
10-day old	304.5±12.8	295.0±21.5	350.9±68.8
5-day old	321.5±77.5	378.3±7.5	377.1±49.4
21-day old	227.7±11.6	272.0±13.3	279.5±38.6
SUBCULTURES			
21-day old	320.1±17.3	331.4±120.3	357.9±15.4
28-day old	355.7±43.0	484.3±65.9	501.8±17.6

[a] Ca^{2+}-independent kinase activity

[b] Ca^{2+}-dependent kinase activity

[c] Ca^{2+}-and phosphatidylserine-dependent kinase activity

Results are expressed as mean ± S.D. from at least three separate experiments.

mixed glial cell cultures with variable results as shown in tables 3.1.1.- 3.1.3. Table 3.1.1. gives the first results obtained using 4-week old glial subcultures. Neither these data nor the finding in table 3.1.2. where rat brain was employed as the source of enzyme seemed very hopeful results. However, results obtained when flask number per assay was increased from two to at least six, were far more encouraging. See table 3.1.3.

The table above shows results obtained using glial cultures of different ages. Most show a slight increase in counts incorporated into type IIAS histone on addition of Ca^{2+} and PS. However, the poor

reproducibility within replicate samples was a major cause for concern and the large standard deviations meant that there was no significant PKC activity detectable.

3.1.b. Redefining Some Experimental Parameters

The substrate employed to measure PKC activity, type IIAS histone, was not very pure and it was replaced by the purer lysine-rich type IIIS histone in the hope that this might give some improvement in replicate reproducibility. Type IIIS histone is not a PKC-specific substrate, since it will also act as a substrate for cAMP-dependent protein kinase for example, so it was still necessary to demonstrate PKC activity by an increase in levels of phosphorylated histone on addition of Ca^{2+} and PS. However, PKM is less active in the presence of type IIIS histone than type IIAS, so background (basal) activity ought to be reduced accordingly. A further modification to the assay was to add the unlabelled and labelled ATP to the assay mixture together in order that the very small levels of labelled ATP would not undergo hydrolysis. The third modification was to add the PS/diolein mixture and then prepare the lipid micelles fresh daily, so overcoming any potential problems of micellar aggregation or oxidation of unsaturated fatty acids in the diolein. The assay was terminated by streaking aliquots of the assay medium onto phosphocellulose paper. The paper was rinsed in water and then Cherenkov counted or dried in acetone and liquid scintillation counted as normal. The results are shown in table 3.1.4.

The disappointing results, coupled with practical difficulties in handling a large number of samples given the radioisotope employed, meant that the phosphocellulose paper method of assay termination was considered to be inferior to the TCA-precipitable protein filtration method using glass fibre filters. The latter method was hence resumed.

It was believed that one of the reasons for the poor replicate

reproducibility and the difficulty in detecting significant PKC activity might be due to a number of 'contaminants' in the 15,000g cytosolic extract, such as gangliosides in plasma membrane fragments. These will inhibit PKC activity (Vaccarino et al., 1987). Hence, in an attempt to improve upon this, an adaptation of the method of Kikkawa et al. (1983) was employed with the main change in technique being the use of a 100,000g supernatant as the enzyme source. This should further eliminate the possibilities of plasma membrane contamination, and led to an improvement in replicate sample reproducibility as shown in table 3.1.5. Of greater importance, however, was the fact that the results showed a significant increase in phosphorylation of type IIIS histone upon addition of Ca^{2+} and PS in a number of different aged mixed glial cell culture extracts. This indicated the presence of PKC in extracts of glial cell cultures.

3.2. INCREASING THE SENSITIVITY OF THE ASSAY

INTRODUCTION

The next step in developing the PKC assay for a mixed glial cell system was to increase the assay sensitivity. The enzyme source employed was a crude one containing contaminating factors which made the task of accurate quantitation of a Ca^{2+}- and phospholipid-dependent enzyme very difficult. Kikkawa et al. (1983) cited the problems of the presence of numerous other kinases which may react with some undefined endogenous substrates, and also of small quantities of calcium and phospholipid which may present difficulties when attempting to demonstrate the absolute requirement of PKC for Ca^{2+} and phospholipid. These authors suggested that the problem of contaminants may be ameliorated by use of DEAE-cellulose (DE-52) column chromatography to purify the 100,000g extract prior to enzyme assay.

TABLE 3.1.4. KINASE ACTIVITY IN 23,000g SUPERNATANT OF RAT CEREBRUM USING HISTONE TYPE IIIS AS SUBSTRATE AND PHOSPHOCELLULOSE PAPER FOR ASSAY TERMINATION

	cpm		
	EGTA	Ca^{2+}	Ca^{2+}/PS
Cherenkov counting	245.6±69.8	263.3±280.6	268.7±56.3
Acetone & liquid scintillation counting	16471.7±5526.5	21027.7±2190.3	22707.5±4237.5

Results are expressed as mean ± S.D. from three separate sets of determinations using the same enzyme source.

TABLE 3.1.5. KINASE ACTIVITY IN 100,000g SUPERNATANT OF RAT BRAIN AND MIXED GLIAL CELL CULTURES USING HISTONE TYPE IIIS AS SUBSTRATE

ENZYME SOURCE	cpm/µg protein		
	EGTA	Ca	Ca^{2+}/PS
Rat cerebrum	111.7±17.5	116.4±9.3	154.7±6.6
14 day primaries	70.6±8.0	106.4±10.7	192.5±8.3
28 day primaries	139.6±3.7	270.6±11.9	361.6±12.6
28 day subcultures	130.2±14.4	520.0±6.8	622.4±12.9

Results are expressed as mean ± S.D. from at least three separate experiments for the glial cells and from three separate sets of determinations using the same enzyme source for the rat brain.

RESULTS AND DISCUSSION

The method of Kikkawa et al. (1983) was followed essentially as detailed in section 2.6 but using a 0-0.3M NaCl gradient with collection of 0.5ml fractions. This technique yielded a broad peak of activity between about 80 and 180mM NaCl (see figure 3.2.1). As this peak was so broad, and this was the final stage of purification prior to assay, it was decided to follow the method of Anderson et al. (1985) which does not involve fractionation of the crude extract, but simply elutes the activity from the DE-52 column in one aliquot (see section 2.6). Results obtained using this method showed a dramatic increase in activity when compared to the crude 100,000g extract but also showed a considerable purification of PKC activity of over 20-fold in some cases (see table 3.2.1). It was now apparent that the assay could be used as a routine method for estimating levels of PKC in mixed glial cell cultures.

Further modifications made to the assay at a later stage were as follows:

(i) cultures were rinsed in homogenisation buffer instead of PBS prior to scraping into and homogenising in the same buffer, as even low salt concentrations in the prepared subcellular fractions may interfere with binding of PKC to DEAE-cellulose (Thomas et al., 1987);

(ii) leupeptin and PMSF were added to the homogenisation buffer at concentrations of 100µg/ml and 2mM respectively. Both compounds inhibit protease action and will minimise the proteolytic degradation of PKC;

(iii) experiments undertaken to assess the optimal NaCl concentration for elution of the DEAE-cellulose-bound enzyme established that a 3ml aliquot of 0.12M NaCl in DE-52 column buffer was adequate. This modified protocol is fully listed in sections 2.5. and 2.6.

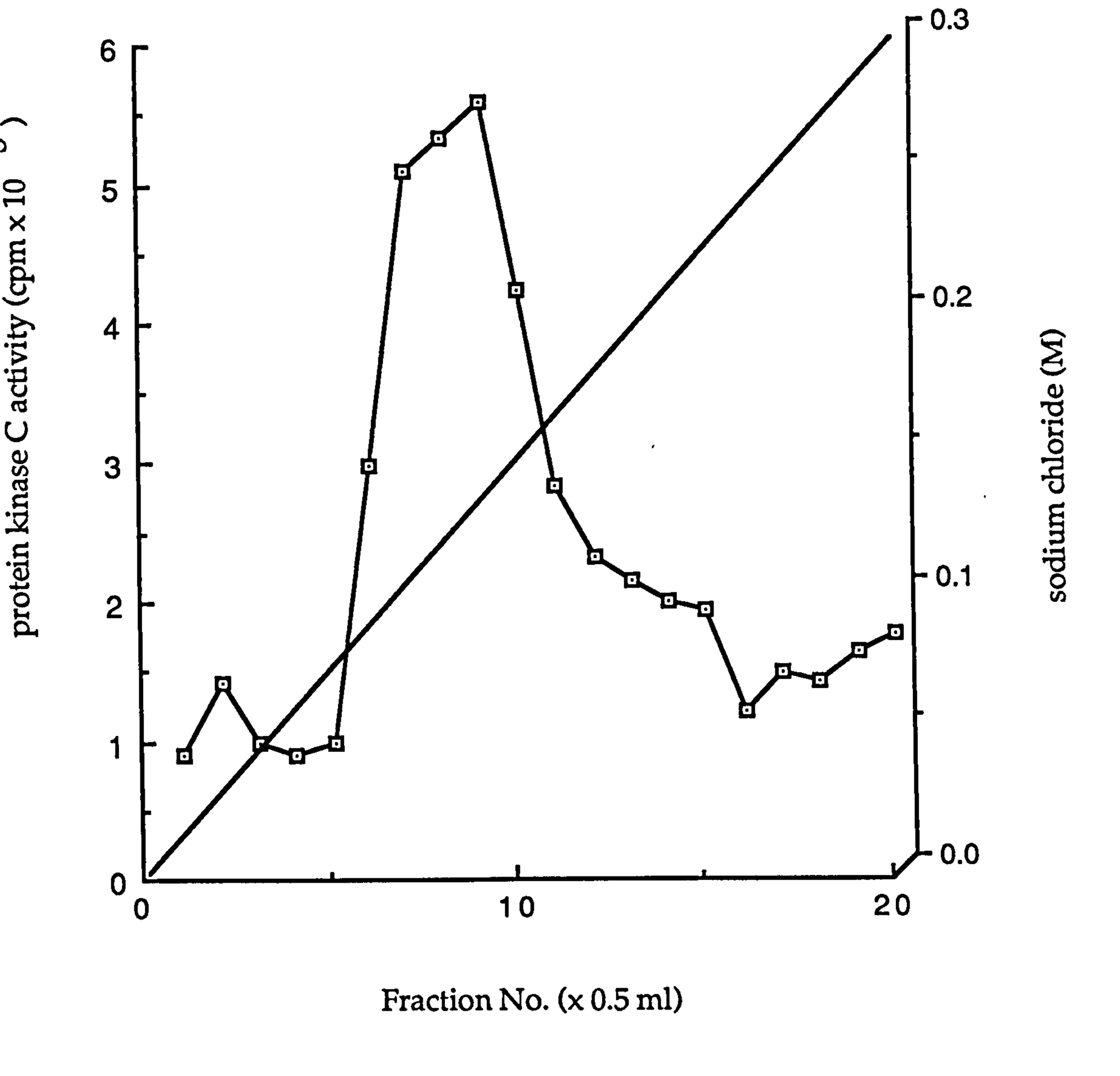


Figure 3.2.1. DE-52 column fractionation of 100,000 g glial cell supernatant.

Method was as detailed in sections 2.4 - 2.6. Each point shown represents the mean of triplicate determinations of Ca/PS - dependent phosphorylation of type III-S histone for each numbered fraction.

TABLE 3.2.1. EFFECT OF DE-52 COLUMN PURIFICATION ON KINASE ACTIVITY IN MIXED GLIAL CELL CULTURE 100,000g EXTRACTS USING TYPE IIIS HISTONE AS SUBSTRATE

DAYS IN PRIMARY CULTURE	ENZYME SOURCE	cpm/µg protein EGTA	Ca^{2+}	Ca^{2+}/PS
17	100,00g SUPERNATANT	129.8±23.6	170.9±7.4	199.3±12.2
	DE-52-PURIFIED FRACTION	155.4±28.4	223.9±56.9	6928.3±161.9
21	100,000g SUPERNATANT	94.2±2.1	174.4±7.4	244.3±7.9
	DE-52-PURIFIED FRACTION	337.9±40.9	498.9±51.9	5456.5±61.6
28	100,000g SUPERNATANT	149.8±6.8	257.2±4.5	323.2±9.4
	DE-52-PURIFIED FRACTION	232.1±36.4	233.9±60.2	1980.6±42.8

Results are expressed as mean ± S.D. from at least three separate experiments.

3.3. WHAT IS THE NATURE OF THE INHIBITORY SUBSTANCE REMOVED BY THE DE-52 COLUMN CHROMATOGRAPHY STEP?

INTRODUCTION

Purification of glial cell supernatants on a DE-52 anion exchange resin resulted in a large increase in measurable PKC activity (see section 3.2). Following this observation, the question to be asked concerned the exact nature of the inhibitory substance removed by the DE-52 column procedure. One possibility was that this substance may be a physiological inhibitor of PKC or that it was possibly related to a 17kDa inhibitor of the enzyme that has been characterised by McDonald et al. (1987).

RESULTS AND DISCUSSION

3.3.a. Crude 100,000g supernatant dephosphorylates phosphorylated histone

Crude 100,000g glial cell supernatant was added to a glial supernatant purified by passage through a DE-52 column. The mixture was then assayed for PKC activity. As shown in table 3.3.1 the crude extract caused a decrease in measurable PKC levels as detected by histone phosphorylation. Although the results are given for levels assayed in 16 day old primary cultures, the same pattern was seen in every age of glial cell culture tested. The crude extract reduced the PKC activity but the mechanism by which this inhibition was achieved was not apparent from these results. Inhibition increased with the amount of crude extract added, beginning to plateau at about 25µl enzyme added. This corresponded to 19µg protein added as shown in figure 3.3.1. Total DE-52 column-purified enzyme present in this assay amounted to 13µg protein.

In an attempt to determine how the crude supernatant produced its

inhibitory action, a column-purified supernatant sample was assayed in the usual manner for 10 min. but, instead of terminating the reaction at this point by addition of 25% (w/v) TCA as described in the Methods section, an aliquot of the crude 100,000g supernatant was added and the reaction continued for a further 10 min. As the data in table 3.3.2. shows, this caused a large reduction in counts appearing as phosphorylated histone on addition of the crude sample. Thus the crude supernatant appears to be exerting its inhibitory action by dephosphorylating the histone phosphorylated by PKC, and therefore does not affect the enzyme itself but inhibits the assay for quantitating PKC activity. Dephosphorylation of PKC-phosphorylated histone by crude supernatant is very rapid, and is all but complete by 3 min. as shown in figure 3.3.2. In this experiment using 26-day old primary glial cells, a 50µl (=62µg protein) aliquot of crude supernatant was added to a 50µl (=21µg protein) aliquot of column-purified supernatant. Again, however, this phenemenon was detected in all cell fractions tested irrespective of culture age, and using protein ratios of crude : column-purified of between 0.25 and 4. Gradient SDS-PAGE of the samples was carried out, and a typical autoradiograph is shown in figure 3.3.3. This clearly shows a decrease in phosphorylation (arrowed) on addition of the 100,000g supernatant of a component which co-migrates with type-IIIS histone.

In an attempt to remove this phosphatase-like activity from the DE-52 column, various concentrations of sodium chloride from 0.15M-3.0M were used and aliquots of eluent added to phosphorylated histone to determine their dephosphorylation ability. However, although the various sodium chloride elutions were clearly bringing different 'impurities' off the column as judged by protein measurements, none of the fractions collected had any significant effect on the

TABLE 3.3.1. EFFECT OF ADDITION OF CRUDE 100,000g SUPERNATANT TO DE-52 COLUMN-PURIFIED EXTRACT ON PKC ACTIVITY IN 16-DAY OLD PRIMARY GLIAL CULTURES

ENZYME SOURCE	PKC ACTIVITY (cpm/µg protein)
100,000g supernatant	111.6± 10.4
DE-52 column-purified supernatant	2627.8±187.0
100,000g supernatant + DE-52 column-purified supernatant	183.8± 20.8

Results are expressed as mean ± S.D. from four separate experiments using the same batch of cells.

TABLE 3.3.2. EFFECT OF ADDITION OF CRUDE 100,000g SUPERNATANT ON HISTONE PHOSPHORYLATED BY GLIAL PKC[a]

SAMPLE ASSAYED	INCUBATION TIME min	PKC ACTIVITY (cpm/µg protein)
100,000g supernatant	10	123.6±19.1
DE-52-purified 100,000g supernatant	10	1735.4±38.9
DE-52-purified 100,000g supernatant + Tris[b]	20	1518.2±63.7
DE-52-purified 100,000g supernatant + 100,000g supernatant[b]	20	261.4±11.8

a = 13-day old primary cultures

b = addition of second-named solution at 10min.

Results are expressed as mean ± S.D. from four separate experiments using the same batch of cells.

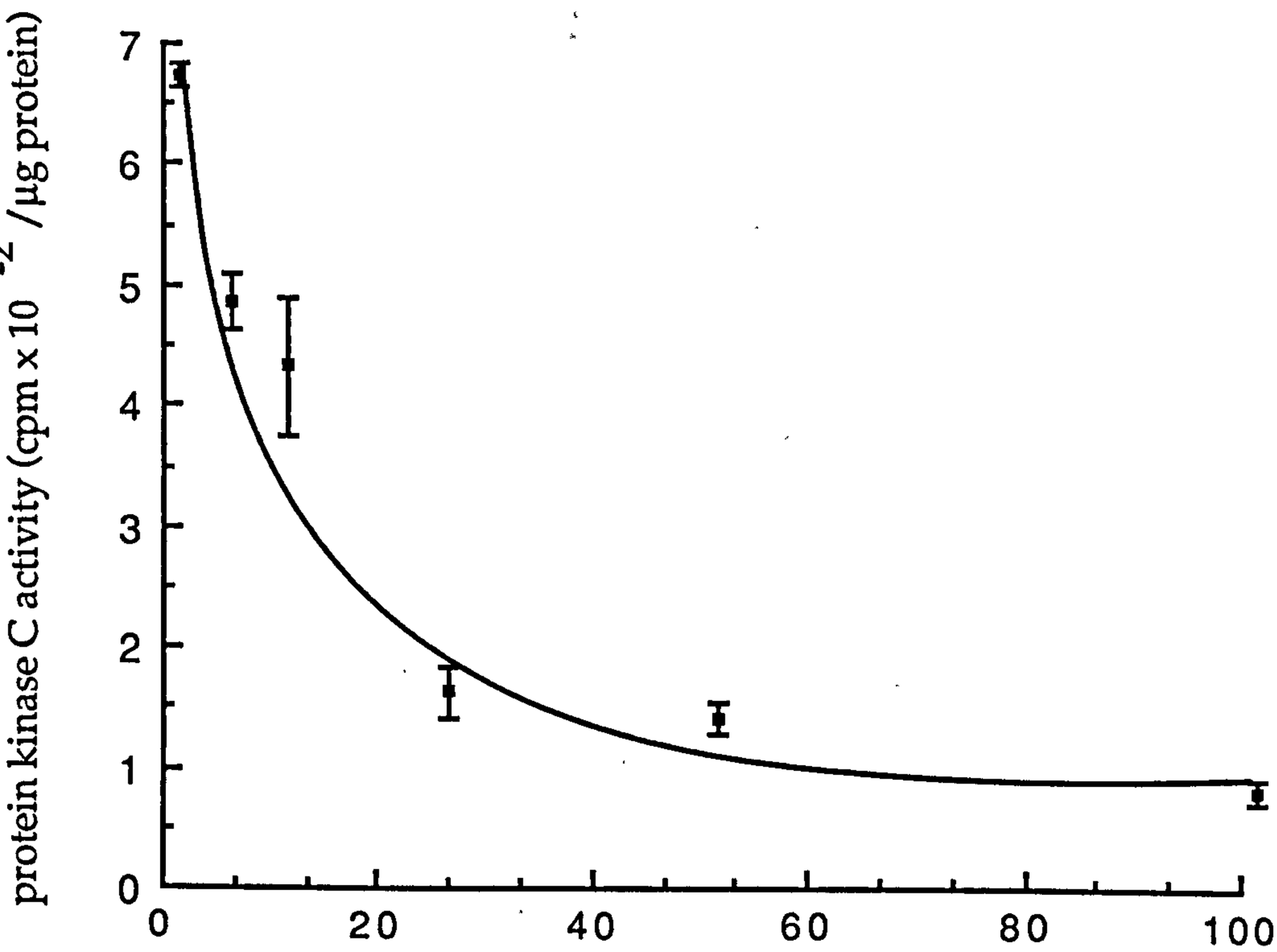


Figure 3.3.1. Effect of addition of 100,000 g supernatant on levels of histone phosphorylated by glial protein kinase C.

Varying amounts of 100, 000 g supernatant were added to an assay mixture containing DE-52 column - purified supernatant and the assay conducted as detailed in section 2.4. Each point shown represents the mean $\pm$ S.D. of triplicate determinations in a single experiment. A similar relationship was seen in four other separate experiments.

phosphorylation state of type-IIIS histone phosphorylation. Neither did any 'inhibitory' activity elute from the column in the wash-through fractions. This contrasts with the recently published work of Bhat (1989), where, using glial subcultures derived from rat brain, a 0.3M NaCl eluate from a DE-52 column previously used to purify a 100,000g supernatant led to a decrease in PKC activity, as judged by type-IIIS histone phosphorylation. Bhat (1989) made no attempt to characterise this 'inhibitor' further however.

3.3.b. Effect of 'inhibitor ' on other substrates

Crude 100,000g supernatant was tested for the ability to dephosphorylate two other substrates of PKC, protamine and myelin basic protein. Assays were conducted with a DE-52 column-purified sample using either histone, MBP (Sigma) or protamine as substrate, with a crude 100,000g supernatant aliquot added at 10min. and the assay continued for a further 10min. at 30°C. The results obtained are presented in figure 3.3.4. The data shows that Ca^{2+}/PS-dependent kinase activity using histone as substrate dramatically increased when the crude 100,000g supernatant was purified by DE-52 column chromatography, and that addition of the crude fractions to histone phophorylated by the DE-52 column purified extract after 10min. of assay caused a large reduction in kinase activity. This has been discussed in the previous section. The data also shows that a similar situation exists for MBP, where Ca^{2+}/PS-dependent kinase activity increased on purification of the 100,000g extract by DE-52 column chromatography. Addition of non-purified 100,000g supernatant to the MBP caused a reduction in kinase activity of approximately 60%. No such relationships were found for protamine. Of interest here also is that MBP appears to be an excellent substrate for Ca^{2+}/PS-independent kinases that are present only in the

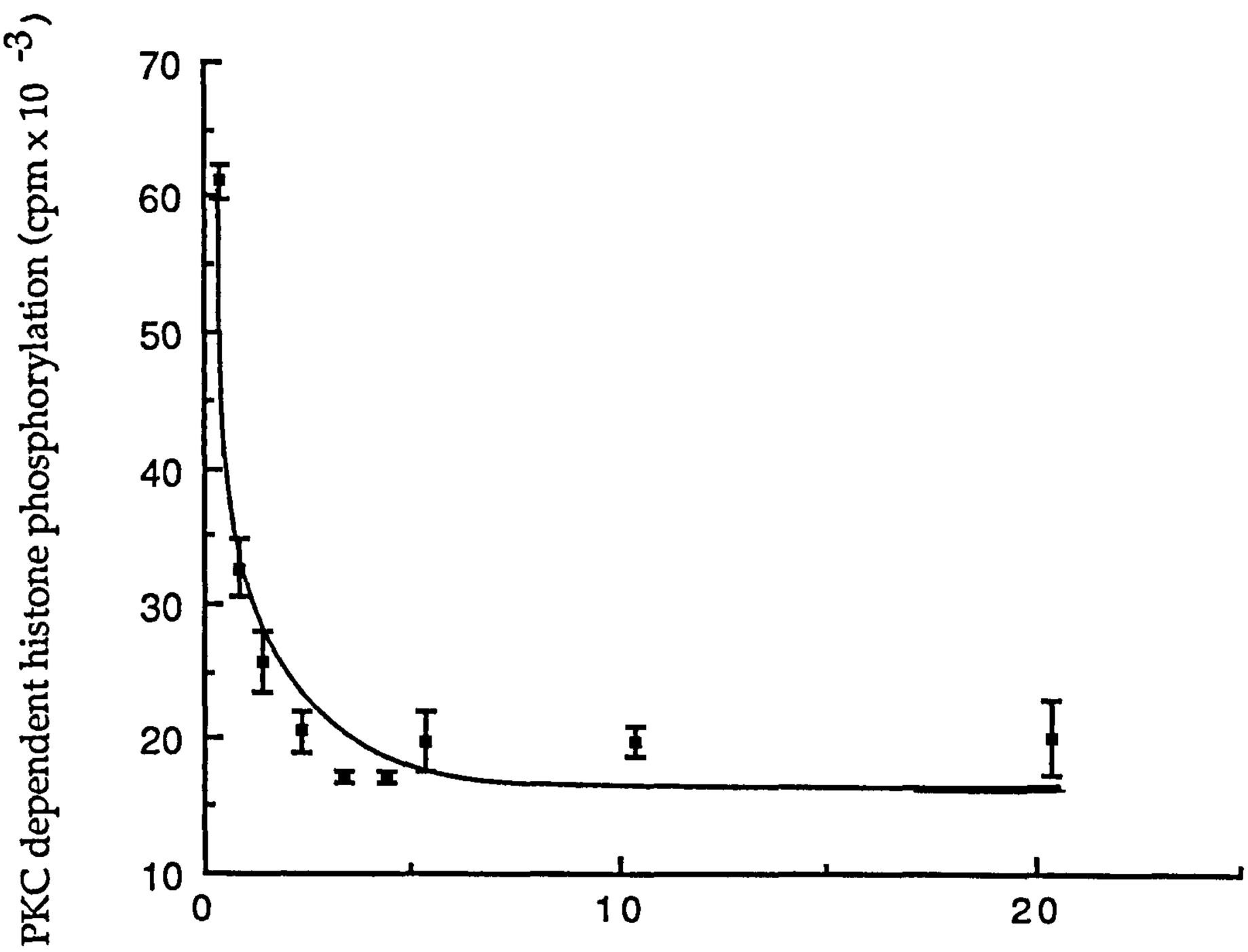


Figure 3.3.2. Time course of dephosphorylation of histone phosphorylated by glial protein kinase C.

Assay was conducted as normal for 10 min. using DE-52 column - purified 100,000 g supernatant. At 10 min. 100,000 g supernatant was added and the assay continued for the times indicated. Each point shown represents the mean $\pm$ S.D. of triplicate determinations in a single experiment. A similar relationship was seen in three other separate experiments.

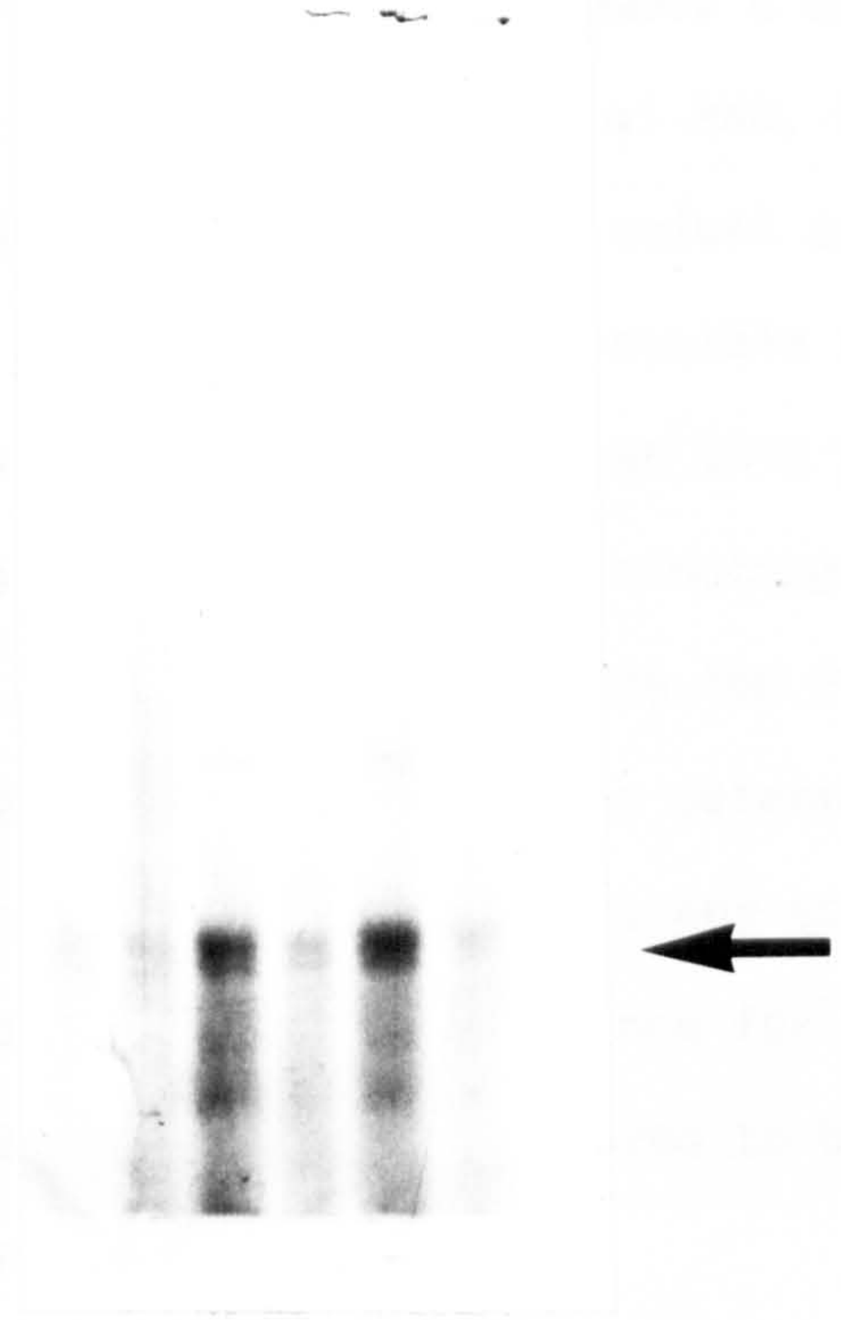


Figure 3.3.3. Autoradiograph of DE-52 column - purified 100,000g glial cell extracts in the presence (2 and 4) and absence (1 and 3) of non - purified extracts as resolved on a 6-18% SDS-polyacrylamide gel.

PKC was assayed as described in section 2.4. using DE-52 column-purified glial cell extract as the PKC source. At 10 min. an aliquot of 0.5M Tris (pH 7.5) (columns 2 and 4) or 100,000g supernatant (columns 1 and 3) were added and the reactions continued for a further 10 min. Reactions were terminated by making the assay mixture 1% (w/v) with respect to SDS. Solubilising buffer was added (1% w/v) and the mixtures boiled at 100°C for 3 min. Protein were resolved by SDS-PAGE.

crude non-purified glial supernatant. Protamine has similar properties which have been previously described (Turner and Kuo, 1986). The use of MBP and protamine as alternative substrates for assay of glial PKC is discussed in more detail in section 3.6.a.

Although the DE-52 column appears to remove a substance that will dephosphorylate histone phosphorylated by glial PKC, the fact that histone has yet to be identified as a physiological substrate for the kinase obviously limits any suggestion of a possible regulatory role for this phosphatase. However, the possibilty that this phosphatase may also be active upon phosphorylated MBP is fascinating since this basic protein is an excellent physiological substrate for PKC (Turner et al., 1984). Further investigations are required to determine whether phosphorylated MBP is indeed such a substrate. Any substantiations of the findings above could have major implications for the process of myelination, the first step of which is believed to be phosphorylation of MBP by PKC (Vartanian et al., 1986).

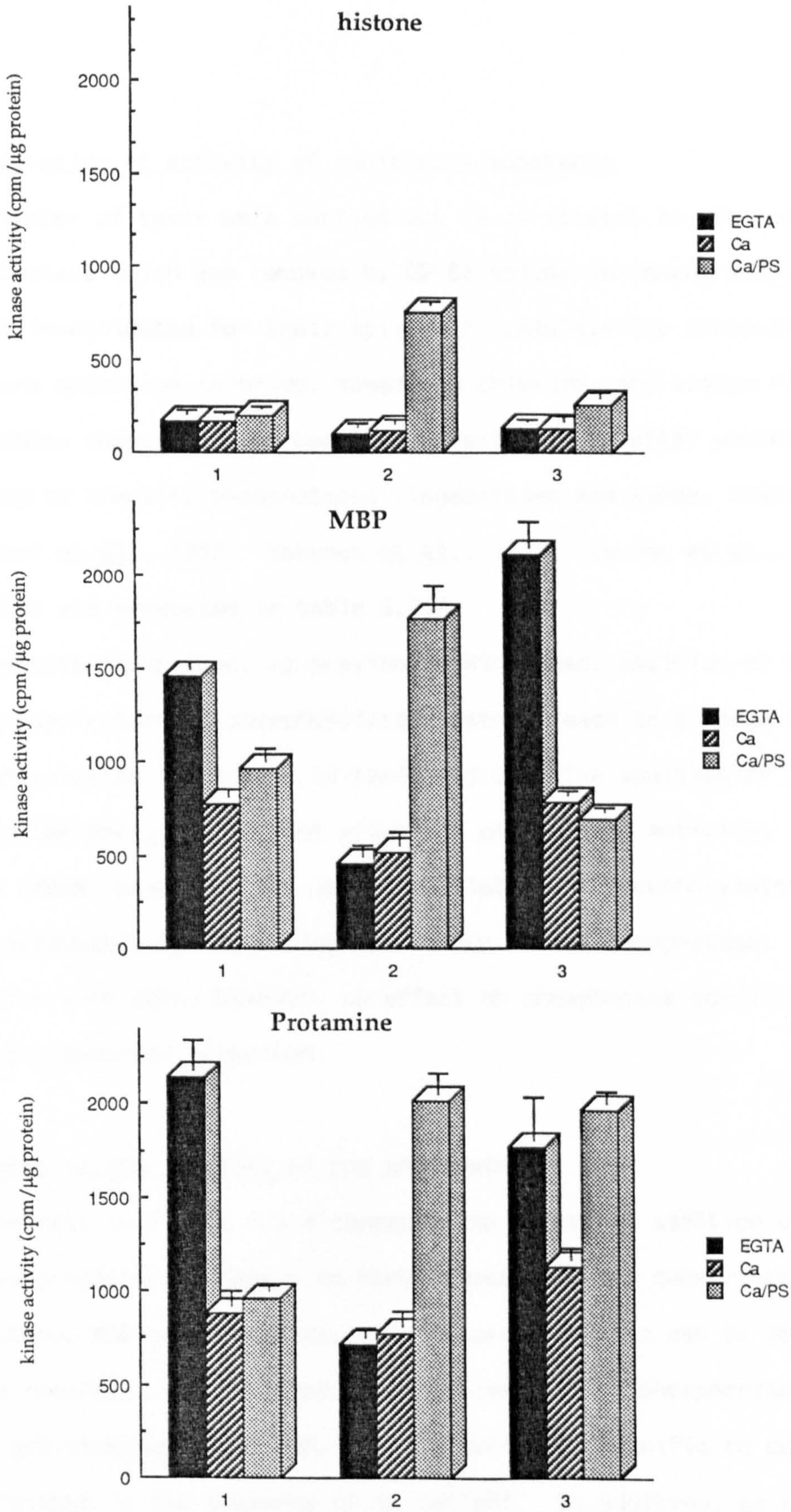


Figure 3.3.4. Effect of addition of 100,000 g supernatant to substrates phosphorylated by glial protein kinase C.

Data is presented as the kinase activities of (1) 100,000 g glial supernatant, (2) DE-52 - purified 100,000 g supernatant and (3) 100,000 g supernatant added to DE-52 - purified supernatant after 10 min. of assay.
Ca-independent (EGTA), Ca-dependent (Ca), and Ca/PS-dependent (Ca/PS) kinase activities were measured. Results are expressed as mean $\pm$ S.D. from at least three separate experiments.

3.3.c.Regulation of activity of inhibitory substance

A number of tests were carried out in an attempt to characterise the phosphatase which was removed by DE-52 column chromatography. Compounds investigated for their ability to regulate the phosphatase action were magnesium chloride, manganese chloride, and sodium fluoride. Each of these compounds have been used previously by other workers attempting to identify phosphatases (Ingebritsen and Cohen, 1983; Ingebritsen et al., 1983; Sahyoun et al., 1983; Levine et al., 1984). The results are presented in table 3.3.3.

The data shows that, as previously discussed, addition of crude 100,000g supernatant to phosphorylated histone leads to a large decrease in phosphorylation (66.1±4.4% in table 3.3.3). The addition of 10mM $MgCl_2$ and 2mM $MnCl_2$ was without effect on phosphatase activity. Sodium fluoride (40mM) prevented the dephosphorylation of phosphorylated histone presumably by inhibiting the action of the phosphatase. In conjunction with EGTA, however, no effect on phosphatase activity was seen on six separate occasions.

3.3.d. What is the identity of the phosphatase?

The data in figure 3.3.4 compares the effect of addition of phosphatase-containing sample to three phosphorylated substrates, type-IIIS histone, MBP and protamine. Two important points can be deduced from the results; the phosphatase is active on both phosphorylated histone and phosphorylated MBP, and its action is specific to substrates phosphorylated in the presence of in Ca^{2+}+PS. In addition, as the data in table 3.3.3 show, the phosphatase is inhibited by 40mM NaF, but 10mM $MgCl_2$, 2mM $MnCl_2$ and 50mM NaF/10mM EGTA have no effect on its activity. The exact localisation of the phosphatase is not certain although two

TABLE 3.3.3. REGULATION OF PHOSPHATASE ACTIVITY

ADDITION	% DECREASE IN Ca^{2+}/PS-DEPENDENT HISTONE PHOSPHORYLATION
None[a]	0
Crude [b]	66.1±4.4
Crude + 10mM $MgCl_2$[b]	64.7±6.0
Crude + 2mM $MnCl_2$[b]	68.3±5.3
Crude + 40mM NaF[b]	4.2±2.8
Crude + 50mM NaF/10mM EGTA[b,c]	63.7±7.2

[a] DE-52-purified sample only

[b] Addition of crude 100,000g supernatant (± test compound) to phosphorylated histone at 10min.

[c] Crude 100,000g supernatant pretreated with 50mM NaF/10mM EGTA for 30min. at 30°C prior to addition

The assay was conducted for 10min. with DE-52-purified 100,000g glial supernatant. Additions were then made as above and the assay continued for a further 10min. Figures given represent the percentage decrease in Ca^{2+}/PS-dependent phosphorylation due to addition to phosphorylated histone at 10min. and are the mean ± S.D. from at least three separate experiments.

separate experiments in which crude 100,000g glial particulate PKC was added to DE-52 column-purified 100,000g glial supernatant showed a decrease in phosphorylated histone comparable to that achieved using crude 100,000g glial supernatant. Such results must be interpreted with care however due to the presence of a known inhibitor of PKC activity, Triton-X-100, in the particulate fraction.

One of the first papers describing a phosphatase specific for phosphorylated histone was that of Meisler and Langan (1969). However, the phosphatase described was also active with respect to protamine dephosphorylation. Clearly this is not the case in the present study (see figure 3.3.4). Sahyoun et al. (1983) have described a specific phosphoprotein phosphatase that acts on histone H1 phosphorylated by protein kinase C. Such characteristics fit the phosphatase under investigation in the present study. This phosphatase did not require manganese or magnesium for its activity, and it was inhibited by 40mM NaF (Levine et al., 1984). However, the phosphatase described was prepared from rat liver (rat brain was not investigated) and only different types of histone were investigated as potential substrates. But the phosphatase was specific for PKC-phosphorylated histone and so may be (similar to) the phosphatase under investigation in the present study.

Four protein phosphatases have been shown to account for virtually all the protein phosphatase activity towards a number of proteins involved in the regulation of glycogen metabolism, glycolysis/gluconeogenesis, fatty acid synthesis, cholesterol synthesis and protein synthesis (Ingebritsen et al., 1983). These are termed protein phosphatases 1, 2A, 2B and 2C. Each has specific properties which may aid in the classification of the phosphatase in this study. Type 2B phosphatase is a Ca^{2+}/calmodulin dependent enzyme (Ingebritsen

et al., 1983) so this can be dismissed in the present case as the phosphatase is clearly specific for PKC-phosphorylated substrates (see figure 3.3.4). Protein phosphatases 1, 2A and 2C have broad and overlapping substrate specificities. Although never previously assessed in glia, over 60% of total brain phosphatase activity is type-2A with types 1 and 2C contributing approximately 18% and 3% of total activity respectively. However, type-2A phosphatase is inhibited by a 30min. pretreatment with NaF and EGTA as is type 1 phosphatase, albeit to a lesser extent (Ingebritsen et al., 1983). The final deduction would thus be that the phosphatase under investigation in the present study is a type 2C phosphatase. Protein phosphatase 2C has a wide substrate specificity and substrates upon which it is highly active ought first to be investigated using the phosphatase in this study. Examples of such substrates are HMB-CoA reductase and HMG-CoA reductase kinase (Ingebritsen and Cohen, 1983) involved in cholesterol synthesis. It is possible however that the phosphatase removed by DE-52 column purification of 100,000g glial cell supernatant is either a mixture of two or more of the phosphatases mentioned or it may be an as yet undefined phosphatase phosphoprotein. Whatever phosphatase it may be, it would clearly have major implications if shown to dephosphorylate phosphorylated MBP and if histone H1 is important in regulation of dephosphorylation of nuclear proteins.

3.4. DOES PKC ACTIVITY IN GLIA FOLLOW A PATTERN THAT IS AGE-RELATED?

INTRODUCTION

The PKC assay finally described in sections 2.4.-2.6 was now used to determine how levels of the enzyme changed over time during glial cell development in culture and whether or not any pattern existed that might mirror the development of function of the cell types involved.

RESULTS AND DISCUSSION

Crude 100,000g cytosolic extracts and DE-52 column-purified cytosolic and particulate extracts were assayed. The results are presented in figures 3.4.1 and 3.4.2. In all cases activity in the column-purified particulate fraction was much lower than that in the column-purified cytosolic extract, implying that glial PKC is predominantly localised in the cytoplasm. In some cases the cytoplasmic localisation approached 90%, a figure similar to that found by Neary et al. (1988) in astrocytes using a different method. Kikkawa et al. (1982) investigating PKC distribution in adult rat brain estimated that approximately 45% of PKC activity was particulate but found that platelet and lymphocyte PKC was approximately 99% particulate. Cardiac tissue which tends to have a much lower PKC activity than any of the enzyme sources mentioned above (Katoh and Kuo, 1982) has approximately 73% of its PKC located in the cytosol. Thus it can be seen that not only do tissues and cells vary with respect to levels of PKC activity, but each has a specific ratio of distribution between the membrane and the cytosol, presumably reflecting the role of the enzyme in the tissue or cell type in question.

Figure 3.4.1. shows no single peak activity but a number of peaks of enzyme activity appear at 9, 11, 19 and 27 days in culture. This pattern is emphasised in DE-52 column-purified cytosolic fractions (see fig. 3.4.2.) which show peaks of activity at 11, 19, 21 and 27 days.

Such peaks do not appear to correspond to any morphological change in culture. Mixed glial cell cultures at 9 days have formed a confluent layer of type-1 astrocytes upon which grow small, rounded, phase-bright cells, predominantly of the O-2A cell type. This covering layer reaches confluence by 18-21 days, after which time the cultures alter very little in appearance. Thus the precise reason for such a pattern in PKC levels is not apparent although clearly a significant level of enzyme is required at these times during the development of the cells. It is unlikely that this increased level of PKC is required to instigate or take part in myelinogenesis as the oligodendrocyte-enriched subcultures which begin to elaborate a myelin-like membrane by about 21 days in subculture show only low levels of the enzyme (figures 3.4.1. and 3.4.2.). One possiblity for such a pattern would be the induction of various genes necessary for the normal development of glia, but equally many other hypotheses may be advanced all of which might merit further investigation.

In a single experiment undertaken to compare levels of PKC in glia immediately after isolation from neonatal rat brain with levels present in neonatal rat cerebrum, a surprising enzyme distribution was seen (see table 3.4.1). Although cerebral levels of PKC were higher than those found in the isolated glial cells, approximately 80% of total cerebral PKC activity was detected in the cytosolic fraction, clearly at odds with the findings of Kikkawa (1982). However, Kikkawa (1982) employed whole adult rat brain as enzyme source whilst neonatal rat cerebrum was employed in this study. Whole adult rat brain will not only be a more

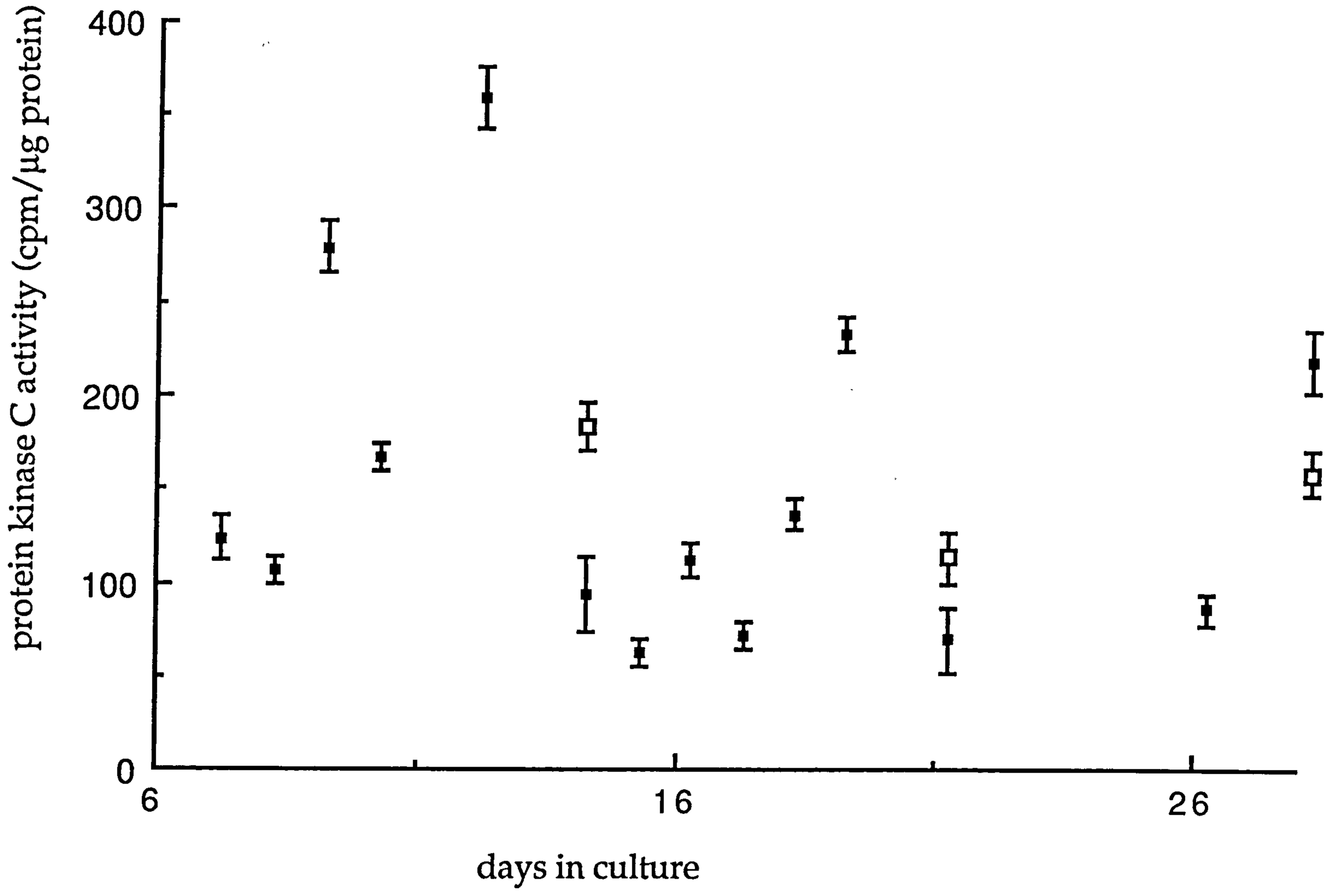


Figure 3.4.1. Protein kinase C activity in 100,000 g cytosolic extracts of mixed glial cells in primary culture (■—■) and subculture(□—□). Each point represents mean ± S.D. of triplicate determinations of PKC activity as detailed in sections 2.4 - 2.6.

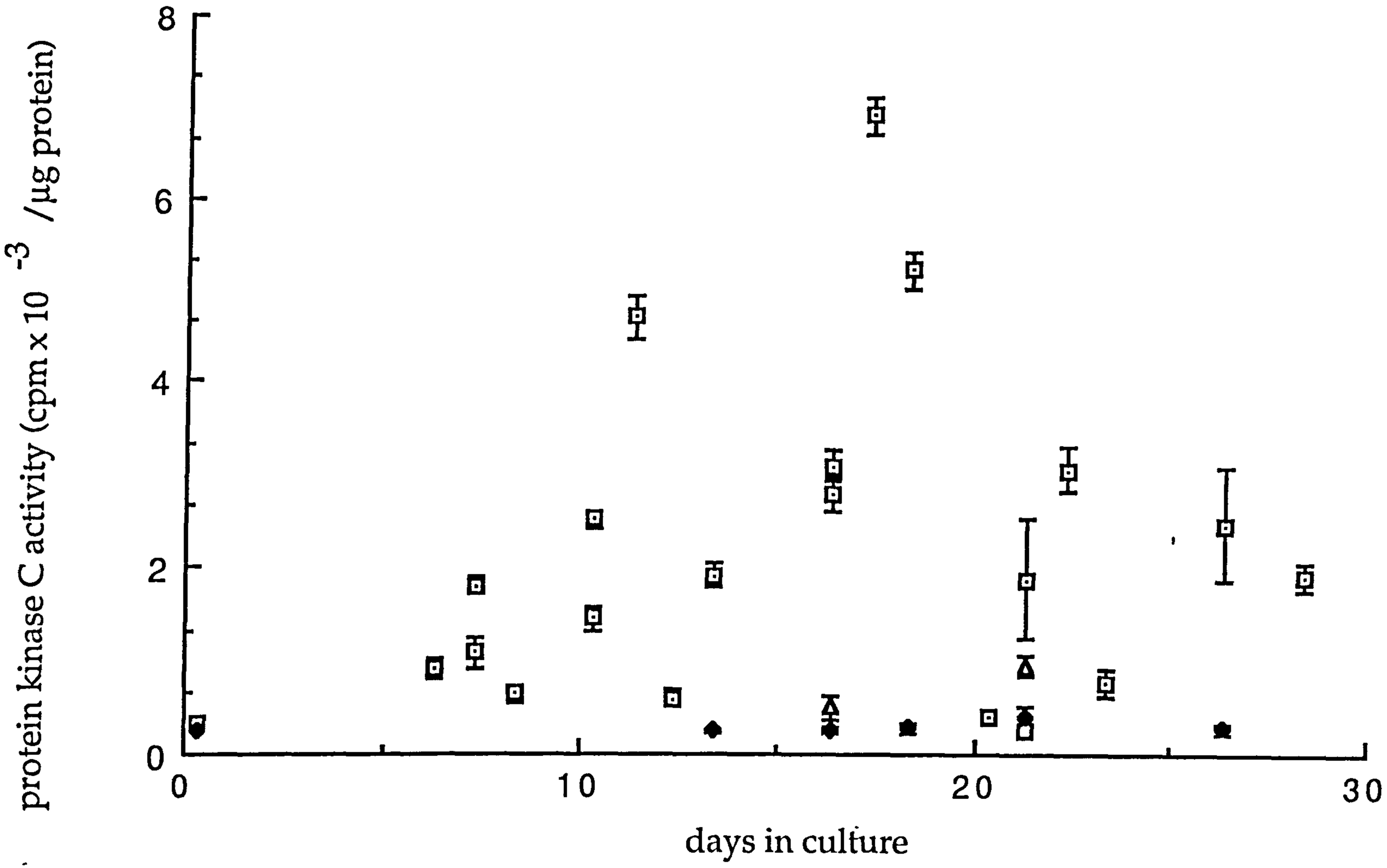


Figure 3.4.2. Protein kinase C activity in DE-52 column - purified 100,000 g extracts of glial cultures Cytosolic and particulate levels of PKC were assayed in primary cultures (□——□ and △——△ respectively) and subcultures (◆——◆ and □——□ respectively). Points shown represent mean ± S.D. of triplicate determinations of PKC activity.

fully developed, and hence a more intricate system, but will also contain a large neuronal population. This ought not to be the case in neonatal cerebrum and so lower levels of PKC activity are to be expected. Huang et al. (1987b) have recently shown the large contribution made by the cerebellum to total PKC activity in rat and monkey brain. In addition, Yoshida et al. (1988) have described the developmental expression of PKC in rat brain. Very low levels of PKC were apparent at three days old, with barely detectable levels of a predominantly particulate subspecies of PKC (γ). These observations support the findings of a reduced level of PKC in the neonatal cerebrum in comparison to adult brain and, to an extent, the distribution of the enzyme. Since levels of particulate PKC in the glial cells isolated and in the cerebra are similar, presumably the differences in levels of cytosolic PKC can be attributed to the cell population that is removed during the glial cell preparation.

Distribution levels within cells or tissues may vary according to the method used for preparation of the cell extracts. Thus methods exist in which 23,000g, 50,000g or 100,000g cellular extracts are used as the cytosolic source of PKC, usually with a DE-52 column purification step (Donnelly et al., 1985; Neary et al. 1986a; Kikkawa et al., 1986). Some authors then attempt to further separate the remaining fractions into microsomal, nuclear and plasma membrane fractions (e.g. Masmoudi et al., 1989), whereas most workers simply treat the fraction remaining following the preparation of crude cytosolic extract as the source of membrane-bound PKC (e.g. Neary et al., 1986b). Methods also vary considerably as to how the membrane-based PKC is extracted and measured. The majority of authors use an extraction step to solubilise the PKC from the membrane. Triton-X-100 at concentrations of up to 1% (v/v)

TABLE 3.4.1. COMPARISON OF PROTEIN KINASE C ACTIVITY IN NEONATAL RAT CEREBRUM WITH ISOLATED GLIAL CELLS

ENZYME SOURCE		PKC ACTIVITY (cpm/µg protein)
Neonatal rat cerebrum	cytosolic	615.4±14.6
	particulate	124.9± 4.9
Isolated glia	cytosolic	191.1±15.9
	particulate	125.0± 6.2

Glial cells were isolated as described in the Methods section from neonatal rat brain, and DE-52 column-purified cytosolic and particulate fractions compared to these of neonatal rat cerebrum. Figures given represent mean ± S.D. from triplicate samples.

is most common but in some cases millimolar concentrations of EGTA are used, sometimes in conjunction with Triton-X-100. Following an extraction step, usually of approximately 30 min. on ice, the extract is either assayed immediately, or centrifuged and the supernatant employed as the particulate source of PKC. DE-52 column purification may then take place prior to assay. Thus because of the wide variation in methods when estimating cellular PKC levels it is important to note which method is being employed when making comparisons between tissues or cell types. In comparing techniques for measurement of membrane-bound PKC activity in glia, a solution of 1% (v/v) Triton-X-100 in homogenisation buffer was found to be most effective. Although this prevented measurement of PKC levels in the crude extract, as Triton-X-

100 in the presence of PS will inhibit the assay if present in a concentration greater than 0.0075% (v/v) (Katoh and Kuo, 1982), this detergent was removed by DE-52 column chromatography so allowing the assay to proceed unhindered (Thomas et al., 1987).

The system used in the present work is a mixed glial cell system derived from 1-2 day old neonatal rat cerebra. Cells that were present in a complex microenvironment with numerous potential regulating factors have been transferred into a system in which the potential for regulation has been hugely decreased, as only type-1 and type-2 astrocytes, oligodendrocytes and O-2A progenitor cells persist in any significant number (Walker et al., 1985). Although clearly the glia themselves have a complex interrelationship which involves intercellular communication and feedback control (see section 1.1) and cells are involved in contact signalling, the majority of the regulatory factors present in this in vitro system are likely to be derived from the culture medium. This in itself presents a problem. Variability in batches of foetal calf serum means that whilst the cultures can be maintained, undefined factors may exist when different batches are used for cell culture and when these cultures are tested for, and compared for, PKC activities. A completely defined medium may alleviate such a problem. There is the added problem that a primary cell system may vary slightly from time to time which an established line would not do. Working with tissue taken from the original system however, by, for example, extraction of whole rat brain at various ages and assaying for PKC, might be argued to be a simpler and more reliable way of investigating age-related changes in PKC activity. Such a tissue will have been continually regulated by the relevant controlling factors during its 'lifetime', with the cells in question carrying out their in vivo role in response to this regulation. However, as the brain is a more complex system, any activity levels quoted here will include the

highly active neuronal population. Using this approach, and examining brain slices from 6-, 12- and 24-month old rats, Friedman and Wang (1989) have shown a 45% decrease in levels of total cellular PKC activity in the oldest animal group, together with a loss of ability to translocate PKC from cytosol to membrane in response to K^+ depolarisation or phorbol ester stimulation. However, in such experiments where tissues are taken from animals at advanced stages of development, only long-term changes in PKC activity are detected and short-term developmental changes have not been measured. Again the *in vitro* system differs from fully developed rat brain as the latter, by definition, will have matured fully whereas neonatal brain will be in a critical period where huge levels of cell growth and division are taking place, processes in which PKC has been implicated (Nishizuka, 1986). Glial cells are cultured over a short time period only, and during this time cellular development is still taking place, so perhaps this will mask any slight changes in PKC levels which may occur.

A further problem with the process of extraction of cellular PKC for biochemical assay is the need to perform such an extraction in the presence of a calcium chelator such as EGTA to prevent proteolysis of the enzyme by the Ca^{2+}-dependent protease, calpain. This means that the precise intercellular topography of the enzyme cannot be determined. Indeed Phillips *et al.* (1989) suggested that the distribution of the enzyme between the cytosol and the plasma membrane is a dynamic equilibrium controlled by levels of free Ca^{2+}. If this is the case, then the use of a calcium chelator in the extraction buffer will radically alter the equilibrium and so lead to results that may not accurately reflect the situation that exists within the living cell. Hence determinations of precise intracellular topography now adopt immunocytochemical approaches to overcome such a problem.

3.5. LOCALISATION OF PROTEIN KINASE C ACTIVITY IN GLIA

INTRODUCTION

Sections 3.1 and 3.2 established the presence of PKC in mixed glial cell cultures. However, these were mixed cultures and in the following section an attempt is made to look at PKC activity in the different glial cell types present. A number of methods were investigated for their ability to separate out the astrocytes, oligodendrocytes and O-2A progenitor cells in sufficient quantity and purity to allow a PKC assay to be performed on a relatively homogeneous cell population.

RESULTS AND DISCUSSION

3.5.a. Unsuccessful techniques

A number of techniques were investigated with unsuccessful results.

(i) Magnetic beads coated with anti-IgG-fluorescein conjugate were employed in an attempt to select out a desired subpopulation. Cells were treated as for normal immunocytochemical staining (see Methods section) except that labelling took place in solution with cells that had been trypsinised and cells were not fixed. Instead of incubation with the usual secondary antibody, the magnetic beads were added and the incubation carried out for the required length of time. Cells were then washed and those coated with magnetic beads (i.e. the required subpopulation) 'collected' by placing a high-power magnet underneath the tube or flask. Unbound cells could then be pipetted off. This method was unsatisfactory however due to poor recovery of required cells and lack of required purity in the population. In addition, the need to trypsinise the cells off the flask may have caused problems with the method.

lack of required purity in the population. In addition, the need to trypsinise the cells off the flask may have caused problems with the method.

(ii) The use of poly-L-lysine-coated flasks was investigated to see if this had any influence on the percentage constituency of the glial cell cultures. Although cells plated onto poly-L-lysine coated flasks usually settled down more quickly, no change in any of the subpopulations was evident as judged by immunocytochemical staining.

(iii) Density gradient centrifugation using Percoll and sucrose was attempted with cells isolated directly from rat brain or taken from culture flasks. Although a number of methods exist for Percoll density gradient isolation, (for example, Hirayama et al., 1983; Koper et al., 1984) it was found to be unsuccessful for separation of glial cells. However the sucrose gradients were more promising. Two separate methods were investigated, the Chao and Rumsby (1977) adaptation of Norton and Poduslo's (1970) method for preparation of oligodendrocytes from rat brain, and the method of Snyder et al. (1980) for preparation of oligodendrocytes from rat brain (see Appendix for methods). Both methods allowed for a relatively pure population of cells (≥75%) but not in sufficient number to allow PKC assays to be conducted. For such assays to proceed a large volume of starting material would have been required. Attempts to get around this problem by growing up the isolated cells and then performing PKC assays were unsuccessful.

3.5.b. Use of subculturing techniques to separate sub-populations

Cultures of 9-day old primary glial cells were treated as detailed in the Methods section (2.1.b) for subculturing. This technique removes the majority of small, rounded, predominantly phase-bright cells from

TABLE 3.5.1. PROTEIN KINASE C ACTIVITY IN GLIA BEFORE AND AFTER SUBCULTURING

ENZYME SOURCE	PKC ACTIVITY (cpm/µg protein)
9-day old mixed glial cells (i.e. before subculturing)	1329.0±145.2
Type-1 astrocytes (i.e. layer left after subculturing)	703.4± 50.6
Progenitor cell population (i.e. cells removed by subculturing)	580.5± 86.8

Cells were treated as detailed in the Methods section for assay of PKC. All fractions were DE-52 column-purified prior to assay. Results are expressed as mean ± S.D. from five separate experiments.

the monolayer of type-1 astrocytes beneath. Thus the mixed glial system is separated into two sub-populations, one of type- 1 astrocytes and the other of predominantly O-2A progenitor cells. Small, rounded cells not lifted off by shaking were removed by striking the flask against a solid surface three times and washing with DMEM. By taking a batch of glial cells at nine days in culture, keeping some for PKC assay and subculturing the rest, the PKC activity of the mixed glial culture prior to subculturing, of the cells shaken off, and that in the type-1 astrocyte layer left behind could be estimated. The results of such work are shown table 3.5.1. As described previously (Murphy et al., 1988) the type-1 astrocyte layer remaining after subculturing contains significant levels of PKC activity as does the predominantly O-2A progenitor cell population shaken off. The data thus implies the presence of PKC in all cell types present in the culture system. Immunocytochemical investigations by Walker et al. (1985) in this lab have shown that at nine days in culture 61±11% of cells exhibit GFAP

immunoreactivity and 31±5% exhibit immunoreactivity to anti-A2B5 antibody, with GC-positive and NF-positive cells accounting for only 6±3% and 2±1% of total cells respectively. Bearing in mind a small proportion of the cells shaken off during subculturing are GFAP+ A2B5+ (i.e. type-2 astrocytes), the data shows that GFAP+ cells contain appreciable levels of PKC activity . A number of workers (for example, Burgess et al., 1986; Girard et al., 1985) have identified PKC activity in neuronal cultures and neglected to examine glial cells, despite the often inferred presence of PKC in glia. Burgess et al. (1986) allowed glia to proliferate in primary neuronal cultures and noted a large increase in PKC levels. It is highly unlikely that the PKC activity measured here was significantly affected by the presence of NF+ cells (i.e. neurones). Not only do they contribute a very small percentage of the total cell population, but, as they appear to have very high levels of PKC activity (Burgess et al., 1986; Miller, 1986) a decrease in their numbers, as occurs as the glial cultures age, ought to show a large decrease in PKC activity. This is not the case as has been seen previously (section 3.4). The data shown in table 3.5.1 giving activity in type-1 astrocytes at nine days in culture of 703.4±50.6 cpm/µg protein (specific activity of approximately 4,000 pico moles ^{32}P transferred/10mins/mg protein) is only in the region of 25% of the activity reported by Neary et al. (1986b). However the culture conditions and assay system employed differ considerably. Treatment of the astrocyte monolayer with cytosine arabinoside followed by one or more passages did not lead to any increase in PKC levels in cells cultured for up to three week. In agreement with Neary et al. (1988) however was the finding that over 90% of PKC activity in astrocytes was contained in the cytosolic fraction (data not presented).

3.5.c.Use of a Defined Medium

A number of defined media have been described for the culture of glial cells (Bottenstein and Sato, 1979; Eccleston and Silberberg, 1984; Saneto and de Vellis, 1985; Sykes and Lopes-Cardozo, 1988). Defined media have been developed to replace serum-containing media since the addition of serum to culture medium may affect experimental reproducibility due to variation between batches of sera. Perhaps more important are the unknown effects that the undefined serum is having and the possibility that the serum is either masking experimentally-induced effects or preventing such effects from occurring. Therefore the use of defined media has become very popular recently and is commonly used in investigations involving glial cells and other cell types. It is also a method which can be employed for influencing the percentage cell constituency of a given cell type in a mixed cell system.

An attempt was made to use a defined medium to increase the percentage of oligodendrocytes in the mixed glial cell cultures. The idea was based on the findings of Raff et al. (1983) that glial progenitor cells in vitro will develop into type-2 astrocytes in the presence of foetal calf serum and into oligodendrocytes in a defined medium. Initial experiments which compared a number of serum-free or serum-substitute media for their ability to encourage the growth of glial cells in primary culture and subculture led to the selection of the medium of Espinosa de los Monteros et al. (1988). This is a chemically defined medium for the culture of mature oligodendrocytes. When mixed glial cell primary cultures were grown under normal conditions (in 10% FCS in DMEM) for six days and then switched either into the oligodendrocyte defined medium (OLDEM) (Espinosa de los Monteros et al. 1988) or into a reduced level of FCS in DMEM for a further four days, a change in PKC levels was apparent. See table

3.5.2. The data shows that a four-day incubation with OLDEM lead to an increase in PKC levels of approximately twofold. Reduction in levels of FCS when compared to the 10% level regularly employed led to a small decrease in PKC activity, possibly due to a reduction in the level of available nutrients. Murphy et al. (1987) have previously described an arrest in cell growth upon reduction of FCS levels in astrocytes, and this, together with an observation by Adamo et al. (1986) that proliferating cells display higher PKC activity than quiesient cells, may offer suitable explanation for the data seen in table 3.5.2. Confirming the results of Espinosa de los Monteros et al. (1988) cells treated with OLDEM contained floating debris not seen in cells treated with FCS; this presumably reflected the death of cells which did not survive OLDEM treatment (primarily astrocytes). Curiously immunocytochemical analysis did not observe significantly increased numbers of GC-positive cells although in some cases a decrease in GFAP-positive cells in the order of 10% was seen.

A second study, similar to that above, switched cells to OLDEM or maintained them in 10% FCS in DMEM at day six in primary culture. PKC assays and immunocytochemical analyses were carried out at 3 and 6 days following the medium change. Results are presented in tables 3.5.3 and 3.5.4. The data in table 3.5.3 again shows that a switch to OLDEM led to an increase in PKC activity in DE-52 column-purified fractions of 100,000g cytosolic fractions. In both cases the OLDEM-treated cultures appeared to have PKC activity levels approximately 60% greater than cultures grown in 10% FCS in DMEM. Analysis of particulate levels of both culture types showed no difference in PKC activity, with PKC associated with the membrane accounting for less than 10% of total cellular activity. Results in table 3.5.4 show that after 3 days

TABLE 3.5.2: PROTEIN KINASE C ACTIVITY IN GLIAL CELLS GROWN IN CULTURE IN VARIOUS CONCENTRATIONS OF FOETAL CALF SERUM OR IN AN OLIGODENDROCYTE DEFINED MEDIUM

CULTURE CONDITIONS	PKC ACTIVITY cpm/µg protein
0.5% FCS in DMEM[a]	1797.3 ± 23.6
2% FCS in DMEM	1834.8 ± 52.5
10% FCS in DMEM	2373.1 ± 84.2
OLDEM[b]	5065.8 ± 95.8

[a]DMEM = Dulbecco's modified Eagle's Medium

[b]OLDEM = Oligodendrocyte defined medium (Espinosa de los Monteros et al., 1988)

Cells were grown for six days in primary culture in 10% FCS in DMEM before medium change and then grown for a further four days in the media listed. Figures given are mean (± S.D.) from three separate experiments using the same batch of cells.

treatment, the immunocytochemistry of both culture types was very similar. There may perhaps be increased A2B5 staining in the 10% FCS in DMEM-treated cultures but this may also be due to cross reactivity (as type-2 astrocytes will exhibit immunoreactivity towards A2B5 and GFAP). Following 6 days treatment however, there was increased A2B5 staining and decreased GFAP staining in OLDEM-treated cells with a slight increase in cells staining for GC. This finding fits in with observations of Raff et al. (1983) and Espinosa de los Monteros et al. (1988). The presence of the defined medium would, according to these two studies, push O-2A progenitor cells (i.e. A2B5+) along the oligodendrocyte (i.e. GC+) differentiation pathway with the astrocytes dying off. However, Espinosa de los Monteros et al. (1988) described a huge cell proliferation that was not seen in the present study. In fact protein analysis on the treated cultures showed a nearly 4-fold increase in protein levels over the 3-day period in cells treated with 10% FCS in DMEM compared with a mere 2-fold increase in OLDEM-treated cells.

Espinosa de los Monteros et al. (1988) described a system using glial cells isolated at 20 days and grown for a further 30 days in OLDEM, thus selecting for oligodendrocytes. In the present study however, cells were treated with OLDEM at an earlier stage not to select mature oligodendrocytes but to try to push O-2A progenitor cells along the oligodendrocyte differentiation pathway. Also, cells were isolated for analysis after only 6 days treatment with OLDEM. Cells that were treated over longer periods (up to 14 days) had a poor viability rate; the medium appeared not to be able to support growth of a monolayer of cells beyond about 10 days following OLDEM treatment. Viewed under phase contrast, large patches in the monolayer appeared followed by the cells lifting off from the substratum.

TABLE 3.5.3. EFFECT OF OLDEM ON LEVELS OF PKC ACTIVITY IN PRIMARY GLIAL CELL CULTURES.

CULTURE MEDIUM	NO. OF DAYS AFTER ADDITION OF MEDIUM	PKC ACTIVITY (cpm/µg protein)
10% FCS in DMEM	3	2446.5±133.2
10% FCS in DMEM	6	3247.5± 68.5
OLDEM	3	4089.5±450.8
OLDEM	6	5341.9±183.9

Cells were grown for 6 days in primary culture in 10% FCS in DMEM prior to medium change. Cytosolic levels of PKC were estimated at 3 and 6 days after medium change as detailed in the Methods section. Figures given are mean ± S.D. from three separate experiments using the same batch of cells.

TABLE 3.5.4. IMMUNOCYTOCHEMICAL ANALYSIS OF OLDEM-TREATED PRIMARY GLIAL CELL CULTURES

CULTURE MEDIUM	NO. OF DAYS AFTER ADDITION OF MEDIUM	% TOTAL CELLS LABELLED		
		A2B5	GFAP	GC
10% FCS in DMEM	3	37 ± 9	66 ± 1	4 ± 2
10% FCS in DMEM	6	31 ± 12	70 ± 8	5 ± 3
OLDEM	3	30 ± 11	64 ± 9	5 ± 2
OLDEM	6	47 ± 6	54 ± 8	8 ± 1

Cells were grown for 6 days in primary culture in 10% FCS in DMEM prior to medium change. At 3 and 6 days after medium change flasks were stained for A2B5, GFAP and GC immunoreactivity as detailed in the Methods section. Figures given are mean ± S.D. of percentage of cells stained with the requisite antibody from five different fields of view with at least 200 cells counted per field. Results are not significantly different, as determined by analysis of variance tests.

Attempts were made to use OLDEM at the subculture stage; cells that had been shaken off the primary astrocyte layer were replated directly into OLDEM and their development followed. However these cells did not attach to the substratum, send out processes, nor undergo any proliferative response. It is possible that the OLDEM requires an additional factor in order to stimulate the process of substratum adherence.

Why an increase in PKC activity occurred on treating cells with OLDEM is not clear. If it was due to an increase in A2B5+ cells maturing into oligodendrocytes then this was not readily detectable after 6 days treatment. However, this does appear to be the most likely explanation. It is possible that A2B5+ cells were indeed differentiating into oligodendrocytes, and hence the large increase in PKC activity, but that this was not detected using anti-GC antibody. If the cells were differentiating into oligodendrocytes, but had not reached sufficient maturity to express GC, then they would not be detected as oligodendrocytes. This could be tested using the 04 antibody (Sommer and Schachner, 1984), for example, which will detect oligodendrocytes at an earlier stage of development. Another possibility would be that the OLDEM somehow unmasks a level of PKC activity that is not seen when using 10% FCS in DMEM; perhaps the FCS contains a factor which is somehow preventing the 'true' levels of PKC from being accurately estimated. The FCS contains many growth factors which may be causing a downregulation of PKC while the OLDEM, containing few PKC activators, would cause no such effect. In fact, Sykes and Lopes-Cardozo (1988) have recently shown that serum leads to an impairment of oligodendroglial differentiation by the reduction of a number of essential processes, whereas Bhat (1989), for example, has implicated PKC in process formation in glia, an observation confirmed in the present study (see section 3.8). More in-depth studies are required

to elucidate the true explanation.

The use of a defined medium, though a popular choice in many studies, has many difficulties. Characterisation of a defined medium is extremely time-consuming by implication and ought to be examined over a wide age-range in culture. In addition, much discussion is currently taking place in the literature as to which factors are necessary requirements for defined media. Warringa et al. (1987) suggested that hydrocortisone is a necessary requirement for an oligodendrocyte-defined medium in order to drive the conversion of O-2A progentior cells to oligodendrocytes. Insulin appears to be a necessary requirement for the majority of defined media although opinions differ as to how it acts in cultures of O-2A progenitor cells (see section 1.1.b). Most recently Espinosa de los Monteros (1989) and Dubois-Dalcq (1989) have argued for the importance of transferrin and fibroblast growth factor respectively in oligodendrocyte development, while Honegger (1989) has implicated triiodothyronine in oligodendrocyte maturation. The differences in constitution of the various defined media combined with the various uses to which the media is put and the cell types treated, makes this a very complex and unresolved subject at present which will become clearer as more data are obtained.

3.5.d. Use of complement-mediated cytoxicity

In a mixed cell system one of the subpopulations may be removed by labelling of the cells with an external marker specific to that cell type, followed by complement-mediated lysis of cells labelled with the antibody. What concentrations of antibody and complement are to be used, incubation times selected etc., must be determined in an initial set of experiments. In an attempt to determine levels of PKC in the progenitor cell population (and in type-2 astrocytes) the antibody against A2B5 was employed. By examining a 7-day primary glial cell

culture, all but a few cells ought to be either A2B5+ or GFAP+ representing either O-2A progenitor cells and type-1 and type-2 astrocytes. One problem with this approach was the need for large amounts of antibody because upwards of six 25cm² tissue culture flasks of cells were required; this was overcome by increasing the time of incubation with anti-A2B5 antibody, with a proportional increase in dilution of antibody.

Results shown in table 3.5.5 indicate that the cells remaining following treatment with complement had appreciable levels of PKC activity. However, this activity was only in the region of 55% of that seen in the untreated mixed glial cell culture.

3.5.e.Localisation of PKC in mixed glial cell cultures

In table 3.5.6. some of the data obtained in sections 3.5.b. to 3.5.d. is summarised. The activities of PKC in the various populations investigations are compared. Specific activities are expressed as picomoles of ^{32}P transferred per 10 minute reaction per milligramme of protein. The main conclusion is that all the major cell subpopulations forming the glial cell population appear to contribute to the overall PKC activity. A further study is required that can separate out all the constituent cells of culture into discrete sub-populations, perhaps by the use of flow cytometry for example, such that measurements can be made of PKC levels in such bulk-isolated cells to examine what percentage each cell type contribues to overall levels of PKC activity. Such a study might also investigate PKC subspecies (discussed further in section 3.7) within these constituent cell types using cDNA probes.

TABLE 3.5.5. COMPLEMENT-MEDIATED CYTOLYSIS OF A2B5-POSITIVE GLIAL CELLS AT 7 DAYS IN CULTURE: EFFECT ON PROTEIN KINASE C ACTIVITY

ENZYME SOURCE	PKC ACTIVITY (cpm/µg protein
Untreated cells[a]	1657.4 ± 95.1
Complement-treated cells[b]	914.2 ± 37.3

7-day old glial cells were treated with (b) or without (a) anti-A2B5 antibody for 3h. followed by assay for PKC levels, using a DE-52 column-purified 100,000g supernatant as the enzyme source, as detailed in the Methods section. Figures given are mean ± S.D. from three separate determinations. Non-specific complement-mediated cytolysis was ≤5% as determined by nigrosin dye exclusion.

TABLE 3.5.6. METHODS EMPLOYED IN PROTEIN KINASE C LOCALISATION STUDIES IN GLIAL CELL CULTURES

METHOD EMPLOYED	THEORY	PKC ACTIVITY ($pmol^{32}P$ *incorp./10min./mg protein*)		CONCLUSION
Subculturing	Separation of layer of phase-bright rounded cells from protoplasmic astrocyte monolayer	Mixed glia Astrocytes Shaken cells	7442.4 3939.0 3250.8	Considerable levels of PKC activity present both in type-1 astrocytes and in subcultured cells at 9 days in culture
Oligodendrocyte-defined medium	Increased percentage of O-2A progenitor cells differentiate to become oligodendrocytes	10% FCS in DMEM OLDEM (6-day treatment)	18186.0 29914.6	Increased levels of PKC in OLDEM-treated cells, probably associated with an increase in GC+ cells and a decrease in GFAP+ cells.
Complement-mediated cytotoxicity (with anti-A2B5 antibody)	Cytolysis of O-2A progenitor cell population	Mixed glia Cells after treatment	9279.2 5119.5	O-2A progenitor cell population contains significant levels of PKC

Cells were treated as detailed in sections (ii) - (iv) and in the Methods section. Figures given represent the specific PKC activities of the various cell populations calculated from the mean values presented in sections (ii) - (iv).

3.6. CHARACTERISTICS OF GLIAL PROTEIN KINASE C

3.6.a. Alternative in vitro substrates for glial PKC

INTRODUCTION

Whilst type IIIS lysine-rich histone is undoubtedly an excellent assay substrate for measurement of PKC levels, there is no evidence to suggest it is a physiological substrate for PKC, so other potential substrates were investigated. To this end, protamine and myelin basic protein (MBP) were used as substitutes for histone in the PKC assay at concentrations comparable to the level of histone used (i.e. 50µg per assay).

Protamine and MBP were both obtained commercially (Sigma), but MBP was also prepared from bovine white matter by Kim Anderson in this laboratory.

RESULTS AND DISCUSSION

Results showed that both protamine and MBP could substitute for histone in the assay to detect PKC activity but had markedly different properties as phosphoryl acceptor proteins. The different levels of kinase activities obtained using the four substrates are shown in figure 3.6.1. Levels of phosphorylation obtained in the presence of Ca^{2+}/PS are presented as the maximal attainable level (100%). This allowed an interesting comparison of Ca^{2+}-independent and Ca^{2+}-dependent kinase levels to be made. Commercially-obtained MBP and histone appeared to be almost identical when analysing their basal and Ca^{2+}-dependent kinase levels as a percentage of maximal phosphorylation. Ca^{2+}-dependent kinase levels were only slightly higher than basal kinase levels, with a large increase in substrate phosphorylation seen on addition of PS to the assay mixture. Despite these similarities when the results were

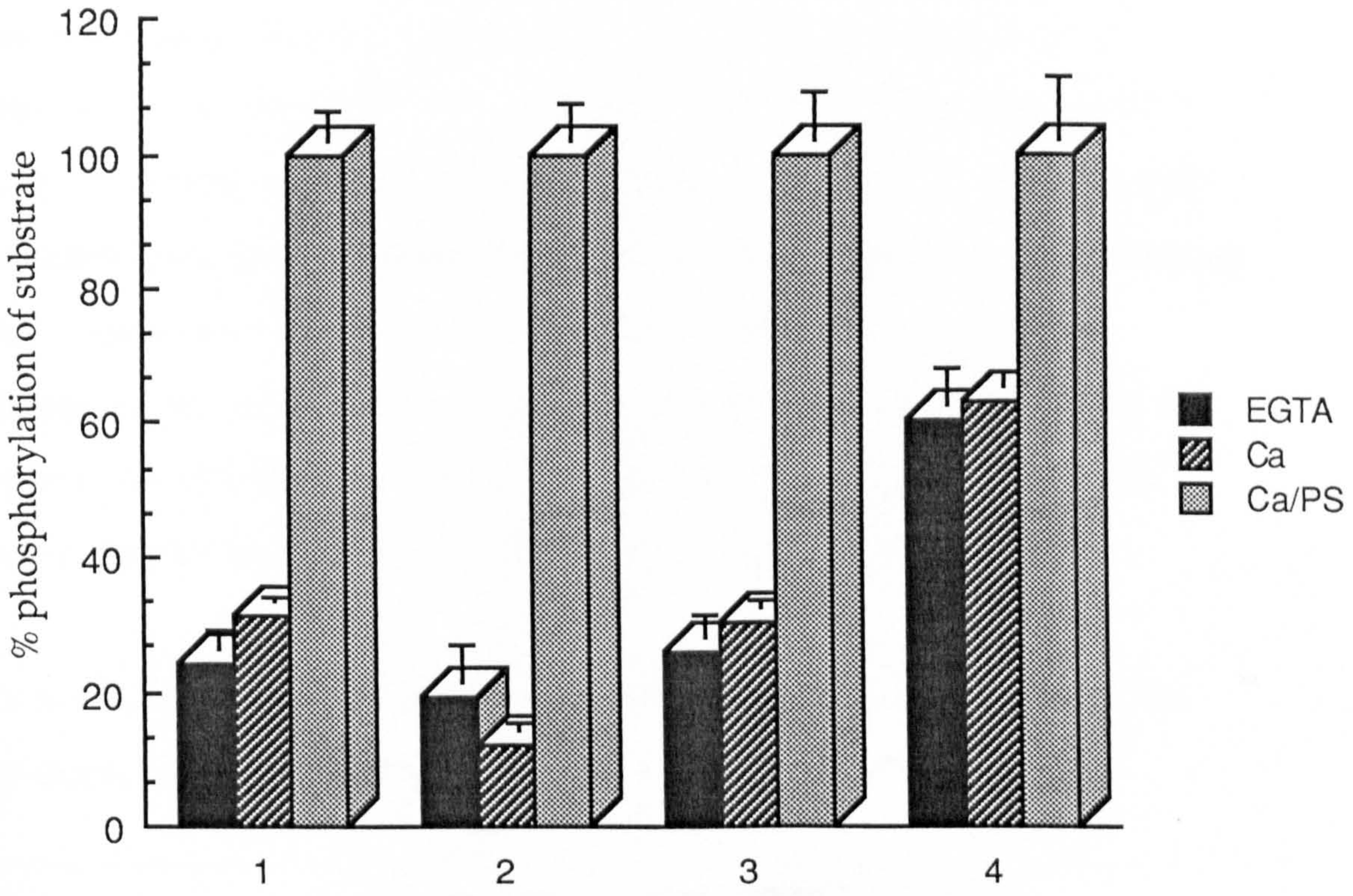


Figure 3.6.1. A comparison of substrate phosphorylation by Ca - independent, Ca - dependent, and Ca/PS - dependent kinases.
Substrates employed were type III-S histone (1), purified myelin basic protein (2), commercially-available myelin basic protein (3), and protamine (4).
Results are expressed as a percentage of the counts obtained in the presence of Ca/PS (100%) and are the mean $\pm$ S.D. from at least three separate experiments.

expressed on the basis of percentage phosphorylation of substrate, however, levels of MBP phosphorylation were in the region of 2.4 times greater in all three kinase categories than the levels of phosphorylation attained with histone, as table 3.6.1. shows. Purified MBP, on the other hand, whilst clearly showing the huge increase in phosphorylation on addition of PS, and indeed having lower basal and Ca^{2+}-dependent activities than the other substrates, had a Ca^{2+}/PS-dependent kinase level of less than one-third of that obtained using histone as substrate. Surprisingly, purified MBP was only approximately 12% as successful as its commercially available counterpart at incorporation of the γ-^{32}P of radiolabelled ATP in the presence of Ca^{2+}/PS, and also appeared to be an 'inferior' substrate for calcium-independent and calcium-dependent kinases when compared on a percentage basis. The latter observation would be something of a positive advantage to an assay system such as that employed for PKC, where the substrate is non-specific and where the lower the levels of kinase activity due to enzymes other than that being assayed the better.

TABLE 3.6.1. EFFECT OF SUBSTRATE VARIATION ON KINASE ACTIVITIES IN MIXED GLIAL CELL CULTURES

	cpm/µg protein		
SUBSTRATE	EGTA	Ca^{2+}	Ca^{2+}/PS
TYPE III HISTONE	127.0±11.2	164.1±5.3	524.0±21.0
PURIFIED MBP	28.8±9.1	17.9±1.2	152.1±7.6
COMMERCIAL MBP	328.0±37.8	376.7±12.5	1254.0±87.8
PROTAMINE	518.1±51.4	543.9±21.6	863.0±77.6

Results are expressed as mean ± S.D. from at least three separate experiments using 7-day old primary glial cell cultures.

The protamine differs completely from the other three substrates. Phosphorylation of protamine in the presence of EGTA and Ca^{2+} is considerable (60% plus) although addition of PS again increased phosphorylation significantly. Although in terms of actual counts incorporated into protamine the substrate does not compare to the high levels of activity incorporated into commercially available MBP, protamine is a 'better' phosphoryl acceptor than either purified MBP or histone. The very high levels of protamine phosphorylation in the absence of Ca^{2+} and PS when using a DE-52 column-purified enzyme fraction may be due to the detection of PKM activity, the proteolytically-activated form of PKC. This proenzyme is Ca^{2+}- and phospholipid-independent and will exhibit activity in the presence of PS. Hence although Ca^{2+} appears not to be required for phosphorylation of protamine by the glial PKC extract (calcium addition has no significant effect), maximal phosphorylation of protamine is only obtained in the presence of PS. Kimura et al. (1987) have obtained similar results showing that although PKC is dependent upon both Ca^{2+} and PS when using histone as a substrate, protamine is significantly phosphorylated even in the absence of both Ca^{2+} and PS. The same authors have shown that addition of DNA to the assay renders PKC phosphorylation of protamine Ca^{2+}-dependent, probably due to neutralisation of positively charged arginine groups in the protamine molecule by the DNA, so preventing Ca^{2+}-independent phosphorylation. The suggestion has been made that charged nitrogen atoms in arginine residues in protamine partially substitute for Ca^{2+} in the phosphorylation of PKC, and these charged groups are neutralised by the negatively charged phosphate of DNA. Protamine appears to be a unique substrate for PKC since it can be activated in the presence or absence of Ca^{2+} (Turner and Kuo, 1986).

Figure 3.6.2 presents data on how the substrates compare when

activity in the presence of EGTA is subtracted from that obtained in the presence of Ca^{2+}/PS. The results give a level of kinase activity due to PKC. Using histone as the 100(±8)% value, purified MBP exhibited only 31±4% of the activity obtained with histone, while protamine exhibited 87±9% and commercially available MBP a massive 234±9% of the histone phosphorylation. Thus it would appear that for the four substrates tested, commercially available MBP is the best substrate for PKC derived from glia. Whilst this may appear to be so some important factors have to be considered.

The assay to measure PKC activity requires that a significant increase in substrate phosphorylation is seen on addition of Ca^{2+}/PS. Using histone as a substrate, such an increase can easily be demonstrated. Commercial MBP certainly acts as a better phosphoryl acceptor than histone in the presence of Ca^{2+} and PS (with a 2.4-fold increased level of phosphorylation) but it shows a similar increase in levels for the other two kinase classes measured, i.e. Ca^{2+}-independent and Ca^{2+}-dependent kinases. This is not sufficient reason for substituting MBP for histone in the assay system regularly employed. In addition, the type IIIS histone is cheaper than the commercially available MBP. The best reason for substituting another substrate for the histone in the assay system employed here would be if the 'new' substrate were shown to be a PKC-specific substrate. No such substrate of PKC appears to exist, although some workers are now replacing histone with synthetic peptides which contain phosphorylation sites for PKC in an attempt to partially circumvent some of the difficulties inherent in the PKC assay. Such peptides can also remove the need for DE-52 column chromatography.

One reason for the use of synthetic peptides is highlighted by the results in figures 3.6.1 and 3.6.2. Whilst commercially available

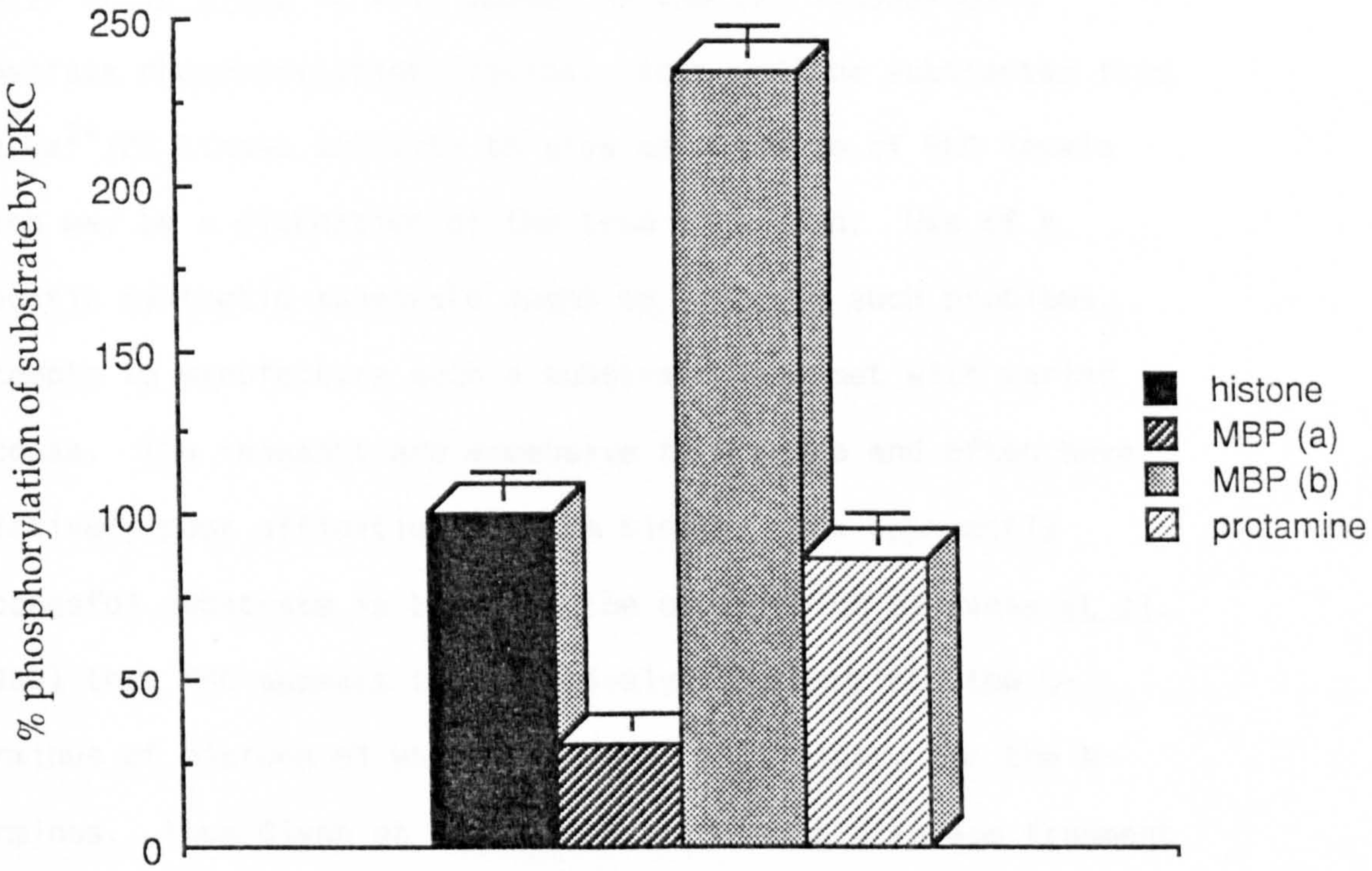


Figure 3.6.2. A comparison of protein kinase C - catalysed substrate phosphorylation.

Figures were obtained by subtraction of mean basal activity from that measured in the presence of Ca/PS, with phosphorylation of type III-S histone taken as the 100% value. S.D.'s shown were calculated using data from three separate experiments.

MBP is most active with respect to both Ca^{2+}/PS-dependent kinase levels (fig. 3.6.1) and PKC levels (fig. 3.6.2.), protamine has a huge Ca^{2+}/PS-dependent kinase activity but appears in the fig. 3.6.2. with a PKC activity lower than that of histone. Such results are arrived at by subtracting the basal activities from the Ca^{2+}/PS-dependent activities. But if some cellular PKC has been modified proteolytically either *in vivo* (although it is not clear where such regulation occurs in cells [Woodgett *et al.*, 1987]) or *in vitro* it will appear in the Ca^{2+}-independent substrate phosphorylation figures. This will be subtracted from the Ca^{2+}/PS kinase activity to give an estimate of PKC levels which may be a distortion of the true situation. Use of a specific synthetic substrate ought to overcome such problems. Attempts to manufacture such a substrate have met with varied success. The reagents are expensive to prepare and often have relatively poor affinities for the kinase. One apparently successful substrate is based on the observation by Iwasa *et al.* (1980) that PKC appears to selectively phosphorylate the C-terminus of histone H1 whereas PKA has an affinity for the N-terminus. Thus Glynn *et al.* (1985) prepared a cleavage fragment of the C-terminal domain of histone H1 by use of N-bromosuccinimide. However, the peptide is inhibitory to kinase activity in concentrations greater than 1mg/ml (Witters and Blackshear, 1987) and optimal conditions with respect to tissue extract concentrations, time and substrate concentration need to be elucidated for each system studied.

Despite the existence of synthetic substrates and others which clearly are *in vivo* and *in vitro* PKC substrates, the large majority of workers continue to employ the PKC assay using type IIIS histone as substrate.

What further complicates the issue of PKC substrate suitability is the apparent contradictions that appear to exist within the literature on this problem. Turner and Kuo (1986) have collated much of the existing data and have compared K_m and V_{max} values for various substrate proteins for PKC isolated from brain, heart and spleen. These authors maintain that MBP is the most effective substrate as determined by V_{max}:K_m ratio. The same group reported that purified MBP incorporates up to five moles of phosphate per mole of MBP in comparison to only two for histone. However, reported K_m and V_{max} values for MBP and histone vary greatly according to the enzyme source, making such an interpretation very complicated.

In an attempt to determine the reason for the differences in levels of phosphorylation exhibited by the two sources of MBP employed, the proteins were separated using SDA-PAGE. See figure 3.6.2.A. The figure shows the expected protein band distribution for protamine and histone. The MBP samples differed greatly however. Both purified bovine and rabbit MBP (the latter was purified in our laboratory by Sally Jenner) exhibited the expected staining pattern; commercial MBP, however, had a single major protein band of much lower molecular weight. The fact that it was a single band implied it to be a preparation of high purity. The likely explanation for such an appearance is therefore that the commercial sample has undergone hydrolysis to produce this lower molecular weight protein. This may then offer some explanation for the differences in levels of phosphorylation seen between the two MBP's. Further investigations are necessary to make a positive identification of this single lower molecular weight protein band, and a comparison of MBP's isolated from

various sources as substrates for PKC might prove to be of interest.

3.6.b. Alternative lipid activators for glial PKC

INTRODUCTION

Despite the fact that PKC activity appears to be regulated by phosphatidylserine in conjunction with Ca^{2+}, there have been reports that other phospholipids may substitute for PS, albeit less successfully (for example, Ku et al., 1981). The possibility of regulation of the enzyme by other phospholipids would have major implications with regard to site of activation of PKC within the cell and could reflect an alteration in the state of the cell such as a change in cellular metabolism. A number of phospholipids were therefore investigated for their ability to substitute for PS both in the presence and absence of diolein for glial cell PKC activation.

RESULTS AND DISCUSSION

When the specificity of fatty acid for activation of glial PKC was investigated both in the presence or absence of unsaturated DAG (diolein), a wide variety of responses was obtained (see table 3.6.2.). In each case listed, the phospholipid added was in place of PS and was at the same concentration as for PS in the routine assay. The results in table 3.6.2. show that PS was the most effective phospholipid for activation of PKC. Other phospholipids also supported enzyme activation but at a greatly reduced rate. Most successful at substituting for PS were phosphatidylinositol, phosphatidylethanolamine and phosphatidic acid, all, like PS, acidic phospholipids. These findings are supported by results

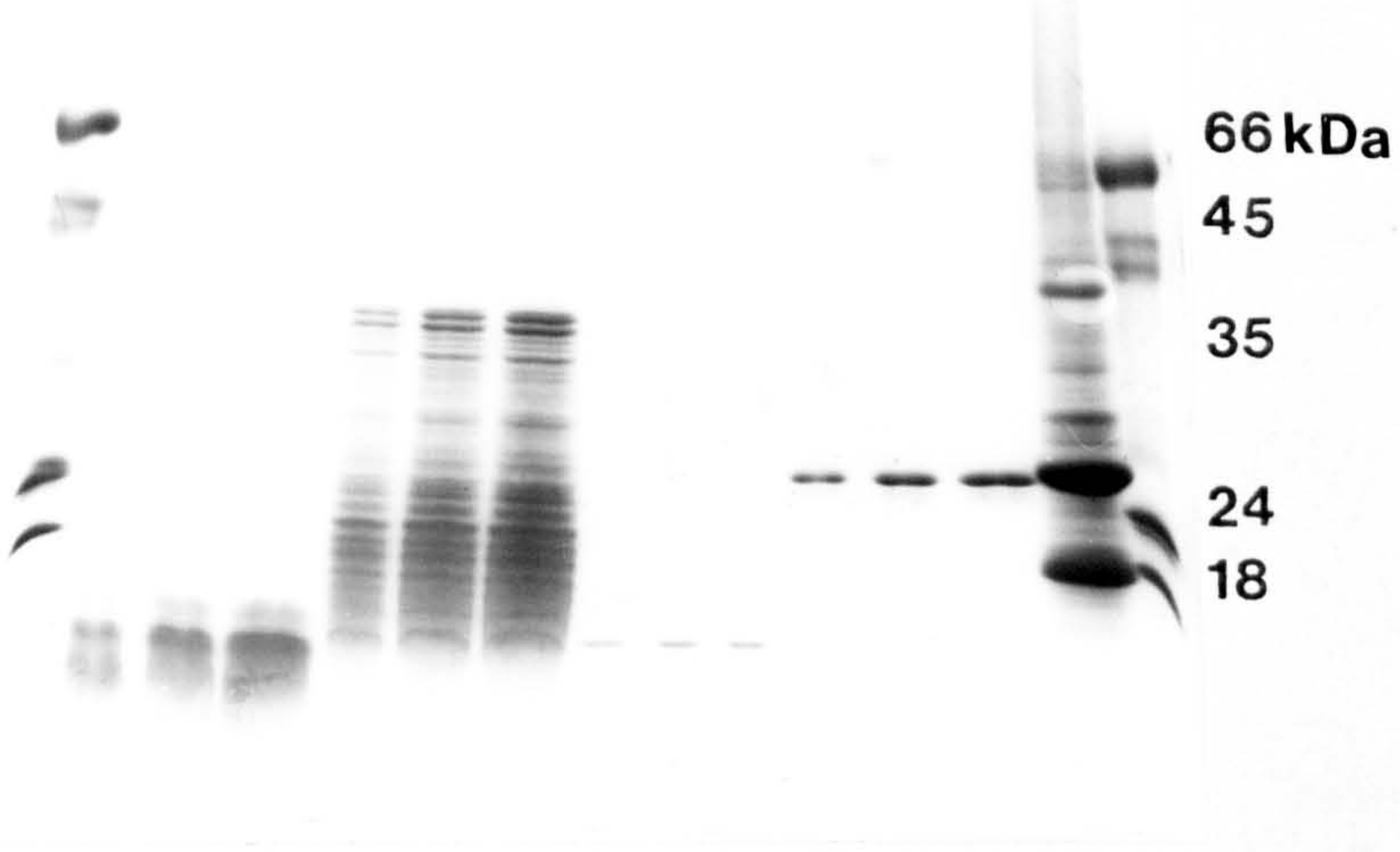


Figure 3.6.2.A. Histone, myelin basic protein and protamine, as resolved on a 6-30% gradient SDS-polyacrylamide gel.

Lanes 1 and 15 - molecular weight marker; lanes 2-4 - protamine; lanes 5-7 - type IIIS histone; lanes 8-10 - bovine myelin basic protein (Sigma); lanes 11-13 - purified rabbit myelin basic protein; lane 14 - purified bovine myelin basic protein. Each sample was run in triplicate, using protein levels of 2.5, 5.0 and 7.5 µg of protein (from left to right); lane 14 contained 5.0 µg of protein.

from other workers (e.g. Ku et al., 1981; Sekiguchi et al., 1988) although comparisons with published material are difficult as each separate investigation uses a different concentration of phospholipid and calcium. Using calcium concentrations identical to those in this investigation, Ku et al. (1981) employing PKC derived from human peripheral lymphocytes obtained activation levels of 78%, 43% and 18% for phosphatidylethanolamine, phosphatidylinositol and phosphatidylcholine respectively, relative to the PS value in the presence of diolein. However, these workers used phospholipid concentrations of only 8µg/ml, one fifth of that employed in the present study. In the absence of diolein Ku et al. (1981) found reduced PKC activation by PS (56%), phosphatidylethanolamine (23%), phosphatidylinositol (22%) and phosphatidylcholine (18%). Thus, many of these figures are similar to those obtained in the present investigation. Ku et al. (1981) concluded that whilst PS is most active in supporting PKC activity, both phosphatidylinositol and phosphatidylethanolamine will do so also, but less effectively, and that phosphatidylcholine, phosphatidic acid, sphingomyelin and lysophosphatidylcholine are inert. However, the data in table 3.6.2. shows that this appears not to be the case in glia, where phosphatidic acid is a useful activator of PKC. This view is supported in later work by the same workers when investigating phospholipid activation of PKC subspecies derived from rat brain (Sekiguchi et al., 1988). Using a phosphatidic acid concentration of 8µg/ml and calcium at 3µM (100µM was employed in this study) in the presence of diolein (0.8µg/ml), these workers found activity of 44%, 36% and 52% for subspecies α, ß and ɣ respectively using PS as the 100% marker. Of interest here is the fact that conversion of DAG to phosphatidic acid by DAG kinase is believed

TABLE 3.6.2. SPECIFICITY OF PHOSPHOLIPIDS AND ARACHIDONATE FOR ACTIVATION OF GLIAL PROTEIN KINASE C

	PROTEIN KINASE ACTIVITY (%)	
PHOSPHOLIPID ADDED	+DIOLEIN	-DIOLEIN
PHOSPHATIDYLSERINE	100	72
PHOSPHATIDYLINOSITOL	46	36
PHOSPHATIDYLETHANOLAMINE	35	25
PHOSPHATIDIC ACID	33	31
PHOSPHATIDYLCHOLINE	16	17
LYSOPHOSPHATIDYLCHOLINE	11	10
SPHINGOMYELIN	19	18
ARACHIDONIC ACID	11	17

Lipids were added at 40µg/ml in the presence/absence of diolein (0.8µg/ml). Results were expressed against PS (+diolein) as 100% in the presence of 100µM calcium and are the mean of at least three separate experiments. Assays were carried out as detailed in the Methods section.

to be a method for switching off PKC activation. If indeed this merely serves to prolong PKC activation then this will completely alter our view of how PKC is regulated. Phosphatidylinositol gave respective activities of 60%, 32% and 65% whilst phosphatidylethanolamine had significantly lower activation potential with only 9%, 15% and 16% for the α, β and χ subtypes. All these figures highlight the necessity for great care to be taken when comparing lipid activation studies as each employs different concentrations of activators, often using PKC derived from different sources. Only recently, with the discovery and characterisation of some of the isoforms of PKC has it become clearer as to why so many studies conflict in their assessments of lipid activation of PKC. The existence of the well characterised α, β and χ subtypes, the less well characterised δ, ε and z subtypes, plus the likelihood of the existence of others, one or more of which may exist in a specific tissue or cell type, and all with subtly different activation characteristics, could give a plausible explanation for such observations.

The data in table 3.6.2 shows, for activation of glial PKC under the conditions described, the presence of diolein is important for supporting PKC activation when using PS, but that it appears to have somewhat less of a potentiating effect when using phosphatidylinositol or phosphatidylethanolamine, and little or no effect in the presence of the other phospholipids listed. It is also of interest that arachidonic acid appears to preferably activate PKC in the absence of diolein, albeit at a very low level. Recent work by Nishizuka's group involving characterisation of the PKC subspecies has centred on the potential activation properties of arachidonic acid. In the

presence of 300μM calcium and the absence of diolein, γ-PKC was activated to about 50% of maximal activity (that obtained in the presence of 8μg/ml PS and 0.8μg/ml diolein) at lower concentrations of arachidonic acid (approx. 50μm) but was inactive at concentrations above 200μM (Sekiguchi et al, 1988). α-PKC, however, exhibited nearly 100% activity under such conditions up to 400μM arachidonic acid, with β-PKC having similar activation properties though at a lower level. These and other observations have led Nishizuka (1988) to propose that different PKC subspecies may be activated by the series of phospholipid metabolites such as DAG and arachidonic acid that are produced in successive phases of the response of the cell to stimulation of a cell-surface receptor.

Adopting an approach which assayed for PKC activity over varying concentrations of arachidonic acid both in the presence and absence of calcium, with no diolein added in either case, led to two very different results (see figure 3.6.3). In the presence of 100μM Ca^{2+}, kinase activity increased quickly up to 27% of maximal using 50-100μM arachidonic acid and reduced gradually thereafter. In the absence of Ca^{2+} however, (when 100μM EGTA was added) no increase in kinase activity was seen until 50μM arachidonic acid was used. Levels of activity using 100μM arachidonic acid plus EGTA approached those seen when Ca^{2+} was present, although increasing arachidonic acid to 200μM led to an activity of 48% of maximal. Thereafter any further increase in arachidonic acid concentration led to a sharp drop in kinase activity. Comparing both curves obtained with those of Sekiguchi et al (1988), who demonstrated the differing affinities for arachidonic acid of the PKC subspecies, the curve representing χ-PKC appears to correlate most closely with those seen in this study. Again, however, comparisons are difficult as the two studies were conducted under different

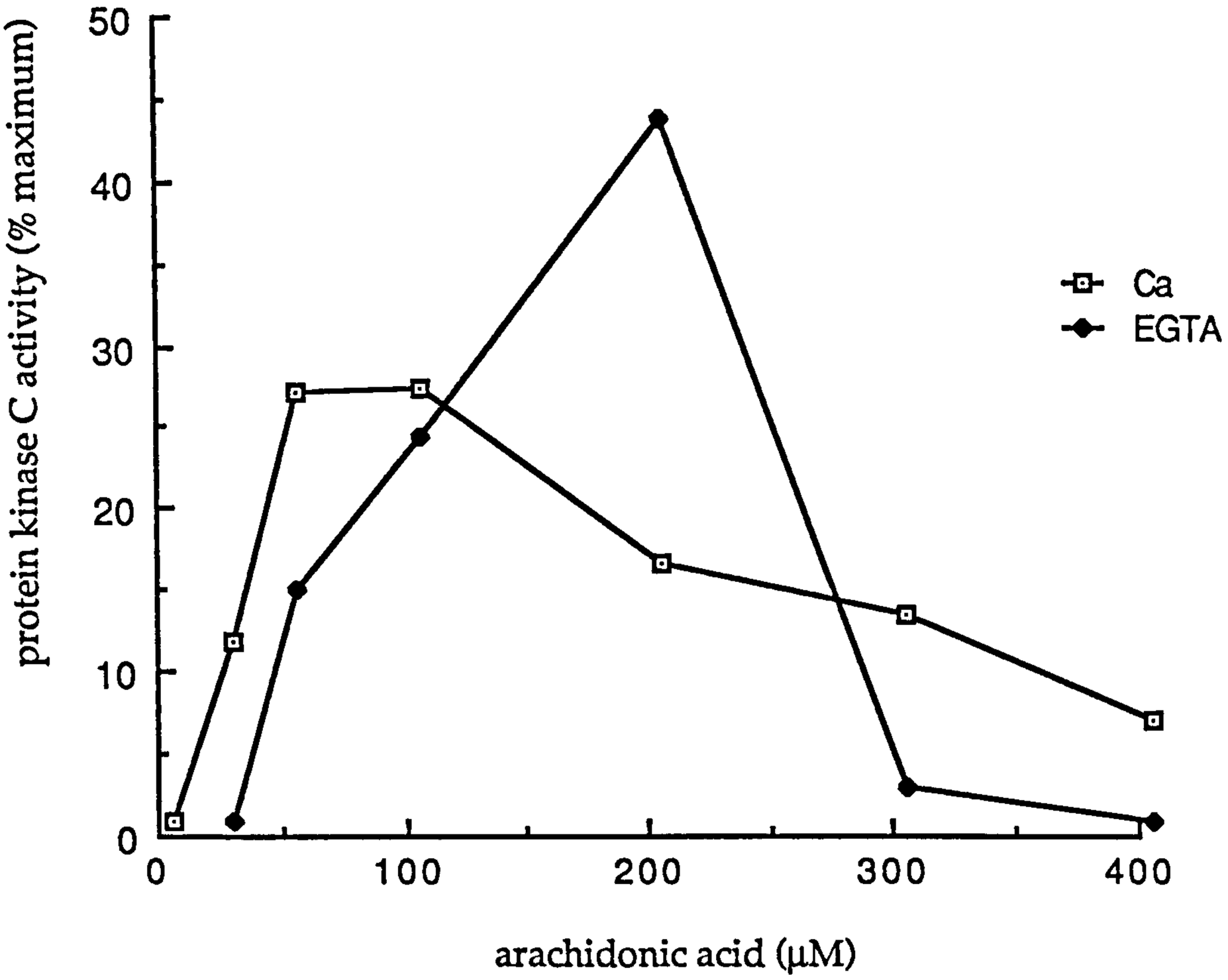


Figure 3.6.3. Activation of glial protein kinase C by arachidonic acid.

Phosphatidylserine was replaced with arachidonic acid and PKC assayed in the presence of EGTA or Ca (400 μM) as detailed in section 2.4. Results are expressed as a percentage of the activity obtained in the presence of phosphatidylserine (40 μg/ml), diolein (0.8 μg/ml) and Ca (100 μM). Each point represents the mean ± S.D. from three separate experiments.

experimental conditions. These results indicate that arachidonic acid may have a role in glial PKC activation, as may some of the other phospholipids listed in table 3.6.2, but any postulations to this effect will require further investigation. Possibilities also exist that although the phospholipids may not substitute for PS and support PKC activation, they may have a considerable influence on modulating activation of the enzyme by PS. Thus different membrane phospholipids play a role by enhancing or inhibiting enzyme activation. Exactly which phospholipids exert a positive cooperativity and which a negative one may reflect the lipid distribution within the cell membrane. Hence, in erythrocytes and platelets, phospholipids stimulatory to PKC activity are localised predominantly to the cytoplasmic face of the lipid bilayer where they may interact with the enzyme, with 'inhibitory' phospholipids located in the outer leaflet of the cell membrane (Jacobsen and Saier, 1984). As individual cell membrane phospholipid composition varies from cell to cell, this could explain differences in PKC activation effects of different phospholipids often reported in the literature. Given these many possibilities, the scope for further work in this area is tremendous.

3.6.c. Use of H-7 as an inhibitor of glial protein kinase C

The ability of H-7, one of the isoquinolinesulphonamide compounds described by Hidaka et al. (1984), to inhibit glial PKC was investigated. This compound is commercially available (Sigma) as a PKC inhibitor and is the most selective of the isoquinolinesulphonamide group of compounds described by Hidaka et al (1984) for inhibition of PKC. The effectiveness of H-7 in inhibiting PKC activity, expressed as percentage inhibition of activity over a range of concentrations of H-7, is shown in figure 3.6.4. The results show that H-7 does not completely inhibit PKC; a maximum of 70% inhibition is observed. H-7 would not therefore be the compound of choice to demonstrate a specific PKC-mediated process, but could be used in conjunction with data obtained by other means. H-7 was found to inhibit Ca^{2+}-dependent kinase activity in glia by approximately 50% at a 50μM concentration (data not shown). Thus, as previously detailed by Hidaka et al. (1984) H-7 is not a specific inhibitor of PKC but will inhibit all ATP-dependent enzymes such as PKA.

3.6.d. Activation of glial protein kinase C in vitro using phorbol ester

Mixed glial cultures were incubated with TPA (a DAG substitute; see section 1.3.d) for various time points to examine the effect of phorbol esters upon the subcellular distribution of PKC. The results in figure 3.6.5 show that as the length of incubation with TPA is increased so the PKC distribution between the cytoplasm and the membrane changes in favour of the latter i.e. cytosolic levels decrease with a proportional increase in particulate PKC levels. This movement of PKC to the membrane is termed translocation and was first reported by Kraft and Anderson (1983). 4-α-phorbol, a non-tumour-promoting phorbol ester, had no effect on PKC distribution (data not presented). Thus activation

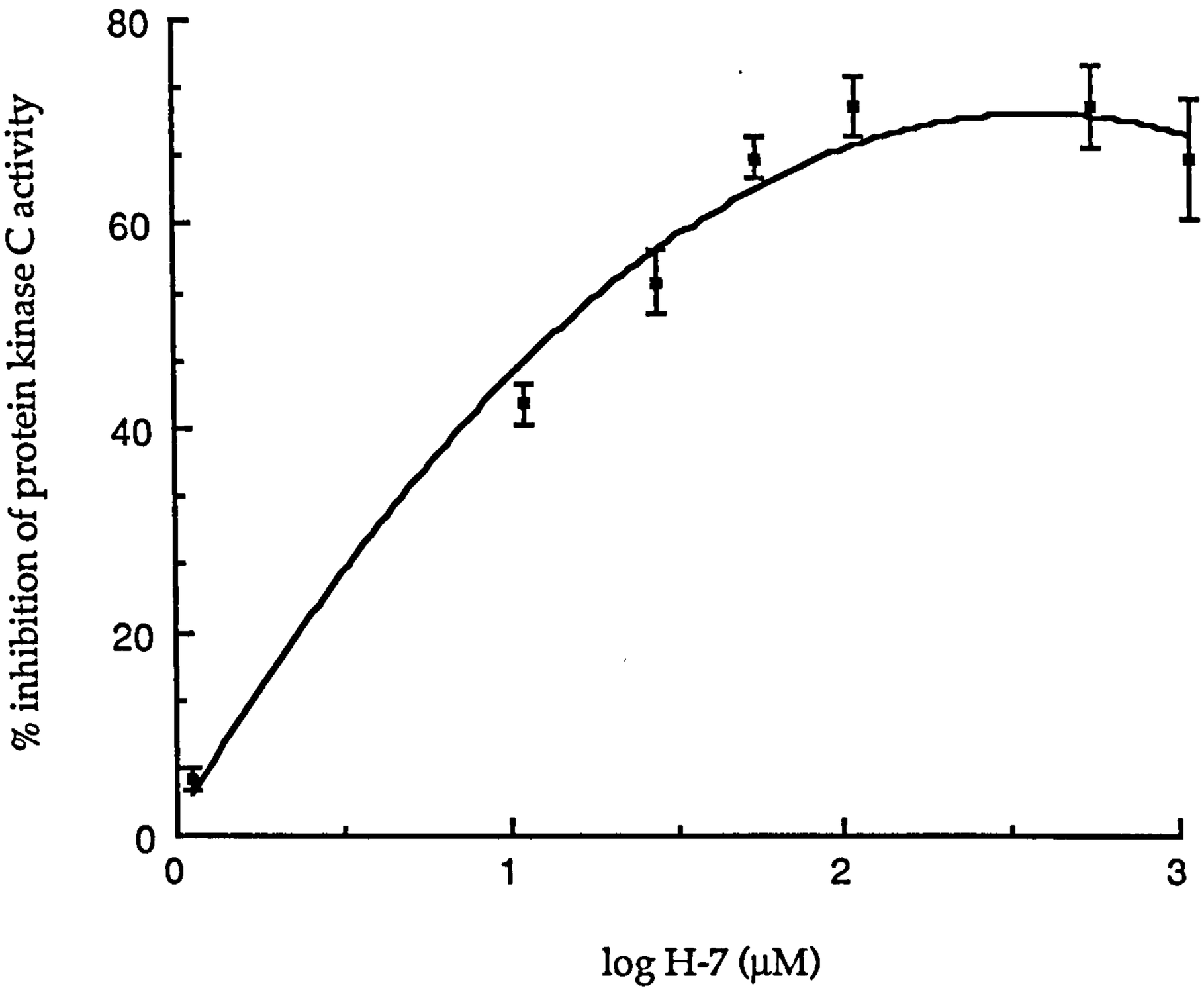


Figure 3.6.4. The effect of H-7, an inhibitor of protein kinase C, on glial protein kinase C activity.

Data is presented as the mean percentage inhibition of PKC activity (± S.D.) from five experiments, using the value obtained in the absence of H-7 as the 100% value. DE-52 - purified supernatant was employed as the source of glial PKC.

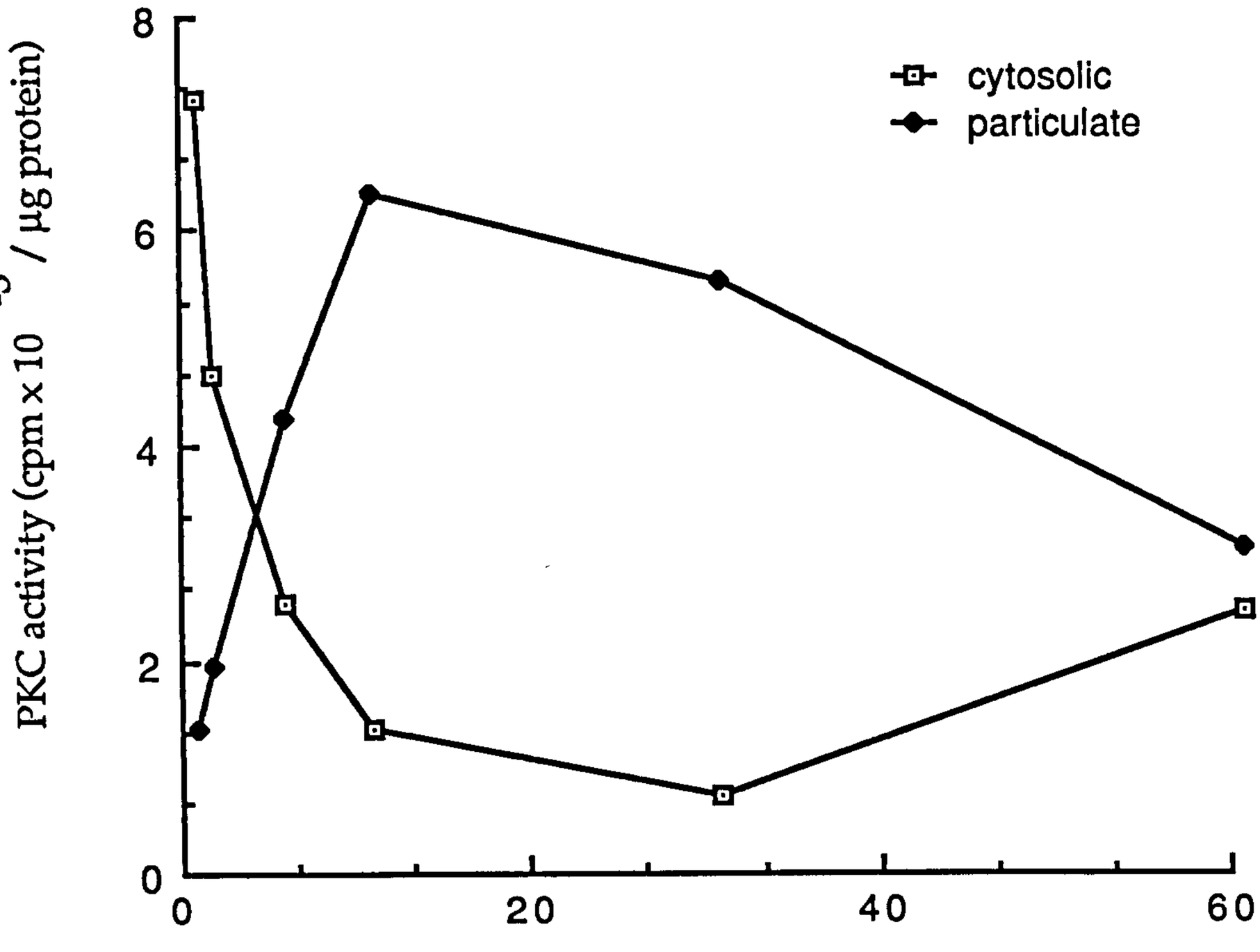


Figure 3.6.5. The effect of phorbol ester on the subcellular distribution of glial protein kinase C.

Mixed glial cell cultures were exposed to 25 nM TPA for the times indicated, washed with ice-cold Tris saline, and then prepared for PKC assay as detailed in sections 2.4 - 2.6. Results are expressed as mean values obtained using a 7-day old culture (all S.D.'s < 10%), but a similar pattern was obtained using cultures of five different ages. 4-α-phorbol was used as a control and had no effect on subcellular PKC distribution.
1000 cpm/ug protein corresponds to a specific activity of approx. 5600 picomoles ^{32}P incorporated / 10 min. / mg. protein.

of PKC by phorbol ester causes translocation of PKC to the membrane where the enzyme can interact with the lipid bilayer to form the active quaternary complex consisting of enzyme, DAG (or TPA), PS and Ca^{2+} (Hannun et al., 1985).

This pattern of redistribution of PKC to the membrane has since been shown by a number of workers in various cell types (for example, Rodriguez-Pena and Rozengurt, 1984; Thomas et al., 1987; Neary et al., 1988). Bhat (1989) has recently shown a similar phenomenon in glial subcultures derived from neonatal rat brain. Neary et al. (1988) investigated TPA-mediated PKC redistribution in primary astrocyte cultures derived from neonatal rat brain and found an increased membrane-association of PKC up to 30 min. of treatment with 100nM TPA, after which time levels of particulate PKC declined. Level of cytoslic PKC decreased sharply up to 30 min. but remained fairly constant thereafter up to 17h. treatment. This observation differs from the data presented in figure 3.6.5 using 25nM TPA, data which is supported by the work of Bhat (1989) using 10ng/ml. (approximately 16.3nM) TPA. The level of particulate PKC follows a similar relationship. An increase over the first 15 min. treatment is followed by a gradual decrease in measurable particulate PKC activity. This decreased activity seen on prolonged incubation with phorbol esters has been shown in a variety of cell types (see Rodriguez-Pena and Rozengurt, 1984; Chida et al., 1986, for example) and is termed 'downregulation'. The process may be mediated by proteolytic degradation of PKC (Chida et al., 1986), although an attractive alternative proposed by Bazzi and Nelsestuen (1988) is that the PKC inserts into the membrane in an irreversible step and produces a Ca^{2+}-independent kinase (see Bazzi and Nelsestuen, 1988, for a discussion on membrane PKC characteristics) hence the proposal of a proteolysis. The different patterns of PKC redistribution seen between studies may represent an inherent difference in the role of PKC

in a given cell type, and hence the level of enzyme that is available for translocation to the cell membrane.

The cytosolic levels of PKC in this study decreased in similar proportion to the increase in levels found to be associated with the membrane (see figure 3.6.5) up to a time of 30 min. TPA treatment. Thereafter, an increase in cytosolic PKC is apparent, albeit only up to a level that is approximately 30% of that seen prior to TPA treatment. Bhat (1989) found that a similar relationship existed, although this increase was not apparent until after 60 min. TPA treatment. However, in that study a lower TPA concentration was used to elicit these effects and so perhaps the time lag seen here is not surprising. Having shown such an increase in cytosolic PKC levels following treatment of glial subcultures for upwards of 60 min. Bhat (1989) made no attempt to explain this phenemenon. It may be that this simply represents a pool of newly- synthesised PKC becoming available for use. Alternatively, some of the membrane-bound PKC may somehow be redistributing to the cytosol.

A paper recently published by Huang et al. (1989) shows that three of the PKC subspecies, α, β and γ, exhibit differential susceptibility to tryptic proteolysis and that the α and β subspecies exhibit differential rates of downregulation in response to phorbol ester. Thus the kinetics of receptor downregulation will vary according to the PKC subspecies present in a specific tissue or cell type.

3.6.e. Effects of heavy metals on glial protein kinase C activity

Heavy metal toxicity is a phenomenon which can result from interaction of heavy metals with a number of cell constituents. Proteins such as PKC may be unusually sensitive however by virtue of the presence of sulphydryl groups that react with heavy metals (Speizer et al., 1989). Also of special interest here are reports by Windebank

(1986) showing that lead inhibits *in vitro* myelination, and arsenic, mercury and thallium inhibit neurite outgrowth in dorsal root ganglion neurones, and by Cookman *et al*. (1988) showing that lead will induce precocious glial differentiation both *in vivo* and *in vitro* leading to potential misrouting of neuronal pathways.

The effects of a number of heavy metals on glial PKC *in vitro* was thus examined in this study. The concentrations of the metals employed was 10μM, chosen with reference to previously published work (Windebank, 1986; Cookman *et al*., 1988; Speizer *et al*., 1989). Those metals under investigation were the divalent cations of arsenic (As), barium (Ba), cadmium (Cd), copper (Cu), lead (Pb), manganese (Mn), mercury (Hg), nickel (Ni) and zinc (Zn). The results are presented in table 3.6.3.

The data shows that As^{2+} alone appeared to cause a substantial activation of PKC activity at 10μM, with a reduced activation seen with Pb^{2+}. An inhibition of PKC activity in the region of 30% was seen on addition of Ba^{2+} and Ni^{2+} to the assay mixture. Neither Cd^{2+}, Cu^{2+}, Mn^{2+}, Hg^{2+}, nor Zn^{2+} appeared to have any obvious effect on glial PKC activity however, when added to the assay mixture and the assay conducted as normal. Thus it could be concluded that As^{2+} and Pb^{2+} stimulate the PKC-catalysed phosphorylation of type IIIS histone whilst Ba^{2+} and Ni^{2+} inhibit it. According to Speizer *et al*. (1989), however, this would be a false conclusion. They argue that any stimulation of PKC activity seen in the presence of heavy metals is due to the fact that the metals liberate Ca^{2+} from the Ca^{2+}-EGTA buffer system, and that Speizer *et al*. (1989) believe that the biphasic effect of stimulation of PKC at low heavy metal concentrations and inhibition at high concentrations seen by others (e.g. Murakami *et al*., 1987) is due to Ca^{2+} liberation at low concentrations of heavy metal and direct inhibition at higher concentrations. Therefore they suggest that any such investigations ought to be carried out in a buffer system free of

TABLE 3.6.3. EFFECT OF HEAVY METALS ON GLIAL PROTEIN KINASE C ACTIVITY

METAL ADDED	% PKC ACTIVITY[a]	S.D.
None	100	-
Arsenic	123	10
Barium	66	4
Cadmium	100	10
Copper	98	3
Lead	110	8
Manganese	103	6
Mercury	103	5
Nickel	73	3
Zinc	98	4

[a] percentage PKC activity was calculated using the activity obtained in the absence of any heavy metal as the 100% value, when DE-52-purified cytosolic extract was used as PKC source.

Divalent metals were added to the assay mixture to make it 10μM with respect to the metal, and the assay conducted as detailed in the Methods section. Figures quoted represent the mean % activity (± S.D.) from three experiments to the nearest whole number.

chelators. Results obtained following this suggestion are presented in table 3.6.4.

Results indicate an inhibition of glial PKC activity in the presence of all of the heavy metals listed, in agreement with the findings of Speizer et al. (1989). The greatest degree of inhibition was seen in the presence of 10μM Ba^{2+} (>50%), while both Cd^{2+} and Cu^{2+} at 10μM concentration had a 45% inhibitory effect on glial PKC activity. This figure for cadmium is lower than that found by Speizer et al (1989) who obtained a 50% inhibition of PKC activity with Cd^{2+} at a concentration of 3μM. Figures obtained for Cu^{2+} and Zn^{2+} were similar in both studies.

Replacement of a Ca^{2+}-EGTA buffering system with a chelator-free system had some direct effects on measurable PKC activity. Homogenisation of glial cells in low concentrations of calcium (i.e. in the absence of EGTA) led to an increase in membrane-associated PKC. In one experiment, cytosolic PKC levels of 1694±136.5 pmol ^{32}P transferred/10min./mg protein obtained in the presence of chelator contrasted with the figure of 231.8±21.7 pmol ^{32}P transferred/10 min./mg protein obtained in the absence of chelator. This reduction may be overcome to some extent by addition of high levels of Mg^{2+} to the homogenisation buffer (30mM) so counteracting the Ca^{2+}-mediated association of PKC with the membrane (Speizer et al., 1989). The use of Mg^{2+} was not investigated in the present study in an attempt to keep all experimental conditions as constant as possible.

The fact that all the heavy metals tested appeared to have an inhibitory effect on glial PKC supports the theory of Speizer et al. (1989) that any stimulation of activity seen in the presence of chelator merely reflects a change in the Ca^{2+}-EGTA buffering system. It also

TABLE 3.6.4. EFFECT OF HEAVY METALS ON GLIAL PROTEIN KINASE C ACTIVITY IN A CHELATOR-FREE ASSAY SYSTEM

METAL ADDED	% PKC ACTIVITY[a]	S.D.
None	100	-
Arsenic	70	6
Barium	47	9
Cadmium	55	10
Copper	57	9
Lead	78	7
Manganese	80	4
Mercury	63	7
Nickel	66	8
Zinc	66	9

[a] percentage PKC activity was calculated using the activity obtained in the absence of any heavy metal as the 100% value when DE-52-purified cytosolic extract prepared in the absence of chelators was used as PKC source.

Divalent metals were added to the assay mixture to make it 10μM with respect to the metal, and the assay conducted as detailed in the Methods section. Figures quoted represent the mean % activity (± S.D.) from three experiments to the nearest whole number.

explains the biphasic effect of stimulation and inhibition of PKC activity at low and higher levels of heavy metal respectively. It is interesting that all the heavy metals inhibit PKC. The site of action of the heavy metals appears not to be on ATP or histone or to be a non-specific denaturation effect since PKA and PKC exhibit differential sensitivity to heavy metal inhibition. Rather the site of action is probably at a sulphydryl moiety on the PKC molecule (Speizer et al., 1989), such as one of the number of cysteine-rich residues present (see fig. 1.3.5). Consistent with this is the finding that β-mercaptoethanol and penicillamine which protect S-S bonds also protect against heavy metal inhibition of PKC activity (Speizer et al., 1989).

Given that PKC has been widely implicated in growth and differentiation processes (Nishizuka, 1986), the results presented here showing an inhibition of PKC acivity in the presence of heavy metals may possibly account for observations of Windebank (1986) that heavy metals inhibit neurite outgrowth . However, such an hypothesis would require investigations of the effects of heavy metals on PKA as well as PKC. This may then lead to a greater understanding as to how certain heavy metals exert their toxic effects, and how these may be counteracted, something which could prove to be of enormous benefit to the large number of persons daily exposed to such elements.

3.7 HYDROXYLAPATITE COLUMN CHROMATOGRAPHY OF GLIAL CELL EXTRACTS

INTRODUCTION

Although once considered as a single entity (see Nishizuka, 1984, for example) gene cloning techniques and enzymological and immunocytochemical analyses have since revealed the existence of multiple subspecies of PKC distributed throughout a number of mammalian tissues (for

example, Ono et al., 1986, 1987; Huang et al., 1987b). Hydroxylapatite (HA) column chromatography was introduced to resolve an apparently homogenous tissue or cell preparation into three PKC subspecies, the α, β and χ isotypes.

Hydroxylapatite is produced by the reaction of Ca^{2+} and phosphoric acid to yield a compound with the chemical formula of $Ca_{10}(PO_4)_6OH_2$. Elution of sample from the column is always by means of a sodium or potassium phosphate gradient. The separation mechanism underlying resolution of solutes on HA remains to be clarified although promotional literature attributes it to a combination of ion-exchange, molecular sieving and recognition of structural difference (the extent to which molecular sieving is important increases with column size). Conventional HA appears flake-like when analysed by X-ray diffraction, but companies marketing the HA chromatographic columns have modified this structure somewhat. The exact shape of the HA crystals gives each specific type of HA column its characteristics with respect to pressure and pH limits and elution profile. Thus given compounds behave differently on different types of HA column.

The earliest methods published for separating PKC into three sub-species were those of Huang et al. (1986) and Kikkawa et al. (1987). Using a Biorad HA column, Huang et al. (1986) separated an extract of monkey brain into three subspecies in a 16h. elution programme. The extent of separation was not as well defined as that achieved by Kikkawa et al. (1987), using a Koken HA column with spherical crystals. These workers obtained three clear well-separated peaks using rat brain extract as a source of PKC, in a programmed elution step of only 4.5h. Hence it is important to be aware of the variability between types of HA column when attempting comparisons. This point is illustrated in figure 3.7.1 where data from a number of published investigations have been collated. Another example of the variability in technique is

illustrated by the fact that the workers indicated in figure 3.7.1, using different HA columns, reported peaks of PKC activity eluted at different concentrations of potassium phosphate. Thus Kikkawa et al. (1987), using a Koken HA column and rat brain PKC as enzyme source, recorded peak activities at 70mM, 90mM and 140mM potassium phosphate; Pelosin et al. (1987) employed a Mitsui Toatso column, and identified PKC peaks at 30mM, 45mM, 60mM and 90mM potassium phosphate; Wooten and Wrenn (1988) used a Biorad column and reported elution of PKC peaks at at 40-60mM, 70-90mM and 100-150mM potassium phosphate, whilst the Mitsui Toatsu column employed by Dianoux et al. (1989) gave peak enzyme activities at 55mM, 65mM and 110mM potassium phosphate.

Initial investigations concentrated on developing a method to resolve PKC isoforms in glia using HA column chromatography. Prior to assay of glial PKC, however, it was necessary to standardise the column for the α, β and γ subspecies using rat brain PKC as enzyme source. Only when three well-defined peaks could be achieved could the glial-derived PKC be investigated to determine which subspecies were present. Unfortunately spherical HA was not available in Britain so initial attempts to resolve the subspecies were made using a Biorad HA column. Later on a Tonen spherical HA column was used.

3.7.a. Results obtained using the Biorad hydroxylapatite column

Initial attempts to resolve PKC subspecies followed the method advised by M. Shearman (personal communication) from Nishizuka's group, as detailed in the Methods section (see section 2.10). The Biorad HA column was borrowed from Dr. A. Freeman (Charing Cross and Westminster Medical School) and was used with a guard column.

Initial problems concerned column pressure levels. As HA columns are very sensitive to pressure it was important to ensure a constant

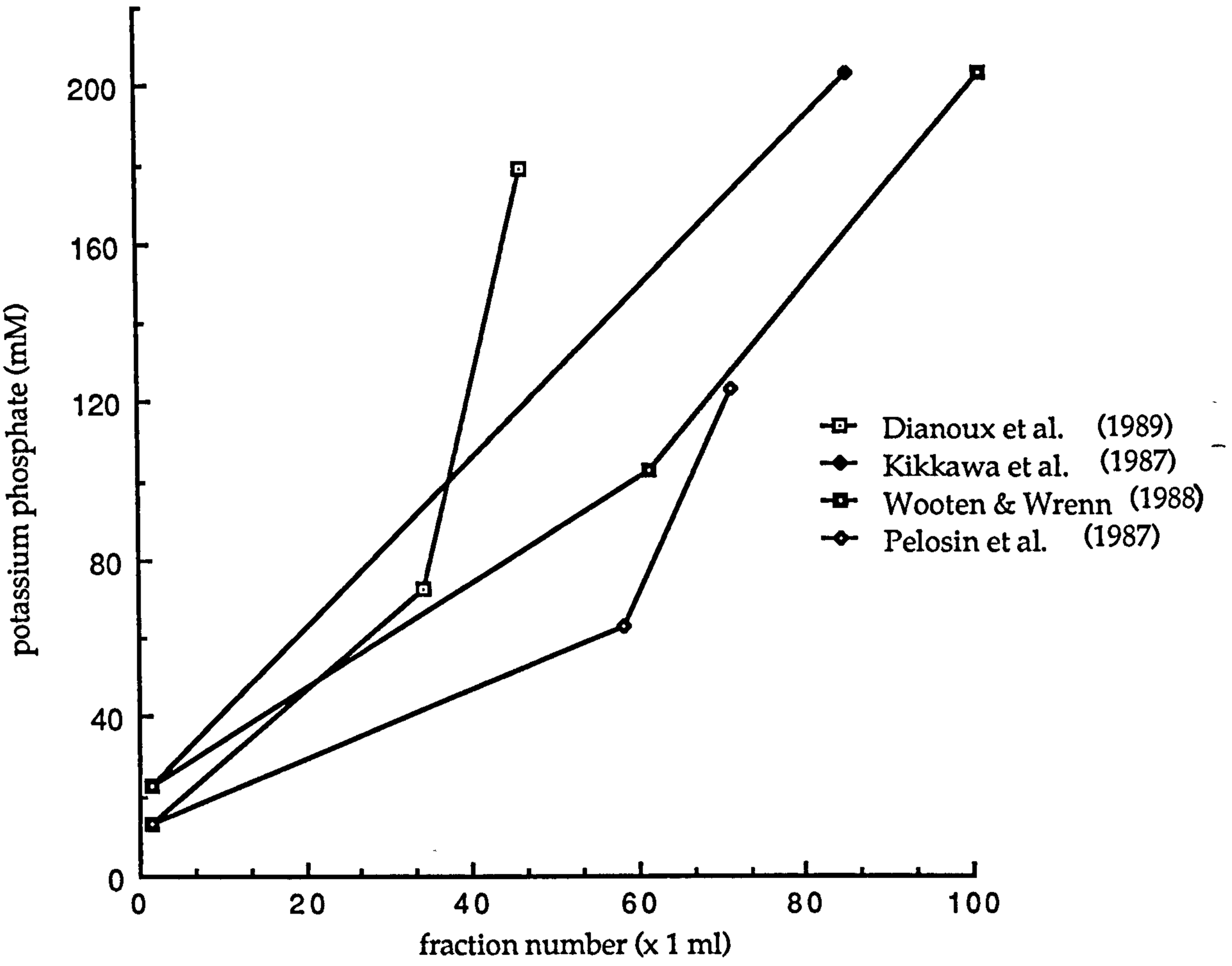


Figure 3.7.1. A comparison of potassium phosphate gradient construction for elution of protein kinase C from hydroxylapatite.

(non-fluctuating) low pressure (approx 3 megapascals); anything greater than this may irreversibly damage the column packing. When the method detailed by M. Shearman was followed, where a filtered DE-52 column eluate was applied to the HA column, the pressure limits were always exceeded. Despite numerous washings of both the guard column and the HA column, each application of the PKC sample had the same effect; an HA column and guard column exhibiting low pressure readings during equilibration with column buffer always showed pressure readings exceeding the permitted level on application of enzyme sample. Hence the column separation could not proceed.

A change in the methodology was introduced as a result of discussions with Dr. A. Freeman. The DE-52 eluate was dialysed overnight against HA column buffer (20mM potassium phosphate) in order to remove the Tris contained in the enzyme sample. In addition, the guard column was removed. Since this was present primarily to ensure that nothing blocked the column, but the DE-52 column eluate was always filtered through a 0.2µm membrane prior to application to the HA column, its presence was felt to be unnecessary. In this revised procedure there was a reduction in pressure on application of the enzyme sample, and a chromatographic separation was possible. However, PKC activity was resolved into only two peaks of activity with perhaps a shoulder on the first peak, indicating the presence of a third subspecies (see figure 3.7.2A). Constructing different phosphate buffer gradients in an attempt to further separate this activity did not improve matters (see figures 3.7.2B-D). Peak activities varied somewhat due to protein concentration of the sample. The two peaks characteristic of liver PKC could be detected (i.e. subtypes ß and α respectively) as shown in figure 3.7.2B. Thus it was not certain whether the first peak obtained

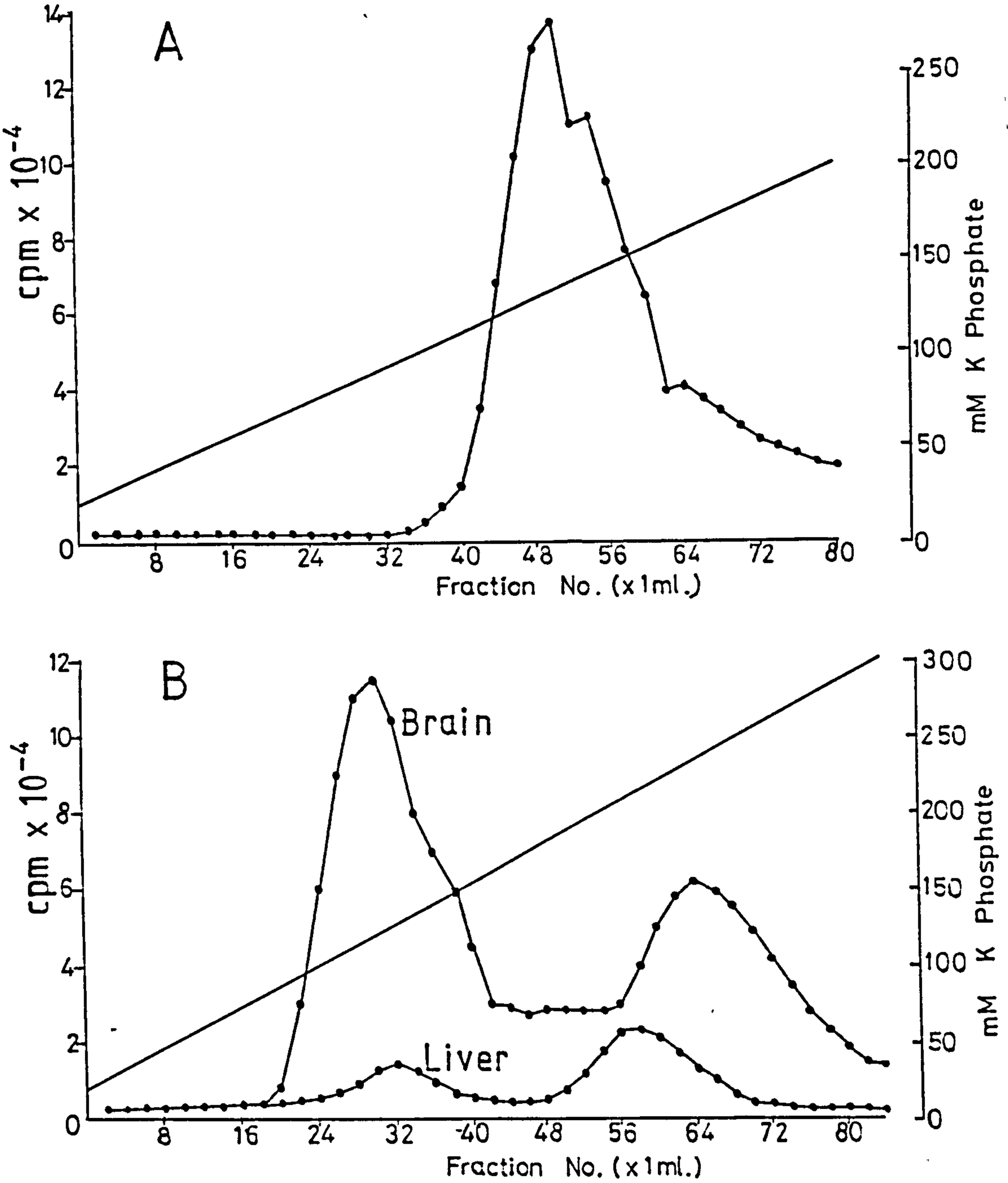


Figure 3.7.2. Resolution of protein kinase C subspecies of rat brain and liver using Biorad hydroxylapatite column chromatography.

DE-52 column-purified extracts were dialysed overnight, applied to the column, and eluted using a potassium phosphate gradient. Enzymatic activity was determined as detailed in section 2.4.

(A) rat brain and (B) rat liver protein kinase C.

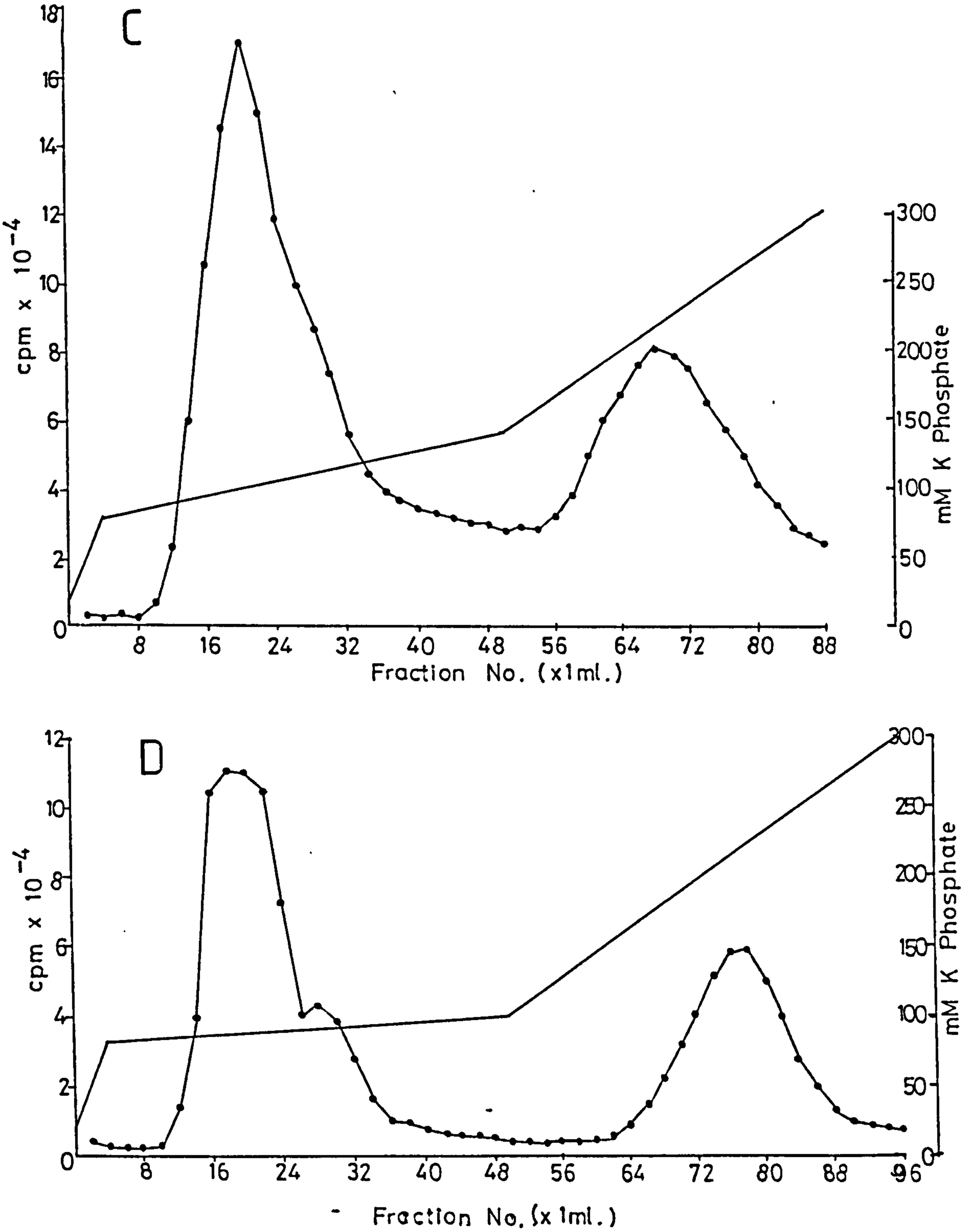


Figure 3.7.2. (cont. from previous page) Resolution of PKC subspecies of rat brain, using a stepped potassium phosphate gradient.

using rat brain PKC represented a fairly equal mixture of subtypes y and ß or whether it merely represented one of the subtypes masking a second with a much lower activity. Lipid activation experiments were undertaken to attempt to characterise these peaks, but comparing them with those obtained using rat liver, shed no further light on the matter. Application of whole glial cells PKC extract or cytosolic extract resulted in no peak activity being detected (see figure 3.7.3), despite the sample being highly active prior to application to the HA column. Neither was the activity run straight off the column nor eluted with 800mM potassium phosphate. There appeared to be a broad spreading out of activity, with all samples exhibiting levels of activity above basal levels seen when using rat brain, and no obvious peak activity was present. Regular analyses of the HA column elution characteristics were carried out using appropriate test samples to ensure that no problems existed.

3.7.b. Results obtained using a Tonen spherical hydroxylapatite column

The Tonen HA column was similar to the Koken column employed by Nishizuka and colleagues (Ono et al., 1987); the packing was a spherical HA of uniform size. A linear gradient elution advised by M. Shearman was used initially. The results obtained for resolution of whole rat brain PKC subspecies are shown in figure 3.7.4 Three clear peaks were obtained corresponding to subspecies χ, ß and α respectively at potassium phosphate concentrations of 120-125mM, 160-180mM and 260-270mM. Such molarities differ from previously published studies, but this is perhaps not surprising since peak enzyme activities appear at various concentrations of potassium phosphate according to the type of HA column used. In addition, no previous investigations have employed a

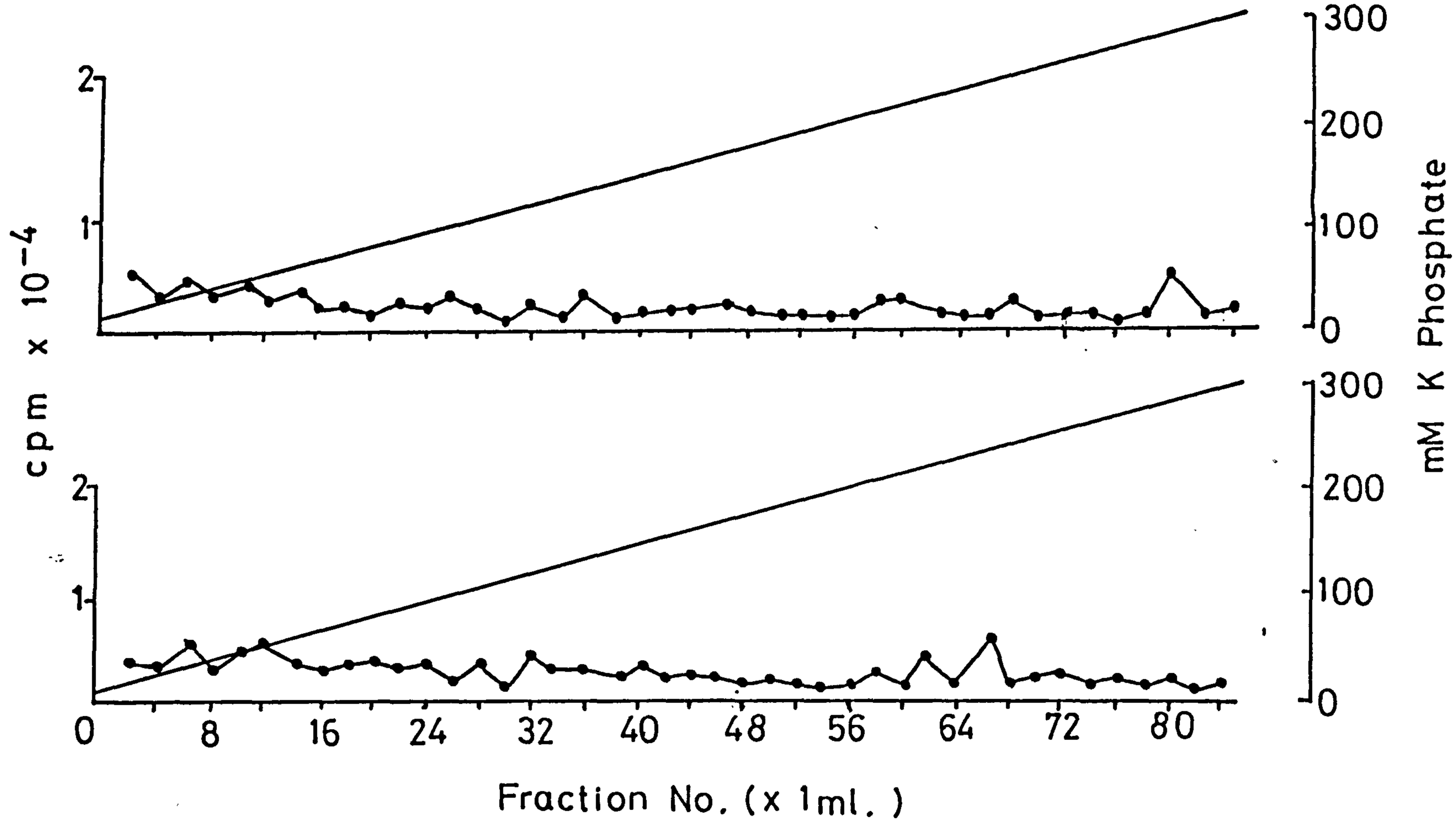


Figure 3.7.3. Resolution of protein kinase C subspecies of rat glial cells using Biorad hydroxylapatite column chromatography.

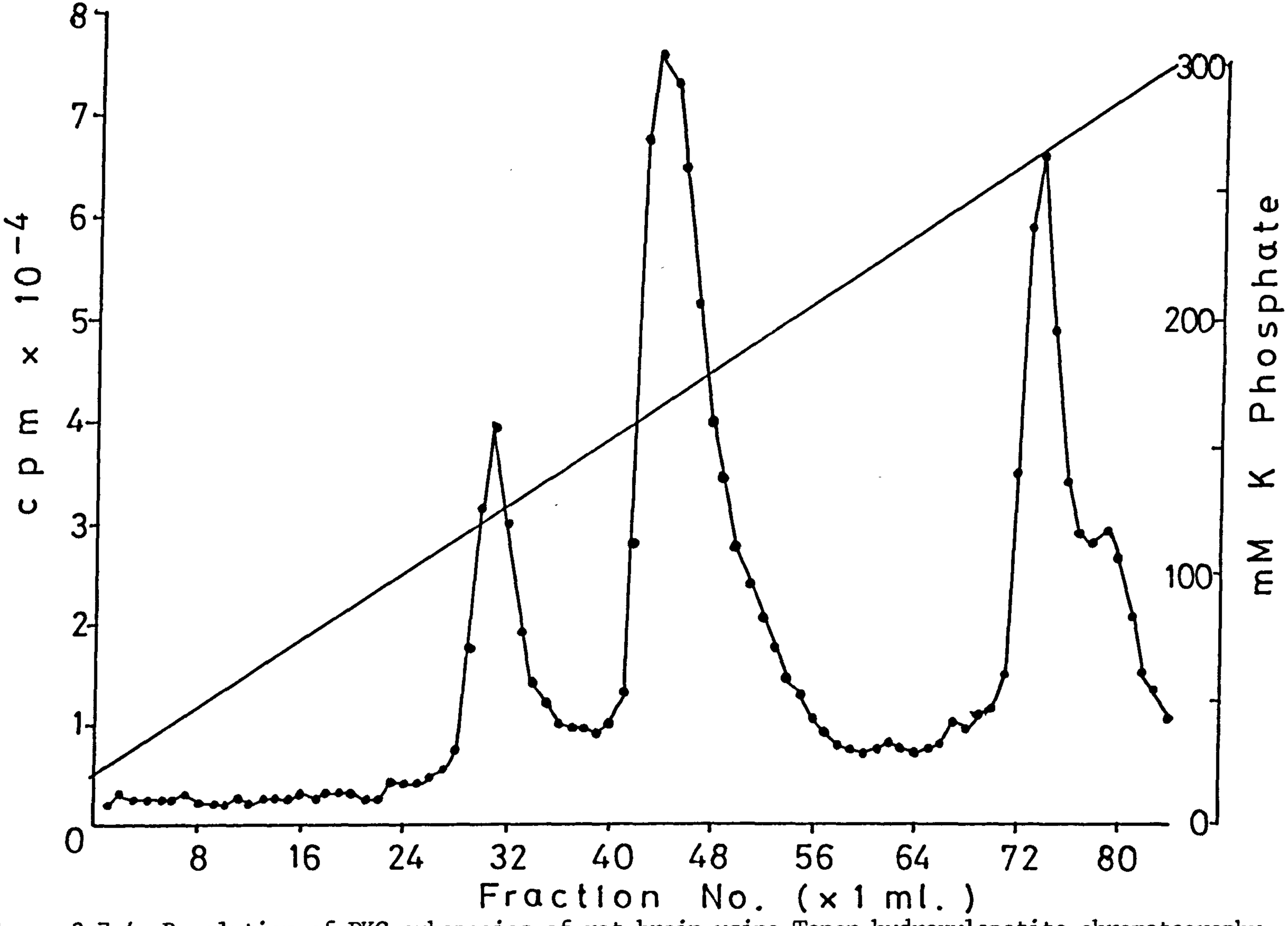


Figure 3.7.4. Resolution of PKC subspecies of rat brain using Tonen hydroxylapatite chromatography.

Tonen HA column, which is new on the market. To date, according to the literature, the best column available in terms of resolving α, β and γ subspecies into three well-defined peaks in a relatively short time period is the Koken HA column, but the resolution obtained in the present study shows the Tonen column to be every bit as good. Both columns have spherical HA packing but the Koken packing is of variable particle size (1-6μm), whereas the Tonen packing is of a regular size of 5μm (not less than 80%) perhaps giving a more uniform separation. For this reason the Tonen column ought to be far superior to those HA columns with irregular particles of HA such as conventional HA or the 'coral-shaped' HA described by Mitsui Toatsu.

The repeated appearance of three peaks of PKC activity when using rat brain as the enzyme source indicated that the Tonen column was resolving well. However, when DE-52-purified supernatants from a mixed glial cell primary culture were resolved, no peaks of activity were obtained (see figure 3.7.5A). Similar results were achieved when using older primary cultures, oligodendrocyte-enriched subcultures and type-1 astrocyte cultures (see figures 3.7.5B-D). Levels of PKC activity obtained were above basal levels as measured by Ca^{2+}-independent kinase activity. In all cases the DE-52-purified supernatant samples contained appreciable PKC activity after overnight dialysis and prior to separation on the HA column, as measured by the standard PKC assay (see sections 2.4-2.6). In addition, regular analyses of the HA column elution characteristics were carried out using appropriate test samples, and rat brain extracts were chromatographed at various times to ensure that the column was resolving the α, β and γ subspecies accurately. It is of course possible that some of the enzyme activity is lost during the column purification because although the level of enzyme purification is high, the actual recovery of protein loaded onto the column is low.

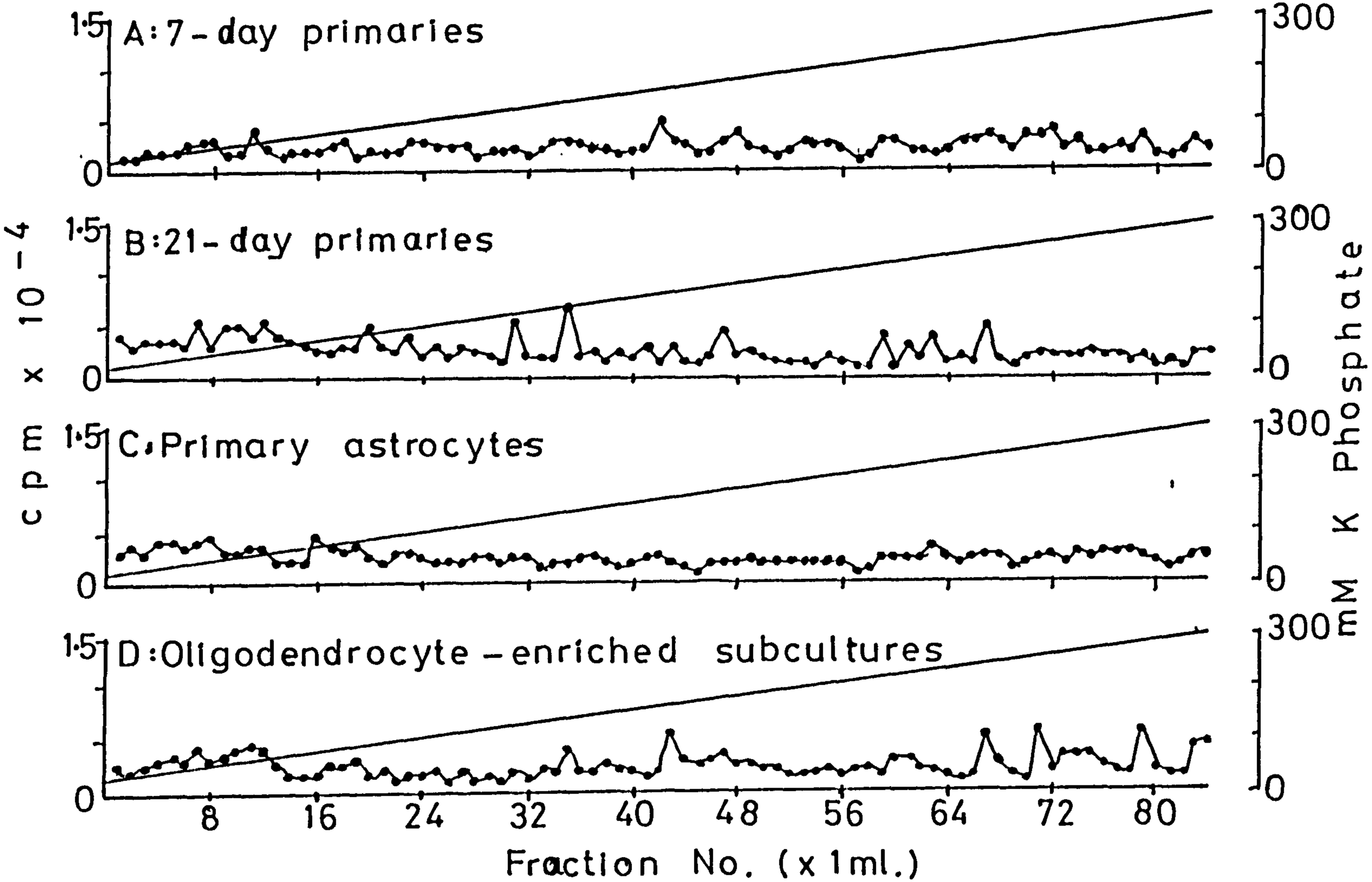


Figure 3.7.5. Resolution of PKC subspecies of glia using Tonen hydroxylapatite chromatography.

These results are, by essence, preliminary investigations but the absence of peaks corresponding to α, β and γ subspecies are very exciting since no cells to date have been identified which do not contain either of these three PKC subspecies (P. Parker, personal communication). The results require verification with the use of monoclonal antibodies for example, but, though requests have been made, no specific antibodies have been made available for use. Another line of investigation would be to use cDNA probes to search for the presence of not only α, β and γ-PKC but also the other subspecies which have been sequenced. Such a study would be very informative and would provide more information on the role of PKC in glia.

3.8. PROTEIN KINASE C AND GLIAL CELL DIFFERENTIATION

INTRODUCTION

Having firmly established the presence of PKC in glia and investigated its characteristics, the question of the precise role of the enzyme within glia arises. PKC has been implicated in a wide variety of processes in various cell types ranging from muscle contraction (Rasmussen et al., 1984) to glucose transport (Kirsch et al., 1985) and synaptic transmission (Nishizuka, 1986). A series of reports have recently appeared in the literature implicating PKC in glial cell differentiation. Honegger (1986) has reported that phorbol esters enhanced the differentiation of astrocytes in serum-free aggregating foetal rat brain cell cultures. Murphy et al. (1987) have shown a similar proliferation of astrocytes in primary culture in response to phorbol esters. Fawthrop and Evans (1987 a,b), however, have induced morphological changes in astrocytes with a calcium ionophore and shown that the changes were independent of PKC activation. Schroter and Althaus (1987) have shown an accelerated regeneration of processes in cultured porcine oligodendrocytes exposed to phorbol ester,

an observation also made by Yong et al. (1988) in bovine oligodendrocytes. More recently Bhat (1989) has shown that TPA increased process formation in one-day old glial subcultures derived from rat brain. In addition, PKC has been implicated in neurotransmitter release in astrocytes (Hansson and Ronnback, 1989) and neurones (Zurgil and Zisapel, 1985).

The effect of in vitro PKC activation on cell morphology was investigated in this study. Cultures were examined for PKC-dependent changes using phorbol ester, calcium-dependent changes using Ca^{2+} ionophore A23187, and a combination of the two compounds to investigate whether any synergistic relationship existed. Cell populations employed were 7-day old primary mixed glial cultures. Cell growth was monitored over a period of 72h. in culture; cell proliferation and morphological changes were recorded.

As a comparison, the effect of PDGF on 7-day old primary mixed glial cultures was investigated. PDGF is secreted by type-1 astrocytes and stimulates O-2A progenitor cells to divide, preventing their premature differentiation into oligodendrocytes (Noble et al., 1988; Richardson et al., 1988). At seven days in culture a large percentage of the glial cells are O-2A progenitor cells (i.e. A2B5+) which should thus be susceptible to the influence of PDGF.

RESULTS AND DISCUSSION

Seven-day old mixed glial cell cultures were given fresh medium containing either 25nM 4-α-phorbol, 25nM TPA, 10μM A23187, or 25nM TPA+10μM A23187. Cultures to be treated with PDGF had been switched to a serum-free defined medium 24h. earlier as described by Richardson et al. (1988). Astrocyte-conditioned medium was used as the source of PDGF (Richardson et al., 1988).

The effects of the various additions to the cultures on cell

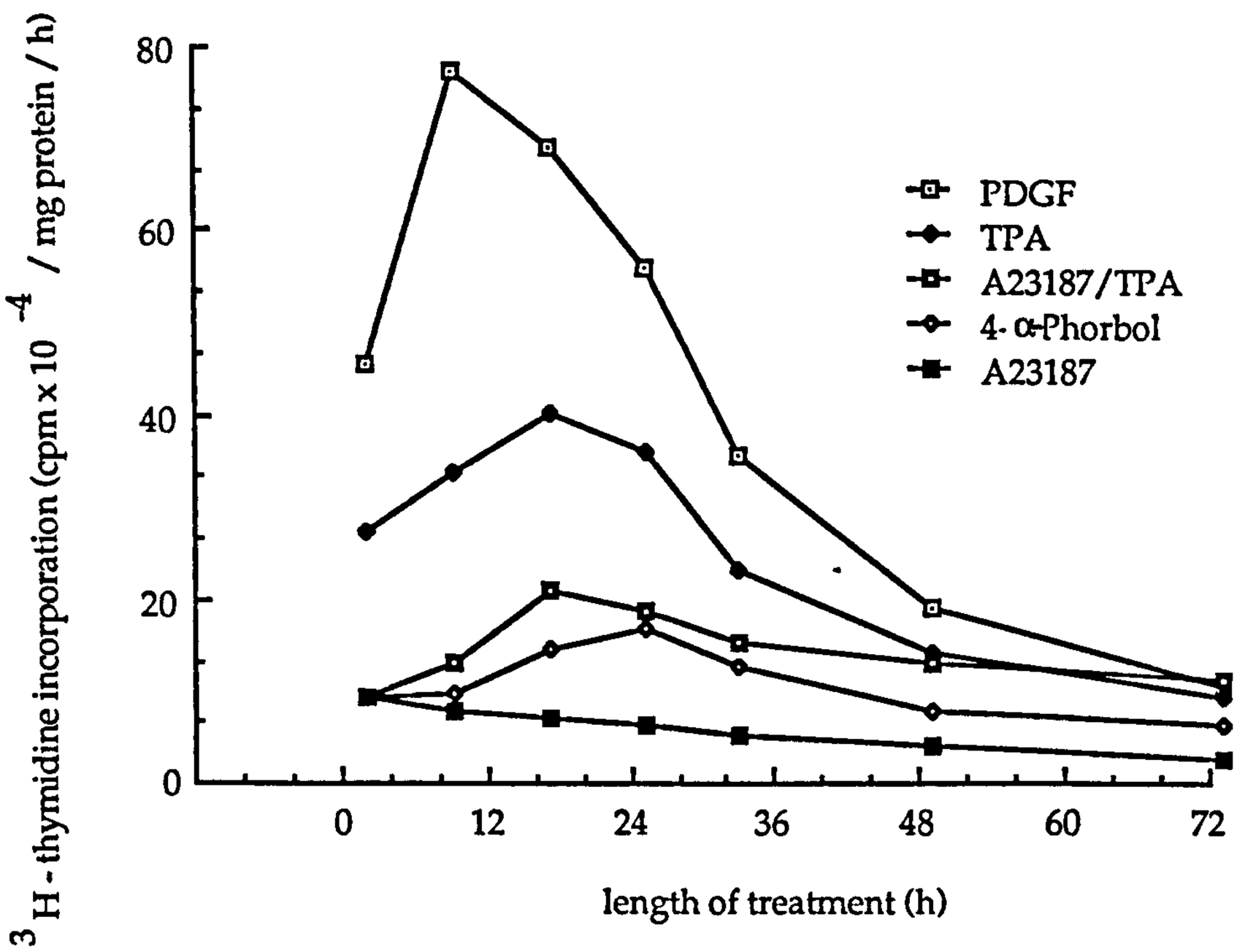


Figure 3.8.1. The effect of various mitogens on cell proliferation in glia.

Seven day old glial primary cultures were treated with the various compound listed above. Astrocyte-conditioned medium (1 : 10 dilution) was used as the source of PDGF. At 1h. prior to the times indicated (1, 8, 16, 24, 36, 48, and 72 h.), duplicate wells of cultures were labelled with 1 uCi/ml ^{3}H-thymidine for 1h. Cells were then harvested and levels of ^{3}H-thymidine incorporated into TCA-precipitable material determined. Each point represents the mean value of triplicate determinations of two culture wells. S.D.'s were <10% in all cases. Other details are contained in the text.

proliferation were assessed using ^{3}H-thymidine incorporation studies at various time points throughout the 72h. period of treatment. Results are presented in figure 3.8.1.

A huge increase in DNA synthesis was seen in PDGF-treated cultures with an approximate four-fold increase over control (4-α-phorbol-treated) cultures. A marked stimulation of ^{3}H-thymidine incorporation was also apparent in TPA-treated cultures although levels were clearly below those seen with PDGF. A23187 caused a reduced level of glial DNA synthesis. This effect was counteracted to some extent by the addition of TPA, but even together levels of incorporation did not reach those found with TPA alone. Thus according to the data in figure 3.8.1., although PKC is clearly implicated in DNA synthesis (as TPA leads to an increased level of ^{3}H-thymidine incorporation over control values) some additional factor(s) is required to account for the greater (and perhaps maximal) response observed. This extra factor(s) apparently present in PDGF-stimulated cultures, is unlikely to be Ca^{2+}-mediated as A23187 has an inhibitory and not a stimulatory effect on ^{3}H-thymidine uptake. It is possible that a tyrosine-specific protein kinase may be involved as the PDGF receptor itself contains an intracellular tyrosine kinase domain (Yarden et al., 1986). Future work investigating such an hypothesis, however, would require a control test sample treated with serum-free medium alone (i.e. without the addition of PDGF) in order that any contribution made by the medium may be examined. This medium contains insulin, a prominent activator of DNA synthesis in astrocytes (Murphy et al., 1985) so it may be contributing to the "PDGF-effect" seen in the present investigation. Also of interest would be to compare the effect of serum-free medium with the usual medium of choice, 10% FCS in DMEM, so that the cells "basal" level of DNA synthesis might be determined. As activation of cell receptors by growth factors stimulates a whole host of cellular

changes (see Williams et al., 1988, for example) investigations into how such growth factors affect DNA synthesis may be rather complex. Many growth factors regularly used in work of this type still await fuller characterisation.

These observations must be considered in light of the morphological changes seen during the treatment regime (see figure 3.8.2.). Figure 3.8.2A is representative of all treatments after 1h. in culture, with cells either clumped or singular, some with processes and some floating cells. By 8h. in culture, cells treated with TPA and PDGF have a similar appearance (figure 3.8.2*C*) with a more elaborate cell network apparent than with 4-α-phorbol (figure 3.8.2*B*). Cells treated with A23187 (± TPA) are little changed (see figure 3.8.2D) with a large number of cells floating in the medium.

By 32h. in culture cells treated with TPA had undergone a huge increase in number with many phase-bright cells apparent, and a dense network of cells had been established (figure 3.8.2F). This was in marked contrast to the 4-α-phorbol-treated cells which were little different from the 8h. treatment point. Cells treated with A23187 (± TPA) appeared, at 32h. in culture, to have a number of phase-bright cells, a number of which were clumped (see figure 3.8.2G). Few floating cells were apparent. The attached cells had begun to elaborate processes which in many cases were connected to other cells. At 32h. PDGF-treated cultures, though apparently not present in the same numbers as cells treated with TPA (see figure 3.8.2H), had formed a distinct network of cells across the substratum, with a similar morphology to the TPA-treated cultures.

At the 72h. timepoint, cells treated with 4-α-phorbol (figure 3.8.2I) had formed a network, but they appeared to be a population of astrocytes predominantly with some small phase-bright cells growing on top. This contrasted with the TPA-treated cultures (figure 3.8.2J) in

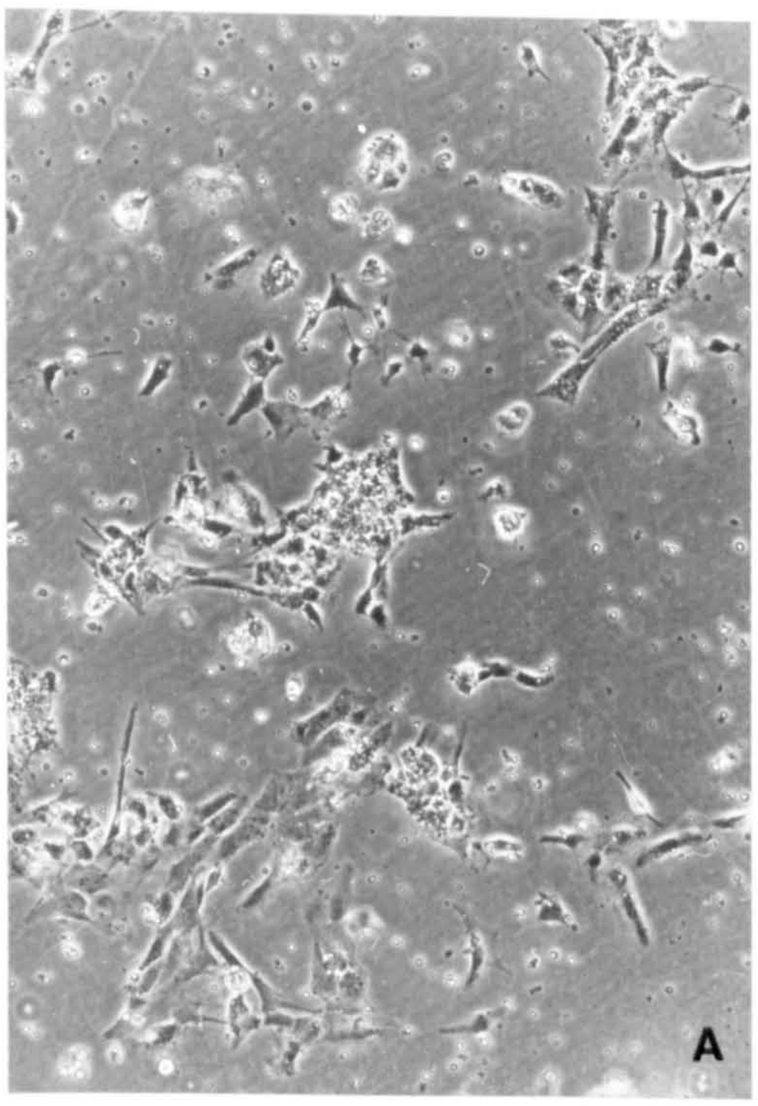
Figure 3.8.2. Morphological effects of various mitogens over a 72h. treatment period on seven day mixed glial cultures. Magnification x 200.

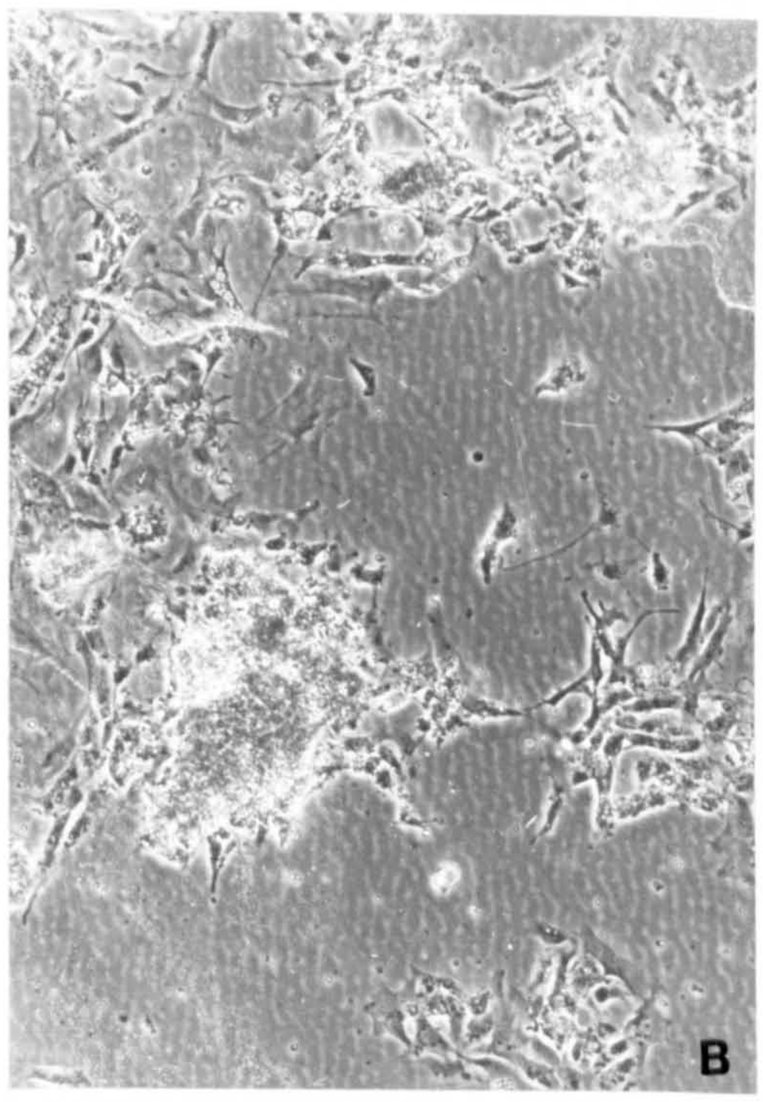
A: 1h. in culture; representative of all treatments

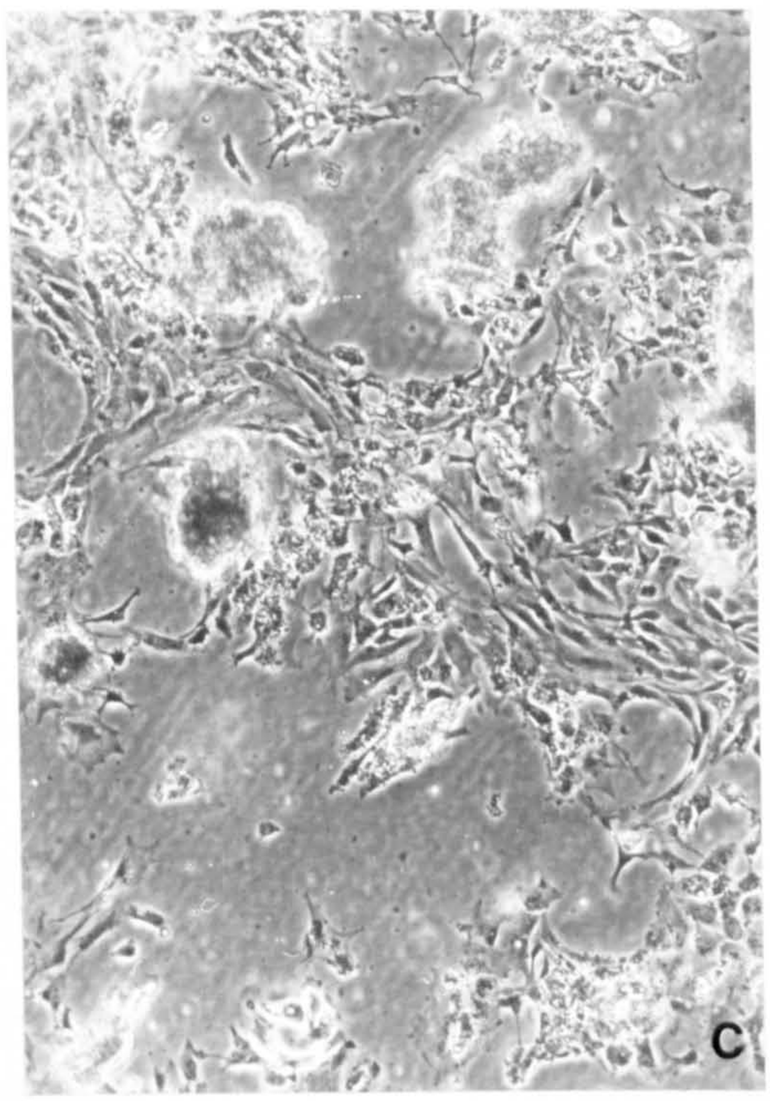
B: 8h. of 25nM 4-α-phorbol treatment

C: 8h. of 25nM TPA (or PDGF) treatment

D: 8h. of 10μM A23187 (or 10μM A23187 + 25nM TPA) treatment


A


B


C

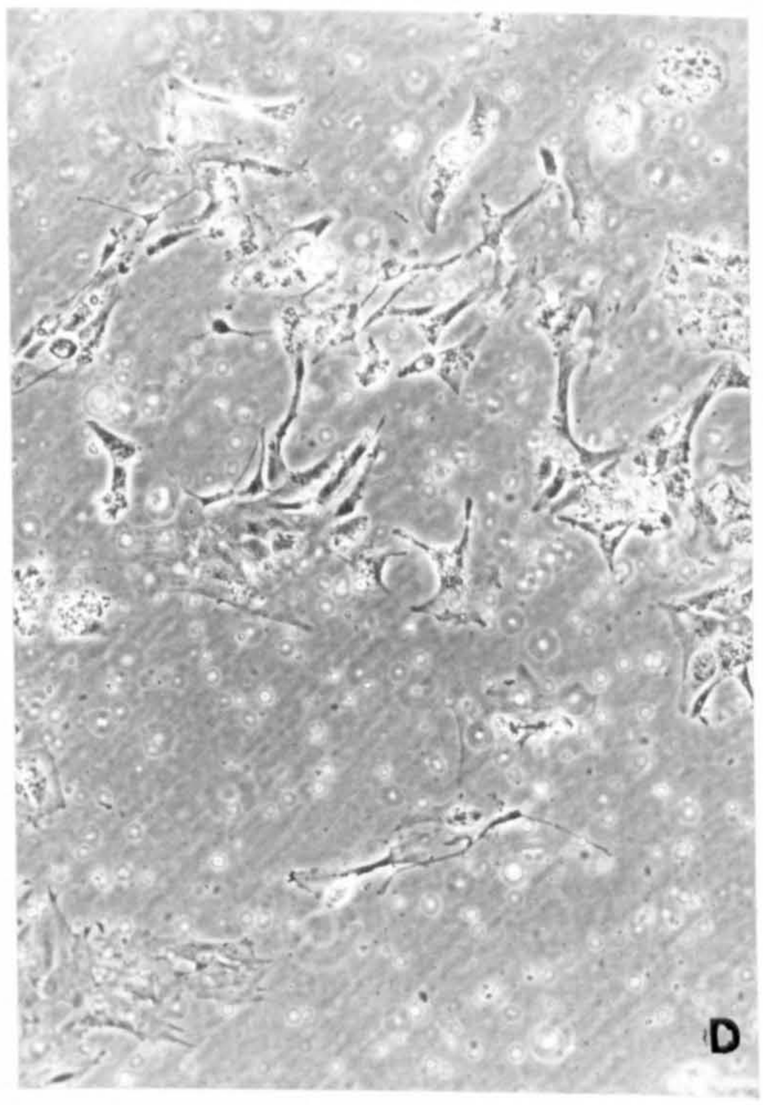

D

Figure 3.8.2. (Cont.)

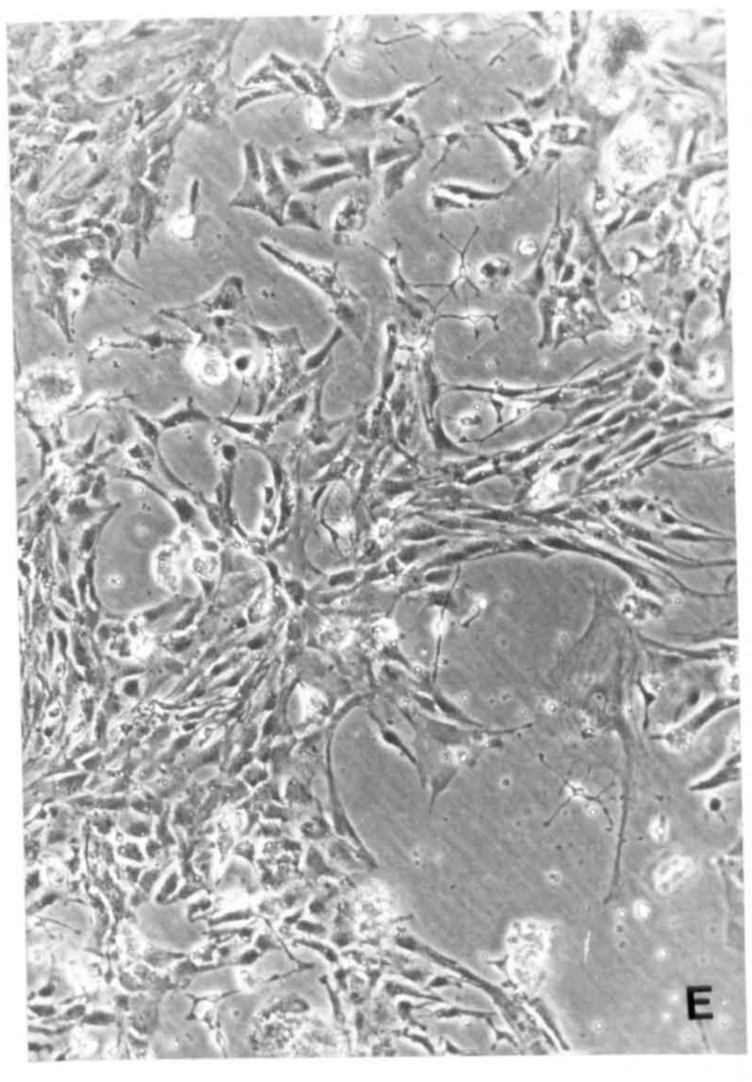
E: 32h. of 25nM 4-α-phorbol treatment

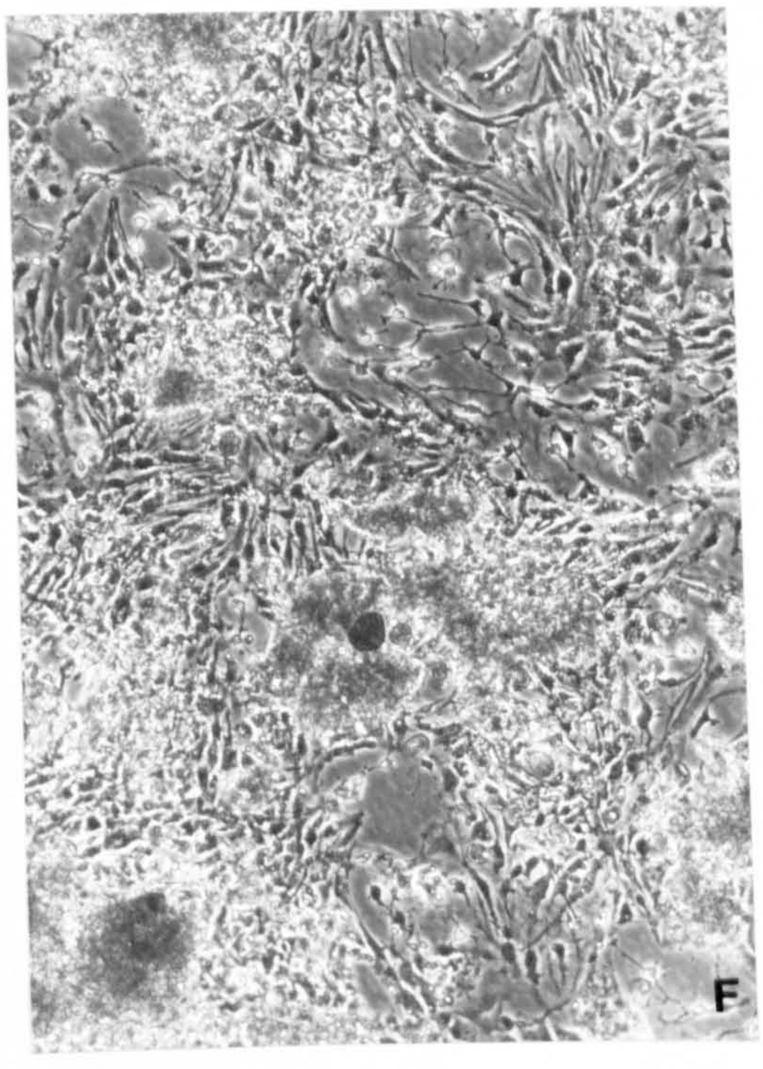
F: 32h. of 25nM TPA treatment

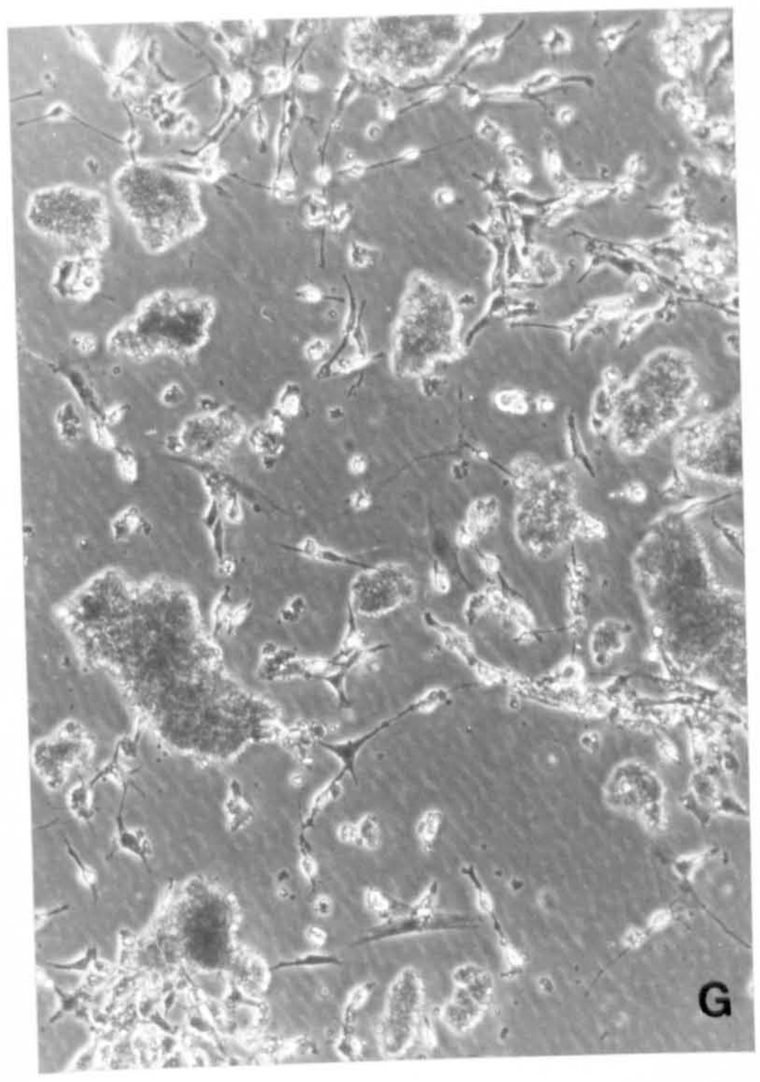
G: 32h. of 10μM A23187 (or 10μM A23187 + 25nM TPA) treatment

H; 32h. of PDGF treatment

(Magnification x 200)


E


F


G

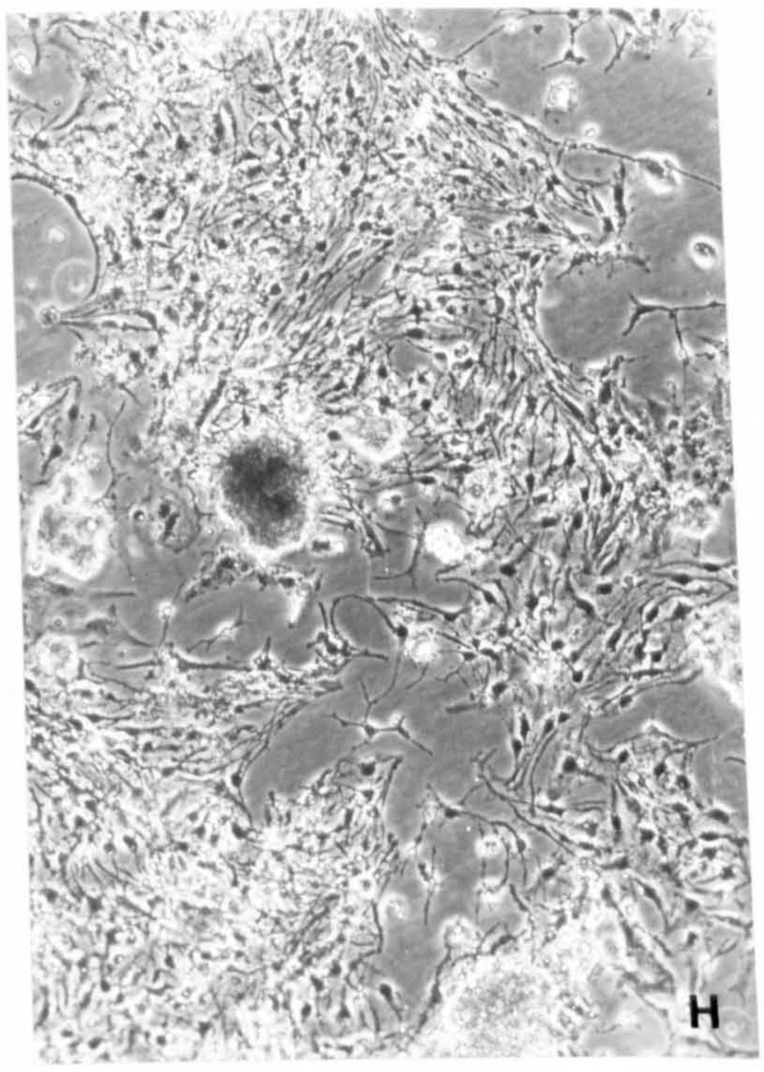

H

Figure 3.8.2 (Cont.)

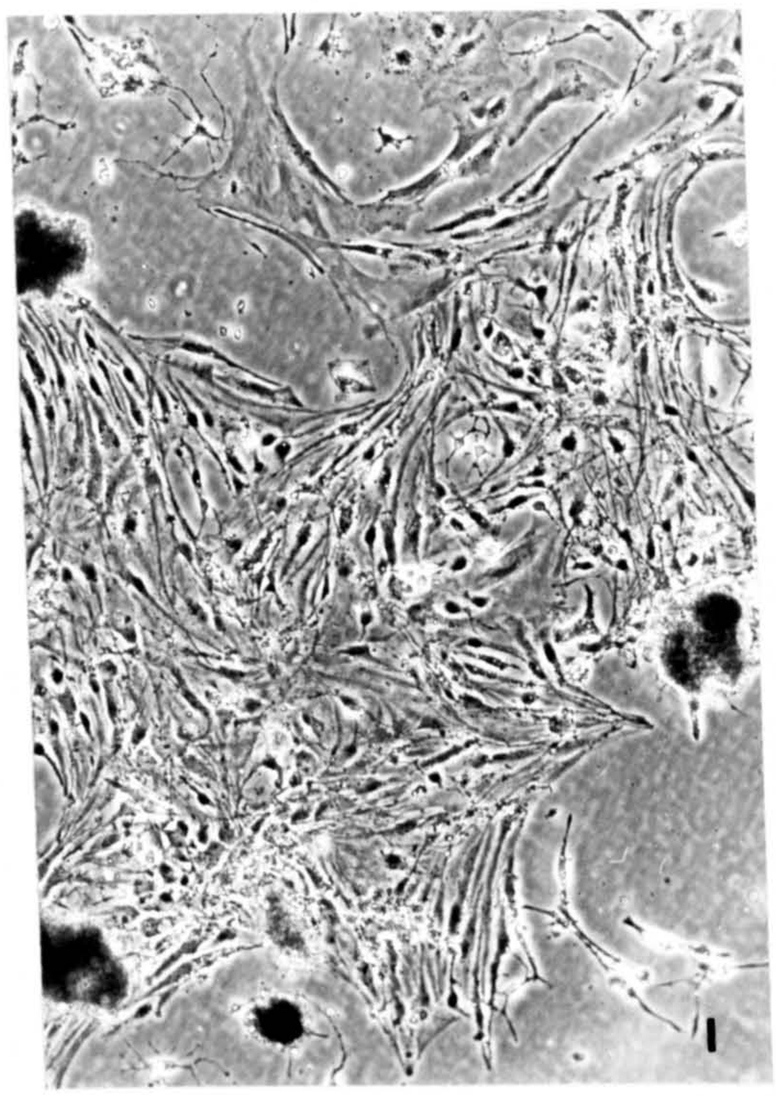
I: 72h. of 25nM 4-α-phorbol treatment

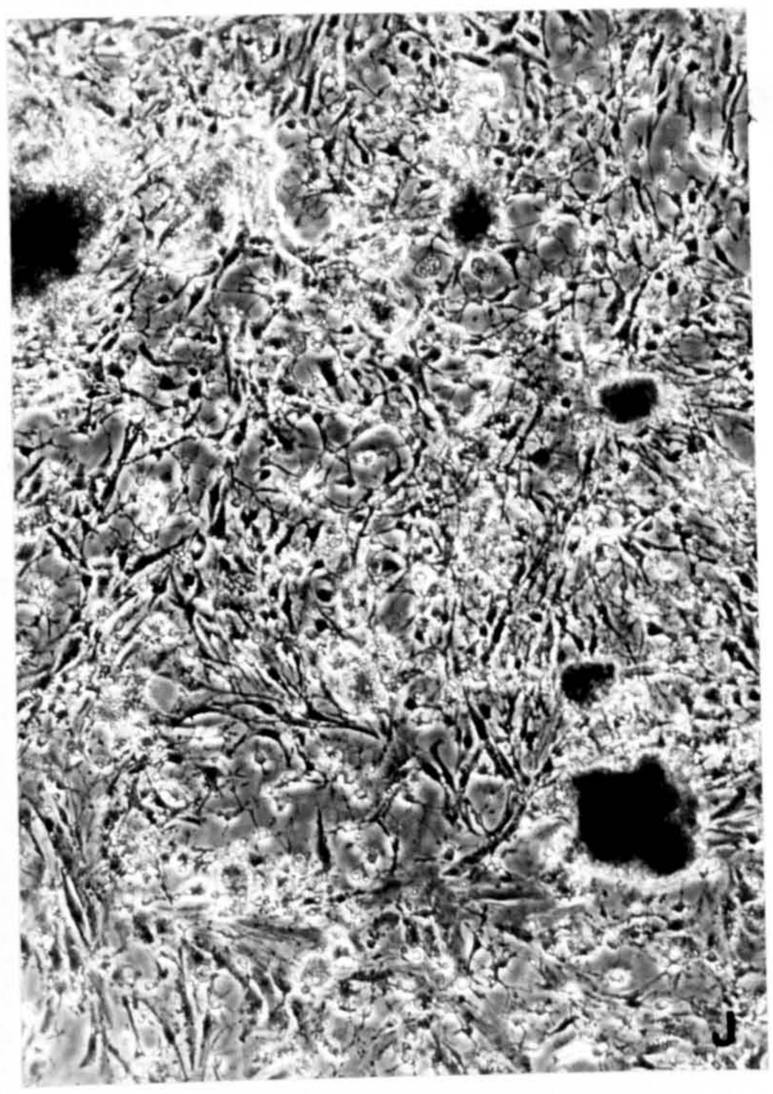
J: 72h. of 25nM TPA treatment

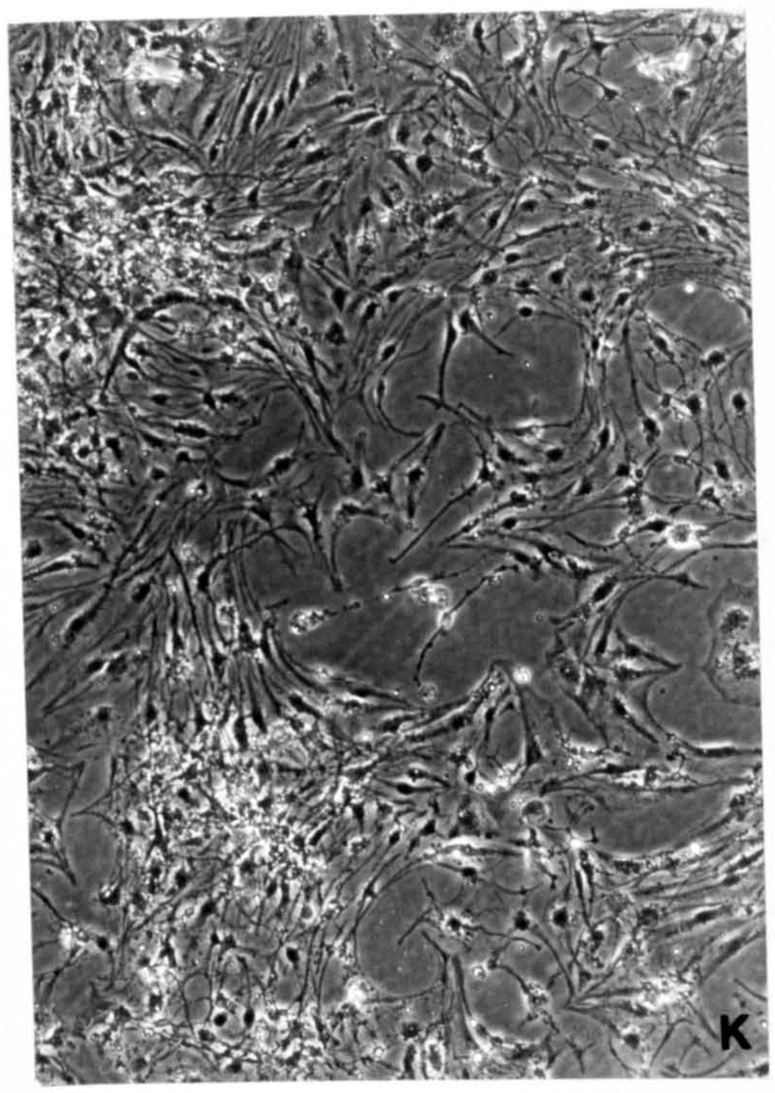
K: 72h. of PDGF treatment

L: 72h. of 10μM A23187 + 25nM TPA treatment

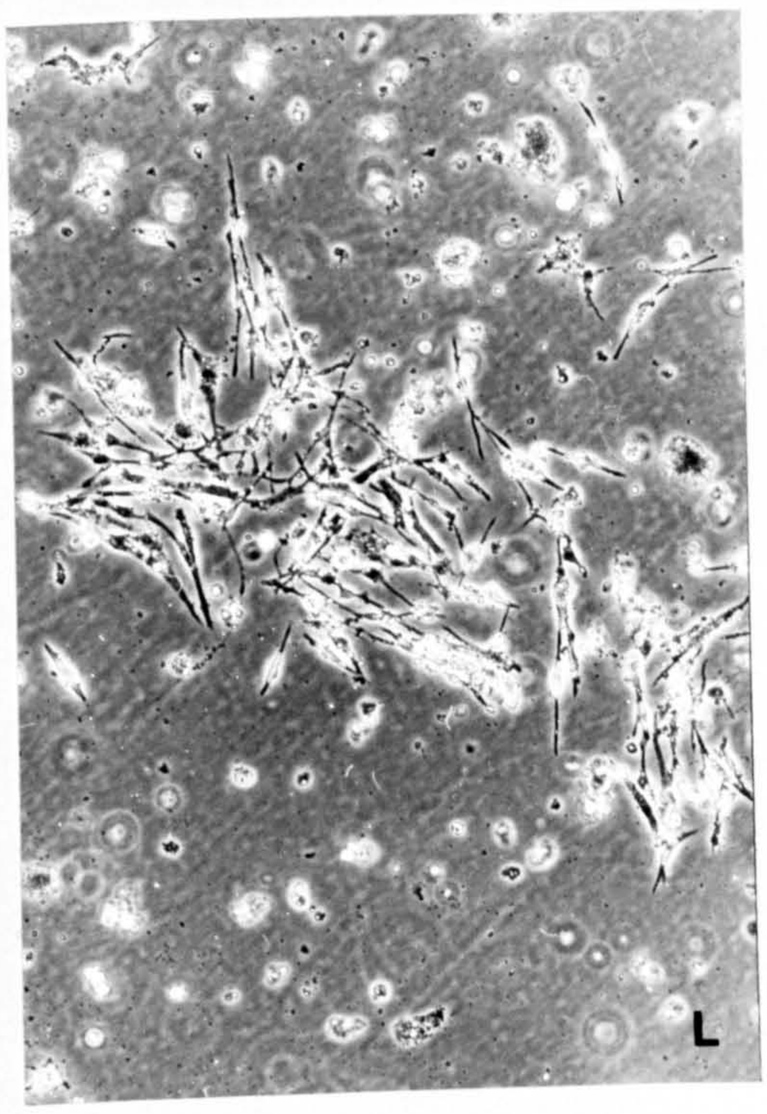
(Magnification x 200)







K



L

which a large number of phase-bright cells could be seen growing over a dense cellular network of cells. There appeared to be no gaps in the cell network and a number of large clumps of phase-bright cells, seen in figure 3.8.2J as dark patches, were visible. By 72h. in culture cells treated with A23187 + TPA had a number of phase-bright cells apparent (figure 3.8.2L). However, in common with those cells treated with A23187 alone, there were many floating cells, indicative of massive cell death. This was substantiated by protein assay and nigrosin dye exclusion. The cells that remained were very elongated in appearance with a different morphology to that seen earlier (figure 3.8.2G). PDGF-treated cells at 72h. in culture (figure 3.8.2K), rather than having an appearance similar to TPA-treated cells (figure 3.8.2J), were more like those treated with 4-α-phorbol (figure 3.8.2I). PDGF-treated glial cultures lacked the same number of gaps in the cell sheets as observed with 4-α-phorbol, but cell morphology was very similar. These cultures did not have the same dense cell growth that was apparent with TPA.

Overall, the following pattern appears to have been established by morphological observations:

(i) TPA-treated cells showed a huge increase in cell number by 32h. treatment and had formed an extremely dense network of process-bearing cells with clumps of phase-bright cells by 72h. in culture;

(ii) PDGF treatment caused cells to increase hugely in cell number by 32h. treatment to form an elaborate network of process-bearing cells by this time, similar to TPA-treated cultures; by 72h. treatment with PDGF, the cells resembled those cultures treated with 4-α-phorbol more closely than those treated with TPA;

(iii) Cultures treated with A23187 (± TPA) showed a massive cell loss,

although some processes were elaborated by the remaining cells.

Taking points (i) and (ii) together, it almost seems as if TPA stimulation leads to a phase of cell proliferation in which cells grow across all the available substratum. The initial large growth phase seen with PDGF, as reflected in the ^{3}H-thymidine incorporation studies, falls off into an apparently more 'ordered' growth pattern. This suggests that TPA-stimulated cultures were perhaps missing a 'controlling' factor which was present in PDGF-treated cultures. Of possible relevance here is a recent observation by Housey et al. (1988) that overproduction of β_1 subspecies of PKC caused a disordered growth control in rat fibroblasts, an interesting phenomenon given that PKC is the intracellular receptor for tumour-promoting phorbol esters (Ashendel, 1985).

Although PDGF (in the form of astrocyte-conditioned medium) elicited a morphological response similar to TPA up to 32h. treatment and showed an increased level of ^{3}H-thymidine incorporation, it is not certain through which mechanisms the growth factor may be acting. PDGF may act via both PKC and IP_3 following PI turnover (Kaplan et al., 1987) and by activation of a tyrosine kinase (Ek et al., 1982). Results obtained here suggest the likelihood that both systems may be operating. However, it was not known whether PDGF activated PKC in glia. The growth factor has been shown to enhance the phosphorylation of an 80kDa band, a marker of PKC activation in intact cells, in fibroblasts and 3T3 cells (Blackshear et al., 1986; Rozengurt et al., 1983). The ability of PDGF to activate glial PKC, as judged by enzyme translocation (Kraft and Anderson, 1983), was investigated. Results presented in table 3.8.1 show that indeed PDGF does bring about PKC translocation; following a 30 min. treatment with astrocyte-conditioned medium, particulate levels of PKC increased by 56%. Therefore it may be supposed that any effects

of PDGF on cell morphology and proliferation could indeed be PKC-mediated, with any differences in response between TPA- and PDGF-treated cultures likely to be due to one of the whole host of cellular processes activated by the binding of PDGF to its receptor (see Williams et al., 1988) e.g. activation of tyrosine kinase or IP_3.

One important distinction to be made concerning TPA- and PDGF-induced PKC activation however relates to the contrast between a non-physiological and a physiological activation of the enzyme. TPA activates PKC directly and is not degraded following enzyme activation, but persists in the membrane as no physiological method exists to terminate its action (Kikkawa and Nishizuka, 1986). The PDGF, however, activates PKC via the PI pathway, the DAG produced, leading to activation of PKC. This DAG will remain only transiently in the membrane prior to its degradation by DAG kinase or lipase (Kaplan et al., 1987; Berridge, 1987). Hence the hypothesis of uncontrolled cell growth may reflect this non-physiological activation of PKC by TPA.

In order to determine the precise relationships between the various kinases in glial cell proliferation and differentiation investigations ought to be carried out using cultures of different ages. This may then give some idea of exactly what stage of growth in culture is critical for processes such as cell adhesion, cell process formation and cell proliferation and which kinases are responsible for, or essential to, those stages. Such investigations ought perhaps to employ a critical physiological activator of PKC (e.g. synthetic DAG) as well as, or instead of, phorbol ester in order that only the effects of a physiological activation of PKC are examined.

An experiment carried out to see if phorbol ester and calcium ionophore had similar effects in older primary glial cell cultures (19 days) found that A23187 (± TPA) led to cells lifting off the substratum after 1h. of treatment and massive cell death following 24h. treatment.

TABLE 3.8.1.EFFECT OF ASTROCYTE-CONDITIONED MEDIUM ON SUBCELLULAR DISTRIBUTION OF PROTEIN KINASE C.

TREATMENT		PKC ACTIVITY (cpm/µg protein)	
Control[a]	Cytosolic	4134.4±367.7	(83%)
	Particulate	848.3± 65.1	(17%)
PDGF[b]	Cytosolic	1311.5±154.3	(27%)
	Particulate	3563.8±278.6	(73%)

Six-day old primary glial cultures were switched to a modified Bottenstein and Sato serum-free medium as described by Richardson et al. (1988). 24h. later cells were incubated with (b) and without (a) astrocyte-conditioned medium (1:10 dilution) for 30min. Cells were then washed in ice-cold homogenisation buffer and cytosolic and particulate PKC levels were determined as detailed in the Methods section. Figures given represent the mean ± S.D. of triplicate determinations from one experiment. Figures given in brackets represent the percentage of total cellular PKC, and were similar in two other experiments.

TPA appeared to have no effects different from those seen with 4-α-phorbol. Such results with regard to TPA are hardly surprising given that older cells are highly differentiated; once again calcium ionophore appeared to have a destructive role, causing cell detachment from the substratum. Fawthrop and Evans (1987a) have reported that A23187 and ionomycin (also a calcium ionophore) cause cell death in cultured astrocytes following a 3h. exposure, with a reversible change in cell morphology apparent in the remaining cell population. In contrast to the effects of phorbol ester apparent in the present investigation using a mixed glial cell population, Fawthrop and Evans (1987b) found no alteration in astrocyte morphology when treated with TPA or synthetic diacylglycerol. Perhaps the difference in response to activation of PKC in these two different culture systems reflects a difference in PKC subspecies. Results obtained using calcium ionophore require careful interpretation as the ionophore is a fairly crude mechanism for increasing internal Ca^{2+} concentrations. Thus this huge non-specific influx of Ca^{2+} ions would not reflect the <u>in vivo</u> situation where IP_3 is an important regulator of intracellular calcium concentration. So it is perhaps not surprising that such an increase in intracellular calcium led to glial cell death similar to that observed by Fawthrop and Evans (1987a).

It would be interesting to compare the effects of PKA on glial cell growth and differentiation with those of PKC. The use of forskolin or dibutyryl cAMP would also allow investigation of any synergy that might exist between these kinases. Richardson (1989) recently reported that O-2A progenitor cells isolated in bulk from rat optic nerve are not affected by activation of either PKC or PKA alone; in conjunction, however, the two kinases have a proliferative effect similar to that seen with PDGF. Which subspecies of PKC may be involved here, however, and which subspecies of PKA it synergises with has recently become more

complicated as Walter (1989) has described the possibility of the existence of eight subspecies of PKA. Despite the fact that PKC or PKA may be implicated in many cellular processes in glia in the absence of the other, it is more likely that the two act together, each having a specific role to play. Pearce et al. (1988) have recently described an interaction between PKA and PKC in the regulation of astrocyte glycogen metabolism and Shafit-Zagardo et al. (1988) have implicated both kinases in regulation of GFAP mRNA levels in astrocytes. In addition, Althaus (1989) has reported that PKC and PKA act together to produce myelin in isolated porcine oligodendrocytes, while Raible and McMorris (1989) reported that though PKC may increase proliferation of O-2A progenitor cells, PKA accelerates their differentiation into oligodendrocytes.

To date, PDGF has not been shown to activate PKA directly. Thus any observations made in this work must be explained by activation of PKC and tyrosine kinase by the growth factor. PDGF activates PKC via the PI pathway to increase cell proliferation in a manner analagous to TPA, but does not cause the massive cell growth seen later in culture. Instead the PDGF-treated cells appeared more like ke the control (4-α-phorbol) cultures after 72h. treatment. This apparent regulation in growth seen with PDGF-treated cultures is somewhat surprising given that PDGF mRNA is present in human tumour cell lines and was detected in all human gliomas tested (Nister et al., 1988a,b). Thus, PKC may be important for the initial cell growth, after which time a slowing down or role of cell maintenance occurs. Althaus (1989) has ascribed a role of cell maintenance to PKA in oligodendroglia. Whether tyrosine kinase may have a similar effect is not yet known as the events following tyrosine-kinase activation remain poorly documented. Weinberg (1989) has suggested that a small number of genes exist on a chromosome to regulate cell growth. PDGF may perhaps induce activation of such an oncogene resulting in a depression of growth functions. Alternatively,

PDGF may cause growth inhibitor signals to be transduced to the nucleus which would cause oncogene activation and downregulation of essential cellular functions, such as transcription of genes required for continued growth. Clearly, much work remains to be done before the molecular mechanisms underlying the process of cell growth can be elucidated.

4. CONCLUDING DISCUSSION

The starting point for this study was the observations of a number of workers a few years ago that the calcium - and phospholipid-dependent protein kinase, protein kinase C (PKC), may be localised in the glial compartment of the CNS. Immunocytochemical studies by Girard and co-workers (1985) suggested the presence of PKC in cells resembling oligodendrocytes. Burgess et al. (1986) showed a significant increase in PKC activity (measured by phorbol ester receptor binding) when glial cells were allowed to proliferate in neuronal cell cultures. Receptor-linked phosphoinositide turnover was demonstrated in astrocytes in culture (Pearce et al., 1986) and levels of PKC in primary astrocytes measured (Neary et al., 1986). Although much interest had been generated concerning the role of PKC in signal transduction, relatively little was known about how the enzyme functioned and in which processes it was involved. The last three years has seen a wealth of literature emerge on this subject, but despite the great advancement in our knowledge concerning PKC and its actions, the picture appears to become more complex rather than simpler.

This study was concerned with the investigation of PKC levels in neuroglia. Problems of neuronal contamination and with obtaining cells in sufficient number for PKC assay meant that CNS tissue was not the system of choice for obtaining the glia. Instead, primary glial cell cultures of dissociated neonatal rat brain were employed, as described by Walker et al., (1985). Such a system contains astrocytes (types-1 and 2), oligodendrocytes and oligodendrocyte -type-2 astrocyte (O-2A) progenitor cells. Neuronal contamination is minimal (Walker et al., 1985).

An assay was developed to measure levels of PKC in these cultures using 100,000g cell extracts as the enzyme source. PKC was detected in

all cultures investigated; use of a DEAE-cellulose (DE-52) anion exchange column led to a dramatic purification of measurable enzyme activity, with PKC values increasing approximately 20-fold (Murphy et al., 1988). Addition of a non-purified extract to a column-purified one caused a huge decrease in enzyme activity as measured by type III-S histone phosphorylation. A similar decrease in levels of phosphorylated histone was observed on addition of the non-purified extract to phosphorylated histone. Thus the crude extract was not acting as an inhibitor of PKC per se, but merely interfered with the assay for measuring levels of the enzyme. This phosphatase, apparently cytosolic in nature, was not regulated by either magnesium or manganese, but was inhibited by 40mM sodium fluoride. An in vivo role for the phosphatase would depend upon the demonstration of PKC-catalysed histone phosphorylation in vivo.

The cell cultures were manipulated using subculturing techniques, complement-mediated cytotoxicity and defined media. Such methods showed PKC to be present in all the constituent cell types of the culture system. A significant increase in PKC levels was apparent when foetal calf serum was replaced with a defined medium, possibly due to the presence of an inhibitor of PKC in the serum.

Much work recently has focussed on the presence of multiple PKC subspecies. At least seven subspecies have been identified to date, three of which (α, β, γ) can be isolated using hydroxylapatite (HA) column chromatography (Ono et al., 1987, 1988). CNS tissue contains all three of these subspecies although the different regions of the brain vary in the ratio of subspecies present, presumably reflecting the function of the given region (Nishizuka, 1988). Although this study separated whole rat brain homogenate into the three subspecies listed above, none of the three were found to be present in various glial cultures using two different HA columns. Various immunocytochemical

studies by other workers are in general agreement with this observation. Huang et al. (1988) found no immunoreactivity to anti-α, β or γ PKC in cerebellar astrocyte cultures, Hosada et al. (1989) found that neurones but not glia stained for β_1-PKC in rat brain, and Saito et al. (1989) found no obvious immunoreactivity to anti-β_{11}-PKC in glia of rat brain. Hidaka et al. (1988), however, found that cerebellar oligodendrocytes in rabbit brain stained for α-PKC. This apparent contradiction of the results of this study may be explained by the species difference, but more likely by the difference in cell source, i.e. cerebellum versus cerebrum. The use of cDNA clones to further investigate these observations is currently underway.

An interesting phenomenon was observed in 6-day primary glial cultures treated with TPA. The cells appeared to undergo a huge proliferation, growing over all available substratum. PDGF-treated cultures underwent a similar proliferative burst initially, but then slowed down and appeared similar to control (4-α-phorbol-treated) cultures after 72h. of treatment. Morphological analysis suggested that some type of growth 'control' had been exerted over PDGF-treated cells which was not present in the TPA-treated cultures. A likely candidate for such control is tyrosine kinase although the effects of its activation remain to be elucidated. Calcium ionophore-treated cells died, probably due to the huge non-specific influx of Ca^{2+} ions. Any future studies ought to include an activator of PKA to determine how it might function alone, and in conjunction with PKC, in the regulation of glial cell growth and differentiation.

In a comparison of in vitro substrates for the PKC assay, type IIIS histone appeared to be a 'better' substrate than either protamine or myelin basic protein (MBP) in terms of levels of PKC-dependent phosphorylation and background (basal) phosphorylation. The two sources of MBP used, however, were found to be very different. Turner and Kuo

(1986) have argued that MBP is the best substrate for PKC, and MBP phosphorylation by PKC has been suggested to be the primary event in myelination (Vartanian et al., 1986). At a recent NATO meeting in Osnabruck (September 1989) it was proposed that a second myelin protein, myelin associated glycoprotein (MAG), may be an in vivo substrate for PKC. Thus PKC may play an important role in myelination. De Rosbo and Bernard (1989) have recently put forward a multistage hypothesis for myelin degradation that involves the activation of PKC, although its role here remains far more tentative than that in myelination.

A whole host of molecules have been cited as substrates for PKC. In few cases however have these proteins been shown to have an in vivo role. Many studies have used cell homogenates, and incubated these with PKC activators to investigate increased levels of phosphorylation in proteins and lipids. Such an approach invariably leads to one or more proposals for PKC substrates. Use of a whole cell system gives a more accurate picture of which proteins act as substrates for the enzyme.

Some of the earliest work employing such a technique was undertaken by Rozengurt et al. (1983) in fibroblasts. These workers showed that activation of PKC led to a rapid increase in phosphorylation of an 80kDa cellular protein, and that downregulation of the enzyme by prolonged phorbol ester treatment prevented such an occurrence. We have shown the presence of a similar cytosolic 80kDa substrate in mixed glial cell cultures and primary astrocyte cultures (Rumsby et al., 1988). Thus this protein appears to be a good marker of PKC activation, although its identity and function remain a mystery. A number of proteins have a molecular weight in the region of 80kDa; one intriguing possibility is that the protein may be diacylglycerol kinase, an in vitro substrate of PKC (Kanoh et al., 1989). DAG kinase degrades DAG, the activator of PKC. Phosphorylation of DAG kinase by PKC would thus represent a form of self-regulation by PKC. Morris and Rozengurt (1988) have

purified an 80kDa substrate of PKC from rat brain and shown it to be highly acidic in nature. Investigations concerning its role are continuing.

Phosphorylation of the 80kDa protein has been shown using neuropeptides such as bombesin and growth factors such as platelet-derived growth factor (PDGF) in fibroblasts (Wolfman et al., 1987). We have observed a similar increase in 80kDa protein phosphorylation in glial cells treated with PDGF (Rumsby et al., unpublished observations), an interesting observation given the importance of the growth factor in glial cell differentiation; could the 80kDa protein be an important determinant here? In addition, preliminary investigations in glia have shown an increase in phosphatidylcholine (PC) metabolites following treatment with PDGF (McNulty et al., unpublished observations) so signal transduction via PC (Pelech and Vance, 1989) may occur in glia.

Figures 4.1 - 4.3 are an attempt to give some indication as to the extent of the knowledge that now exists concerning glial cell regulation. These figures are intended as a simple guide and therefore are not as detailed as they might otherwise be. One problem is the heterogeneity in response to a given mitogen exhibited between cultures of astrocytes according to their source (Cholewinski and Wilkin, 1988); figure 4.1 does not allow for this but simply groups the astrocyte response together as that elicited by a single cell type.

Figure 4.1 details a number of responses that have been described in cultured astrocytes; type-1 astrocytes are not distinguished from type-2 as so little evidence is available on the latter cell type. Cholewinski and Wilkin (1988) have examined the response of cultured rat astrocytes to various peptides and shown that cortical and cerebellar astrocytes exhibited similar responses which differed from those of spinal cord astrocytes. A phosphoinositide-linked response was demonstrated using substance P, neurokinin α and ß, eledoisin, bradykinin, ocytocin and vasopressin. Of these, only bradykinin has

Figure 4.1. Responses of astrocytes to various mitogens

α = α-adrenoreceptor agonist; β = β-adrenoreceptor agonist;

Ang = angiotensin; Benz = benzodiazepine; Dop = dopamine;

Ele = eledoisin; FGF = fibroblast growth factor; Glu = glucagon;

GMF = glial maturation factor; Hist = histamine;

mACH = muscarinic acetylcholine; Neu = neurokinin α, β;

P_2 = purinergic; Som = somatostatin; SubP = substance P;

TK = tyrosine kinase; TXA_2 = thromboxane A_2;

VIP = vasoactive intestinal peptide.

[1] = induces glycogenolysis

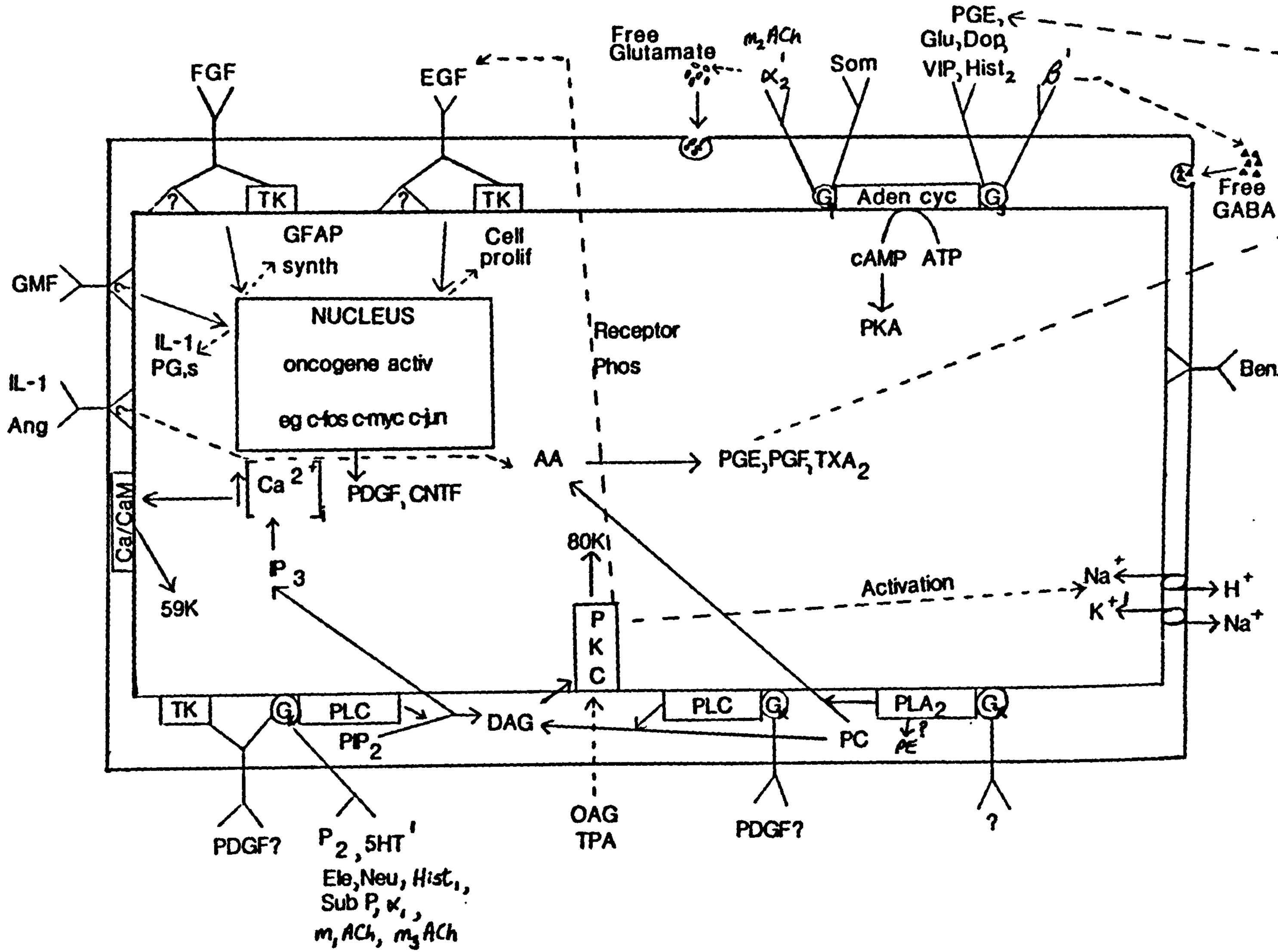
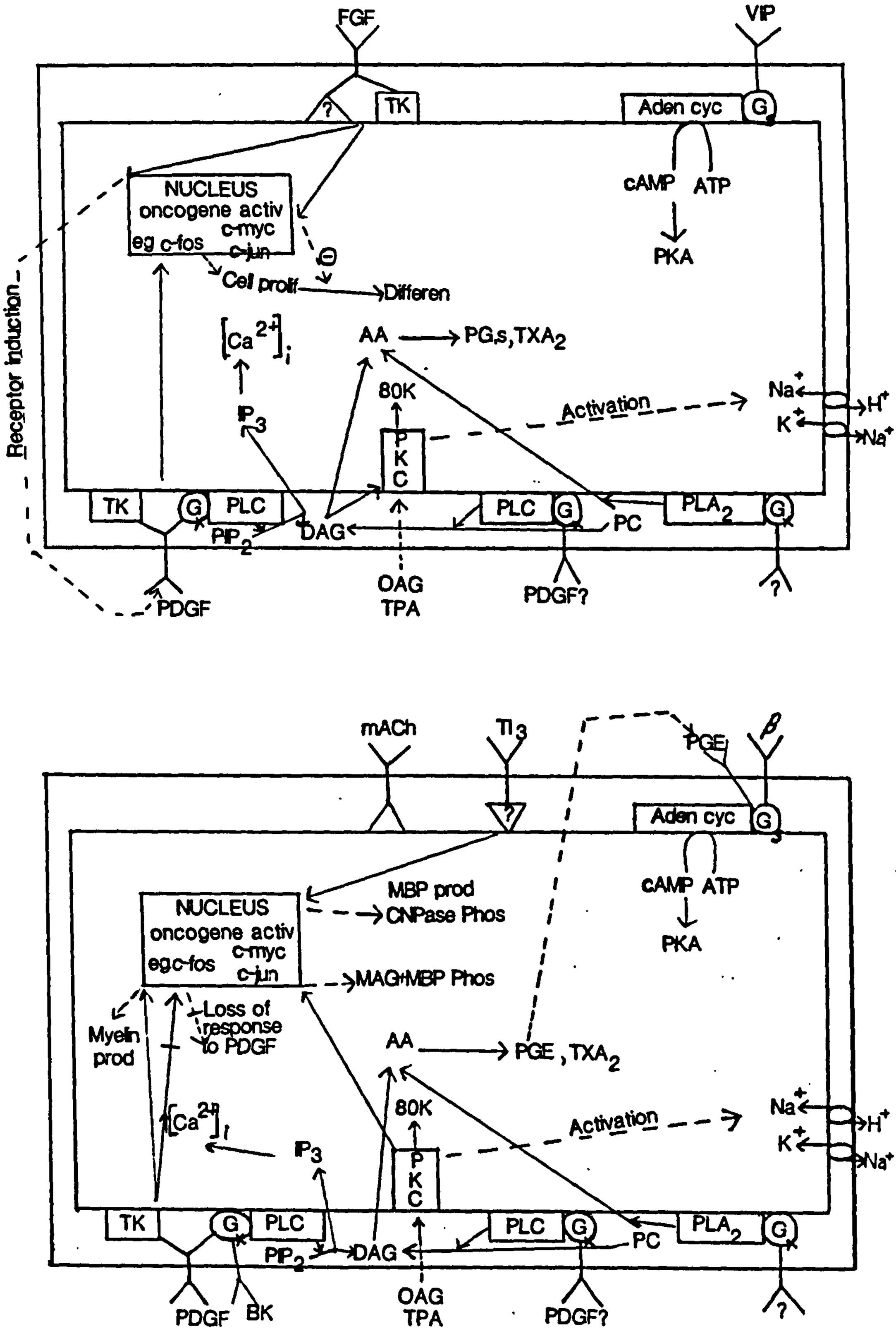

FGF
EGF
Free Glutamate
m_2ACh
α_2
Som
Glu, Dop, VIP, Hist$_2$
β
PGE,
Free GABA
TK
TK
GFAP synth
Cell prolif
G
Aden cyc
G
cAMP
ATP
PKA
GMF
IL-1 PG,s
IL-1
Ang
NUCLEUS
oncogene activ
eg c-fos c-myc c-jun
Receptor Phos
Benz
AA
PGE, PGF, TXA$_2$
Ca^{2+}
PDGF, CNTF
Ca/CaM
59K
IP$_3$
80KI
Activation
Na$^+$
H$^+$
K$^+$
Na$^+$
PKC
TK
G
PLC
PIP$_2$
DAG
PLC
G
PLA$_2$
G
PC
PE?
PDGF?
P$_2$, 5HT
Ele, Neu, Hist$_1$, Sub P, κ_1, m_1ACh, m_3ACh
OAG TPA
PDGF?
?

Figure 4.2. (upper) and 4.3 (lower). Responses of O-2A progenitor cells (upper) and oligodendrocytes (lower) to various mitogens

Bk = bradykinin; TI_3 = triiodothyronine.


FGF
VIP
?
TK
Aden cyc
G
NUCLEUS
oncogene activ
c-myc
eg c-fos
c-jun
cAMP
ATP
PKA
Cell prolif
Differen
Receptor induction
$[Ca^{2+}]_i$
AA
PGs, TXA_2
80K
IP_3
Activation
Na+
K+
H+
Na+
PKC
TK
G
PLC
PIP_2
DAG
PLC
G
PC
PLA_2
G
PDGF
OAG
TPA
PDGF?
?
mACh
TI_3
PGE
β
Aden cyc
G
cAMP
ATP
PKA
MBP prod
CNPase Phos
NUCLEUS
oncogene activ
c-myc
eg c-fos
c-jun
MAG+MBP Phos
Myelin
prod
Loss of
response
to PDGF
AA
PGE, TXA_2
$[Ca^{2+}]_i$
80K
IP_3
Activation
Na+
K+
H+
Na+
PKC
TK
G
PLC
PIP_2
DAG
PLC
G
PC
PLA_2
G
PDGF
BK
OAG
TPA
PDGF?
?

been reported to have a stimulatory effect in oligodendrocytes (Ritchie et al., 1987). Compounds which have been reported to exert their effects on astrocytes via an increase in cAMP include prostaglandin E (PGE), glucagon, dopamine, vasoactive intestinal peptide (VIP), histamine and ß-adrenergic receptor agonists (Hansson, 1988). The latter class of compounds also lead to active take-up of free γ-aminobutyric acid (GABA) (Hansson and Ronnback, 1989). Compounds with an opposite effect to the above list (i.e. causing an inhibition of cAMP increase) include somatostatin and α-adrenergic receptor agonists (Hansson, 1988). Free glutamate is actively taken up following α-adrenergic receptor activation (Hansson and Ronnback, 1989).

In addition, astrocytes have been shown to have receptors for 5-hydroxytryptamine (5-HT), acetylcholine (muscarinic) (mACh), benzodiazepine and secretin, and to be involved in aspartate and taurine uptake (Hansson, 1988). A recent development is the observation that P_2 purinergic receptors, activated by ATP and ADP (possibly from neurones), are present on the cell surface of cortical astrocytes (Pearce et al., 1989). Other interesting observations are the presence of receptors for PGE, given that astrocytes may also produce this molecule, and for interleukin-1ß (IL-Iß) and angiotensin which lead to PGE production in astrocytes (Hansson, 1988). Activation of receptors for glial maturation factor (GMF) promotes the proliferation and phenotypic expression of cultured astrocytes (Lim, 1985) and stimulates IL-1 and prostaglandin production (Fontana et al., 1983; Lim, 1985), and various neurotransmitters induce glycogenolysis in astrocytes (Cambray-Deakin et al., 1988) as indicated in figure 4.1.

Oligodendrocytes appear not to have the same diverse range of receptors on their cell surface (see figure 4.3). Like astrocytes they have receptors for mACh, BK, PGE and ß-adrenergic agonists (Ritchie et

al., 1987; Vartanian et al., 1988). Vartanian et al. (1988) have investigated events immediately following the attachment of isolated oligodendrocytes to substratum. Attachment led to PKC-catalysed phosphorylation of MBP, followed by MBP synthesis. PKC levels then began to decline as PKA levels rose. A ß-adrenergic responsiveness was seen together with an increased responsiveness to PGE. The response to VIP was lost, CNPase phosphorylated and an increase in plasmalogen synthesis was apparent. This presumably represents (some of) the initial stages of myelinogenesis. Triidothyronine (TI_3) may have a role here; treatment of oligodendrocytes with TI_3 led to a huge increase in MBP levels (Honegger, 1989).

Astrocytes secrete both CNTF and PDGF, both of which are important in glial cell differentiation, but quite what the stimulus for their production is uncertain. It is also unknown whether astrocytes have receptors for PDGF on their surface; such receptors certainly exist both on O-2A progenitor cells and oligodendrocytes (Hart et al., 1989). PDGF causes progenitor cells to proliferate, preventing their premature differentiation into oligodendrocytes. At a given time the cells lose their responsiveness to PDGF due to activation of a proposed biological clock. O-2A cells treated simultaneously with both FGF and PDGF will not differentiate however; they will continue to proliferate as long as the co-administration continues. Thus the FGF appears to override the biological clock. In addition, FGF induces GFAP synthesis in astrocytes (Pruss, 1981), and bovine recombinant FGF induces expression of the PDGF-A receptor in O-2A progenitor cells and the MBP gene in oligodendrocytes (Dubois-Dalcq, 1989). Another growth factor implicated in myelin production is insulin-like growth factor (IGF). This is mitogenic for O-2A progenitor cells and increases the conversion of the O-2A cell line into oligodendrocytes (Raible and McMorris, 1989). In addition, McMorris (1989) has reported that a mutant rat overproducing

IGF-1 not only had a greater percentage of the O-2A line as oligodendrocytes, but these oligodendrocytes had a far greater capacity for myelin production as seen by a vastly increased brain size. The epidermal growth factor (EGF) receptor is regulated by PKC. The enzyme phosphorylates the EGF receptor changing it to a low affinity form and hence regulates its activity (Cochet et al., 1984).

Activation of PKC leads to phosphorylation of an 80kDa cytosolic protein in primary astrocyte and mixed glial cell cultures (Rumsby et al., 1988). In addition, Babcock-Atkinson et al. (1989) have reported increased phosphorylation of a 59kDa protein in primary astrocytes treated with dibutyryl cAMP as a result of activation of calcium/calmodulin-dependent protein kinase.

The numerous responses listed above following receptor activation merely detail the beginning and, in some cases, the end point of a given process. Unfortunately, the steps occurring following receptor activation are less well documented. Messages are transferred to the nucleus where (proto)oncogenes are involved as 'third' messengers. Some of the most commonly mentioned oncogenes when discussing signal transduction at the nuclear level are c-fos, c-myc and c-jun. At a recent meeting (Glial Cell Club Inaugural Meeting, London, November 1989) Raff suggested that c-fos and c-jun act cooperatively to transduce message, whilst Schontal et al. (1988) have concluded that fos (and probably jun) serve as primary targets of signal transduction. Further studies involving these oncogenes may give us a clearer understanding of the process of signal transduction.

5. APPENDIX

5.1. PREPARATION OF OLIGODENDROCYTES FROM RAT BRIAN

5.1.a. Chao and Rumsby (1977) method

MATERIALS

(i) Hexose albumin phosphate (HAP), pH 7.4.

5% (w/v) D-glucose, 5% (w/v) D-fructose, 1% (w/v) bovine serum albumin fraction V (BSA), 10mM potassium dihydrogen phosphate.

(ii) Trypsinisation solution

0.1% (w/v) trypsin, 10µg/ml deoxyribonuclease (DNAse) in HAP, but omitting BSA.

(iii) Sucrose solutions

0.9M, 1.2M, 1.55M and 1.75M sucrose in HAP.

(iv) 10µg/ml DNAse in HAP.

METHOD

The brains from Wistar rats aged 10 days or less were dissected into ice-cold HAP, weighed, rolled on filter paper to remove any major blood vessels, and minced finely with a blade on a metal plate on ice. Some HAP was added to facilitate mincing. Tissue was then transferred to a conical flask containing 10ml trypsinisation solution per gram wet weight of tissue. A 1h. incubation at 37° in a shaking water bath was followed by chilling on ice for 5min. and addition of ice-cold FCS at 3ml/g wet weight of tissue. Cells were collected in a Sorvall RC5B ultracentrifuge using an HS4 rotor; the centrifuge was turned on and when the revolution counter indicated 900rpm (150g) the machine was switched off and allowed to retard freely.

Pellets were then suspended in 5ml 0.9M sucrose and filtered through 90µm nylon mesh, followed by three filtrations through 74µm steel mesh. 10ml 1.55M sucrose was pipetted into a Beckman SW27 centrifuge tube and 10ml. 0.9M sucrose carefully layered over it. 10ml of cell suspension was then layered over the top and the tubes spun at 4900g for 10min. in a Beckman L2-65B ultracentrifuge. The band at the interface of the 0.9/1.55M sucrose layers was collected and diluted to 50ml 1.75M sucrose in an SW27 centrifuge tube, and 25ml cell suspension was layered on top. The gradient was then spun at 10,000g for 20 min.

The band at the interface of the 1.2/1.75M sucrose layers was collected and 10µg/ml DNAse in HAP was added dropwise (to prevent cell lysis) to five times the cell volume, with stirring of the suspension during addition and use of a Pasteur pipette to aspirate and mix the cells. Cells were then respun in an HS4 rotor for 2min. at 1000g. Supernatants were removed and cells resuspended in a known volume of HAP and cell viability determined using nigrosin dye exclusion.

5.1.b. <u>Snyder et al. (1980) METHOD</u>

MATERIALS

(i) Isolation medium, pH7.2

Hank's balanced salt solution, 25mM Hepes, 1% (w/v) BSA fraction V.

(ii) Trypsinisation medium

0.1% (w/v) trypsin, 10µg/ml DNAse in (i) but omitting BSA.

(iii) Sucrose solutions

45% (w/v), 53% (w/v) and 70% (w/v) sucrose in isolation medium.

(iv) 10µg/ml DNAse in isolation medium.

METHOD

The brains from Wistar rats were dissected into ice-cold isolation medium, weighed, and minced finely with a blade in a glass dish on ice. The tissue was then transferred to a conical flask containing 2ml trypsinisation solution per gram wet weight of tissue, and incubated for 30min. at 37°C in a shaking water bath. After chilling on ice for 5min., ice-cold FCS at 1ml/g wet weight of tissue was added.

Cells were then collected in isolation medium with a 10min. spin at 190g in a Sorvall RT6000 bench centrifuge. Supernatant was removed and the step repeated twice more. The pellet was then filtered through a 132µm nylon mesh adding isolation medium to a maximum of 10ml/g original tissue to facilitate filtration. The resulting cell suspension was then filtered three times through 75µm steel mesh, and readjusted to 10ml/g original tissue in isolation medium. An equal volume of 70% (w/v) sucrose solution was added and 15ml layered over 4ml 45% (w/v) sucrose which lay over 12ml 53% (w/v) sucrose in a Beckman SW27 centrifuge tube. When brains of animals over 30 days old were used, the 45/53% sucrose interface was blurred using a Pasteur pipette.

The gradient was spun using a Beckman SW27 rotor in a Beckman L2-65B ultracentrifuge for 15min. at 4900g. A red cell layer collects just below the 45/53% interface; everything below this layer, plus the pellet, is retained and diluted five times with isolation medium containing 10µg/ml DNAse. The suspension is then filtered through a 25µm steel mesh to trap capillaries, and adjusted to a known volume with isolation medium prior to determination of cell viability using nigrosin dye exclusion.

5.2 HYDROXYLAPATITE COLUMN CHROMATOGRAPHY - POINTS TO NOTE

All HA columns are supplied with recommendations for use. The following points are either additional to these or a reiteration of them because of their importance.

1. Upon receipt flush the column through with at least ten column volumes of starting buffer followed by a blank gradient prior to first sample load.

2. If possible always operate between pH 6.5-8.0; strong acids and alkalis will irreversibly damage the column.

3. Pay particular attention to solubility of buffers. Some are not very soluble at the flow temperatures often required and particulates or precipitates in samples may damage the column irreversibly or will certainly cause a huge increase in backpressure.

4. For the reasons listed in 3, all samples and buffers must be filtered through 0.2µm membrane filters prior to use.

5. Pressure limits on the operating apparatus (FLPC or HPLC) ought to be carefully set such that the pressure limit on the column is never exceeded, and any large increase in pressure halts the separation so preventing irreversible column damage.

6. If the column is to be washed with a solution or buffer chemically different to the gradient buffer, the two ought to be tested together for production of precipitates to prevent such an occurrence inside the column.

7. It is very important to wash the HA completely free of EGTA/EDTA between runs if the latter are spaced over a few days, as EGTA and EDTA will chelate the Ca^{2+} from the column resulting in a drastic leftward shift of the enzyme peaks (M. Sheaarman, personal communication). Wash the column with pure water containing 0.1mM $CaCl_2$ and store in phosphate buffer containing 0.1mM $CaCl_2$ and 0.02% sodium azide.

REFERENCES

ADAMO, S., CAPORALE, C., AGUANNO, S., LAZDINS, J., FAGGIONI, A., BELLI, L., CORTESI, E., NERVI, C., GASTALDI, R. and MOLINARO, M. (1986) Proliferating and quiescent cells exhibit different subcellular distribution of protein kinase C activity. Febs. Letts. 195, 352-356.

AGRAWAL, H.C., HARTMAN, B.K., SHEARER, W.T., KALMBACH, S. and MARGOLIS, F.L. (1977) Purification and immunohistochemical localisation of rat brain myelin proteolipid. J. Neurochem. 28, 495-508.

ALBERT, K.A., HELMER-MATYJEK, E., NAIRN, A.C., MULLER, T.H., HAYCOCK, J.W., GREENE, L.A., GOLDSTEIN, M. and GREENGARD, P. (1984) Calcium/phospholipid-dependent protein kinase (protein kinase C) phosphorylates and activates tyrosine hydroxylase. Proc. Natl. Acad. Sci. USA. 81, 7713-7717.

ALTHAUS, H. (1989) Role of protein kinase C for oligodendroglial regeneration. In: Abstracts of NATO conference on "Cellular and Molecular Biology of Myelination", September 1989, Osnabruck, FRG, Plenum Press, New York. In press.

ANDERSON, D.J. (1989) New roles for 'PDGF' and 'CNTF' in controlling the timing of glial cell differentiation in the optic nerve. Trends Neurol. Sci. 12, 83-85.

ANDERSON, W.B., ESTIVAL, A., TAPIOVAARA, H. and GOPALAKRISHNA, R. (1985) Altered subcellular distribution of protein kinase C (a phorbol ester receptor). Possible role in tumour promotion and the regulation of cell growth: relationship to changes in adenylate cyclase activity. Adv. Cyc. Nuc. Prot. Phosph. Res. 19, 287-306.

ANTONIADES, H.N. (1981)

Human platelet-derived growth factor (PDGF): purification of PDGF-I and PDGF-II and separation of their reduced subunits. Proc. Natl. Acad. Sci. USA. 78, 7314-7317.

ANTONIADES, H.N. (1983)

Human platelet-derived growth factor: amino-terminal amino acid sequence. Science 220, 963-965.

ASHENDEL, C.L. (1985)

The phorbol ester receptor: a phospholipid-regulated protein kinase. Biochim. Biophys. Acta. 822, 219-242.

BABCOCK-ATKINSON, E., NORENBERG, M.D., NORENBERG, L.O.C. and NEARY, J.T. (1989)

Calcium/calmodulin-dependent protein kinase activity in primary astrocyte cultures. Glia 2, 112-118.

BALLESTER, R., FURTH, M.E. and ROSEN, O.M. (1987)

Phorbol ester- and protein kinase C-mediated phosphorylation of the cellular Kirsten ras gene product. J. Biol. Chem. 262, 2688-2695.

BATTY, I.R., NAHORSKI, S.R. and IRVINE, R.F. (1985)

Rapid formation of inositol 1,3,4,5-tetrakisphosphate following muscarinic receptor stimulation of rat cerebral cortical slices. Biochem. J. 232, 211-215.

BAZZI, M.D. and NELSESTUEN, G.L. (1988)

Constitutive activity of membrane-inserted protein kinase C. Biochem. Biophys. Res. Commun. 152, 336-343.

BELL, R.L., KENNERLY, D.A., STANFORD, N. and MAJERUS, P.W. (1979)

Diglyceride lipase: a pathway for arachidonate release from human platelets. Proc. Natl. Acad. Sci. USA. 76, 3238-3241.

BERRIDGE, M.J. (1984)

Inositol trisphosphate and diacylglycerol as second messengers. Biochem. J. 220, 345-360.

BERRIDGE, M.J. (1987)

Inositol trisphosphate and diacylglycerol: two interacting second messengers. Ann. Rev. Biochem. 56, 159-193.

BHAT, N.R. (1989)

Role of protein kinase C in glial cell proliferation. J. Neurosci. Res. 22, 20-27.

BILLAH, M.M., LAPETINA, E.G. and CUATRECASAS, P. (1980)

Phospholipase A_2 and phospholipase C activities of platelets. Differential substrate specificity, Ca^{2+} requirement, pH dependence and cellular localisation. J. Biol. Chem. 255, 10227-10231.

BISHOP, R., MARTINEZ, R., NAKAMURA, K. and WEBER, M.J. (1983)

A tumour promoter stimulates phosphorylation on tyrosine. Biochem. Biophys. Res. Commun. 115, 536-543.

BLACKSHEAR, P.J., WEN, L., GLYNN, B.P. and WITTERS, L.A. (1986)

PKC-stimulated phosphorylation in vitro of a Mr80,000 protein phosphorylated in response to phorbol esters and growth factors in intact fibroblasts. J. Biol. Chem. 261, 1459-1469.

BOTTENSTEIN, J.E. and SATO, G.H. (1979)

Growth of a rat neuroblastoma cell line in serum-free supplemented medium. Proc. Natl. Acad. Sci. USA., 76, 514-517.

BOUSCAREL, B. and EXTON, J.H. (1986)

Regulation of hepatic glycogen phosphorylase and glycogen synthase by calcium and diacylglycerol. Biochim. Biophys. Acta 888, 126-134.

BRADBURY, M.W.B. (1979)

Why a blood-brain barrier? Trends Neurol. Sci. 2, 36-38.

BROWN, E.M., REDGRAVE, J. and THATCHER, J. (1984a)

Effect of the phorbol ester TPA on PTH secretion. Evidence for a role for protein kinase C in the control of PTH release. Febs. Letts. 175, 72-75.

BROWN, J.E., RUBIN, L.J., GHALAYINI, A.J., TARVER, A.P., IRVINE, R.F., BERRIDGE, M.J. and ANDERSON, R.E. (1984b)

Myo-inositol polyphosphate may be a messenger for visual excitation in Limulus photoreceptors. Nature 311, 160-163.

BURGESS, S.K., SAHYOUN, N., BLANCHARD, S.G., LEVINE, H. III., CHANG, K-J. and CUATRECASAS, P. (1986)

Phorbol ester receptors and PKC in primary neuronal cultures: development and stimulation of endogenous phosphorylation. J. Cell. Biol. 102, 312-319.

CABOT, M.C., WELSH, C.J., CAO, H.T. and CHABBOT, H. (1988a)

The phosphatidylcholine pathway of diacylglycerol formation stimulated by phorbol diesters occurs via phospholipase D activation. Febs. Letts. 233, 153-157.

CABOT, M.C., WELSH, C.J., ZHANGH, Z.C., CAO, H.T., CHABBOT, H., and LEOWITZ, M. (1988b)

Vasopressin, phorbol diesters and serum elicit choline glycerophospholipid hydrolysis and diacylglycerol formation in nontransformed cells: transformed derivatives do not respond. Biochim. Biophys. Acta. 959, 46-57.

CAMBRAY-DEAKIN, M., PEARCE, B., MORROW, C. and MURPHY, S. (1988)

Effects of neurotransmitters on astrocyte glycogen stores in vitro. J. Neurochem. 51, 1852-1857.

CANTRELL, D.A., DAVIES, A.A. and CRUMPTON, M.J. (1985)

Activators of protein kinase C downregulate and phosphorylate the T3/T-cell antigen receptor complex of human T lymphocytes. Proc. Natl. Acad. Sci. USA. 82, 8158-8162.

CARROLL, R.C., BUTLER, R.G., MORRIS, P.A. and GERRARD, J.M. (1982)
Separable assembly of platelet pseudopodal and contractile cytoskeletons. Cell 30, 385-393.

CASTAGNA, M., TAKAI, Y., KAIBUCHI, K., SANO, K., KIKKAWA, U. and NISHIZUKA, Y. (1982)
Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumour-promoting phorbol esters. J. Biol. Chem. 257, 7847-7851.

CHAN, K.F.J. (1987a)
Ganglioside-modulated protein phosphorylation in myelin. J. Biol. Chem. 262, 2415-2422.

CHAN, K.F.J. (1987b)
Ganglioside-modulated protein phosphorylation. Partial purification and characterisation of a ganglioside-stimulated protein. J. Biol. Chem. 262, 5248-5255.

CHAO, S. and RUMSBY, M.G. (1977)
Isolation of oligodendrocytes and other cell lines from whole rat brain tissue. Biochem. Soc. Trans. 5, 194-196.

CHIARUGI, V.P., RUGGIERO, M. and PORCIATTI, F. (1987)
Oncogenes and transmembrane cell signalling. Cancer Invest. 5, 215-229.

CHIDA, K., KATO, N. and KUROKI, T. (1986)
Downregulation of phorbol diester receptors by proteolytic degradation of protein kinase C in a cultured line of foetal rat skin keratinocytes. J. Biol. Chem. 261, 13013-13018.

CHOLEWINSKI, A.J. and WILKIN, G.P. (1988)
Astrocytes from forebrain, cerebellum, and spinal cord differ in their responses to vasoactive intestinal peptide. J. Neurochem. 51, 1626-1633.

COCHET, C., GILL, G.N., MEISENHELDER, J., COOPER, J.A. and HUNTER, T. (1984)

C-kinase phosphorylates the EGF receptor and reduces its EGF-stimulated tyrosine protein kinase activity. J. Biol. Chem. 259, 2553-2558.

COCKCROFT, S. and ALLAN, D. (1984)

The fatty acid composition of phosphatidylinositol phosphatidate and 1,2-diacylglycerol in stimulated human neutrophils. Biochem. J. 222, 557-559.

COFFEY, R.G. and HADDEN, J.N. (1983)

Phorbol myristate acetate stimulation of lymphocyte guanylate cyclase and cyclic guanosine 3', 5'-monophosphate phosphodiesterase and reduction of adenylate cyclase. Cancer Res. 43, 150-158.

CONN, P.M., GANONG, B.R., EBELING, J., STALEY, D., NIEDEL, J.E. and BELL, R.M. (1985)

Diacylglycerols release LH: Structure-activity relations reveal a role for protein kinase C. Biochem. Biophys. Res. Commun. 126, 532-539.

CONNOLLY, D.M., LAWING, W.J. and MAJERUS, P.W. (1986)

Protein kinase C phosphorylates human platelet inositol 5'-phosphomonoesterase, increasing the phosphatase activity. Cell 46, 951-958.

COOKMAN, G.R., HEMMENS, S.E., KEANE, G.J., KING, W.B. and REGAN, C.M. (1988)

Chronic low level lead exposure precociously induces rat glial development *in vitro* and *in vivo*. Neurosci. Letts. 86, 33-37.

COUSSENS, L., PARKER, P.J., RHEE, L., YANG-FENG, T.L., CHEN, E.,

WATERFIELD, M.D., FRANCKE, U. and ULLRICH, A. (1986)
Multiple, distinct forms of bovine and human protein kinase C suggest diversity in cellular signalling pathways. Science 233, 859-866.

CREMINS, J., WAGNER, J.A. and HALEGOUA, S. (1986)
NGF action is mediated by cAMP and Ca^{2+}/phospholipid-dependent protein kinases. J. Cell Biol. 103, 887-893.

CURTIS, R., COHEN, H., FOK-SEANG, J., HANLEY, M.R. GREGSON, N.A., REYNOLDS, R. and WILKIN, G.P. (1988)
Development of macroglial cells in rat cerebellum. I. Use of antibodies to follow early in vivo development and migration of oligodendrocytes. J. Neurocytol. 17, 43-54.

DAVIS, R.J., JOHNSON, G.L., KELLEHER, D.J., ANDERSON, J.K., MOLE, J.E. and CZECH, M.P. (1986)
Identification of serine-24 as the unique site on the transferrin receptor phosphorylated by protein kinase C. J. Biol. Chem. 261, 9034-9041.

DAWSON, R.M.D., HEMINGTON, N. and IRVINE, R.F. (1985)
The inhibition of diacylglycerol-stimulated intracellular phospholipases by phospholipids with a phosphocholine-containing polar group. Biochem. J. 230, 61-68.

DELBEKE, D., KOJIMA, I., DANNIES, P.S. and RASMUSSEN, H. (1984)
Synergistic stimulation of prolactin release by phorbol ester, A23187 and forskolin. Biochem. Biophys. Res. Commun. 123, 735-741.

de ROSBO , N.K. and BERNARD, C.C.A. (1989)
Multiple sclerosis brain immunoglobulins stimulate myelin basic protein degradation in human myelin: a new cause of demyelination. J. Neurochem. 53, 513-518.

DIANOUX, A-C., STASIA, M-J. and VIGNAIS, P.V. (1989)
Purification and characterisation of protein kinase C from bovine neutrophils. Biochemistry 28, 424-431.

DICKER, P. and ROZENGURT, E. (1978)
Stimulation of DNA synthesis by tumour promoter and pure mitogenic factors. Nature 276, 723-725.

DONNELLY, T.E. Jr., SITTLER, R. and SCHOLAR, E.M. (1985)
Relationship between membrane-bound PKC activity and calcium-dependent proliferation of BALB/c 3T3 cells. Biochem. Biophys. Res. Commun. 126, 741-747.

DOWNES, C.P. (1988)
Inositol phosphates: a family of signal molecules. Trends Neurol. Sci. 11, 336-338.

DUBOIS-DALCQ, M. (1989)
Oligodendrocyte lineage in development and remyelination. In: Abstracts of NATO conference on "Cellular and Molecular Biology of Myelination", September 1989, Osnabruck, FRG, Plenum Press, New York. In press.

du PONT, J.J.H.H.M. and FLEUREN-JAKOBS, A.M.M. (1984)
Synergistic effect of A23187 and a phorbol ester on amylase secretion from rabbit pancreatic acini. Febs. Letts. 170, 64-68.

ECCLESTON, P.A. and SILBERBERG, D.H. (1984)
Differentiation of oligodendrocytes in a serum-free hormone-supplemented medium. Dev. Brain Res. 16, 1-9.

EK, B., WESTERMARK, B., WASTESON, A. and HELDIN, C.H. (1982)
Stimulation of tyrosine-specific phosphorylation by platelet-derived growth factor. Nature 295, 419-420.

ELLIOTT, D.C. and KOKKE, Y.S. (1987)
Partial purification and properties of a PKC type enzyme from plants. Phytochemistry 26, 2929-2935.

ENG, L.P., VANDERHAEGHEN, J.J., BIGNAMI, A. and GERSTLE, B. (1971)

An acidic protein isolated from fibrous astrocytes. Brain Res. 28, 351-354.

ESPINOSA de los MONTEROS, A., ROUSSEL, G., NESKOVIC, N.M. and NUSSBAUM, J.L. (1988)

A chemically defined medium for the culture of mature oligodendrocytes. J. Neurosci. Res. 19, 202-211.

ESPINOSA de los MONTEROS, A. (1989)

Oligodendrocyte subpopulations. In: Abstracts of NATO conference on "Cellular and Molecular Biology of Myelination", September 1989, Osnabruck, FRG, Plenum Press, New York. In press.

FAROOQUI, A.A., FAROOQUI, T., YATES, A.J. and HORROCKS, L.A. (1988)

Regulation of protein kinase C activity by various lipids - an overview. Neurochem. Res. 13, 499-511.

FAWTHROP, D.J. and EVANS, R.J. (1987a)

Morphological changes in cultured astrocytes following exposure to calcium ionophores. Neurosci. Letts. 81, 250-256.

FAWTHROP, D.J. and EVANS, R.J. (1987b)

The morphological differentiation of cultured astrocytes induced by ionomycin: lack of dependence on PKC activation. Neurosci. Letts. 81, 257-262.

FEUERSTEIN, N., MONOS, D.S. and COOPER, H.L. (1985)

Phorbol ester effect in platelets, lymphocytes, and leukaemic cells (HL-60) is associated with enhanced phosphorylation of class-1 HLA antigens. Biochem. Biophys. Res. Commun. 126, 206-213.

ffRENCH-CONSTANT, C. and RAFF, M.C. (1986)

The oligodendrocyte-type-2 astrocyte cell lineage is specialised for myelination. Nature 323, 335-338.

FONTANA, A., WELSER, E., GROB, P.J., LIM, R. and MILLER, J.F. (1983)
Dual effect of the glia maturation factor on astrocytes: differentiation and release of interleukin-1 like factors. J. Neuroimmunol. 5, 261-269.

FRIEDMAN, E. and WANG, H-Y. (1989)
Effect of age on brain cortical PKC and its mediation of 5-hydroxytryptamine release. J. Neurochem. 52, 187-192.

FUJIKI, H., TANAKA, Y., MIYAKE, R., KIKKAWA, U., NISHIZUKA, Y. and SUGIMURA, T. (1984)
Activation of calcium-activated, phospholipid-dependent protein kinase (protein kinase C) by new classes of tumour promoters: teleocidin and debromoaplaysiatoxin. Biochem. Biophys. Res. Commun. 120, 339-343.

FUJIKI, H., YAMASHITA, K., SUGANUMA, M., HORIUCHI, T., TANIGUCHI, N. and MAKITA, A. (1986)
Involvement of sulfatide in activation of protein kinase C by tumour promoters. Biochem. Biophys. Res. Commun. 138, 153-158.

FUJITA, S. (1980)
Cytogenesis and pathology of neuroglia and microglia. Path. Res. Pract. 168, 271-278.

GANONG, B.R., LOOMIS, C.R., HANNUN, Y.A. and BELL, R.M. (1987)
Regulation of protein kinase C by lipid cofactors. In: Cell Membranes, Methods and Reviews. Volume 3. Eds. Elson, E., Frazier, W. and Glaser, L. pp. 183-214. Plenum Press, New York.

GARTNER, S., MARKOVITS, P., MARKOVITZ, D.M., KAPLAN, M.H., GALLO, R.C. and POPOVIC, M. (1986)
The role of mononuclear phagocytes in HTLV-111/LAV infection. Science 233, 215-219.

GILMORE, T. and MARTIN, G.S. (1983)
Phorbol ester and diacylglycerol induce protein phosphorylation at tyrosine. Nature 306, 487-490.

GIRARD, P.R., MAZZEI, G.J., WOOD, J.G. and KUO, J.F. (1985)
Polyclonal antibodies to phospholipid/Ca^{2+}-dependent protein kinase and immunocytochemical localisation of the enzyme in rat brain. Proc. Natl. Acad. Sci. USA. 82, 3030-3034.

GLYNN, B., COLLITON, J., McDERMOTT, J. and WITTERS, L.A. (1985)
Assay of protein kinase C with an N-bromosuccinimide-cleavage fragment of histone H1. Biochem. J. 1, 489-492.

GOULD, K.L., WOODGETT, J.R., ISACKE, C.M. and HUNTER, T. (1986)
The protein-tyrosine kinase substrate, p36, is also a substrate for protein kinase C *in vitro* and *in vivo*. Mol. Cell. Biol. 6, 2738-2744.

GOUSTIN, A.S., BETSHOLTZ, C., PFEIFER-OHLSSON, S., RYDNERT, J., BYWATER, M., HOLMGREN, G., HELDIN, C-H., WESTERMARK, B. and OHLSSON, R. (1985)
Co-expression of the *sis* and *myc* proto-oncogenes in developing human placenta suggests autocrine control of trophoblast growth. Cell 41, 301-312.

GREENBERG, M.E., GREENE, L.A. and ZIFF, E.B. (1985)
Nerve growth factor and epidermal growth factor induce rapid transient changes in proto-oncogene transcription in PC12 cells. J. Biol. Chem. 260, 14101-14110.

HABENICHT, A.J.R., GLOMSET, J.A., KING, W.C., NIST, C., MITCHELL, C.D. and ROSS, R. (1981)
Early changes in phosphatidylinositol and arachidonic acid metabolism in quiescent Swiss 3T3 cells stimulated to divide by platelet-derived growth factor. J. Biol. Chem. 256, 12329-12335.

HAGAG, N., HALEGOUA, S. and VIOLA, M. (1986)

Inhibition of growth factor-induced differentiation of PC12 cells by microinjection of antibody to ras p21. Nature 319, 680-682.

HALENDA, S.P., ZAVOICO, G.B. and FEINSTEIN, M.B. (1985)

Phorbol esters and oleoyl acetyl glycerol enhance release of arachidonic acid in platelets stimulated by Ca^{2+} ionophore A23187. J. Biol. Chem. 260, 12484-12489.

HAMMACHER, A., HELLMAN, U., JOHNSSON, A., OSTMAN, A., GUNNARSSON, K., WESTERMARK, B., WASTESON, A. and HELDIN, C-H. (1988)

A major part of platelet-derived growth factor purified from human platelets is a heterodimer of one A and one B chain. J. Biol. Chem. 263, 16493-16498.

HANNUN, Y.A., LOOMIS, C.R. and BELL, R.M. (1985)

Activation of PKC by Triton-X-100 mixed micelles containing diacylglycerol and phosphatidylserine. J. Biol. Chem. 260, 10039-10043.

HANNUN, Y.A., LOOMIS, C.R., MERRILL, A.H. Jr. and BELL, R.M. (1986)

Sphingosine inhibition of PKC activity and of phorbol dibutyrate binding *in vitro* and in human platelets. J. Biol. Chem. 261, 12604-12609.

HANSSON, A., SERHAN, C.N., HAEGGSTROM, J., INGELMAN-SUNDBERG, M., SAMUELSSON, B. and MORRIS, J. (1986)

Activation of protein kinase C by lipoxin A and other eicosanoids: intracellular action of oxygenated products of arachidonic acid. Biochem. Biophys. Res. Commun. 134, 1215-1222.

HANSSON, E. (1988)

Astroglia from defined brain regions as studied with primary cultures. Prog. Neurobiol. 30, 369-397.

HANSSON, E. and RONNBACK, L. (1989)

Regulation of glutamate and GABA transport by adrenoreceptors in primary astroglial cell cultures. Life Sci. 44, 27-34.

HART, C.E., FORSTROM, J.W., KELLY, J.D., SEIFERT, R.A., SMITH, R.A., ROSS, R., MURRAY, M.J. and BOWEN-POPE, D.F. (1988)

Two classes of PDGF receptor recognise different isoforms of PDGF. Science 240, 1529-1531.

HART, I.K., RICHARDSON, W.D., HELDIN, C-H., WESTERMARK, B. and RAFF, M.C. (1989)

PDGF receptors on cells of the oligodendrocyte-type-2 astrocyte (O-2A) cell lineage. Development 105, 595-603.

HAYMAKER, W. (1969)

Effects of ionising radiation on nervous tissue. In: The Structure and Function of the Nervous System. Ed. G.H. Bourne. Vol. 3, pp. 441-578. Academic Press Inc., New York.

HELDIN, C-H., JOHNSSON, A., WENNERGREN, S., WERNSTEDT, C., BETSHOLTZ, C. and WESTERMARK, B. (1986)

A human osteosarcoma cell line secretes a growth factor structurally related to a homodimer of PDGF A-chains. Nature 319, 511-514.

HELDIN, C-H. and WESTERMARK, B. (1989)

Platelet-derived growth factor: three isoforms and two receptor types. Trends in Genetics 5, 108-111.

HERTZ, L. (1981)

Functional interactions between astrocytes and neurons. In: Progress in Clinical and Biological Research - Glial and Neuronal Cell Biology. Eds. Vidrio, E.A. and Federoff, S. 1st Edn. Vol. 59A, pp. 45-58. Alan Liss Inc., New York.

HEYWORTH, C.M., WHETTON, A.D., KINSELLA, A.R. and HOUSLAY, M.D. (1984)
The phorbol ester TPA inhibits glucagon-stimulated adenylate cyclase activity. Febs. Letts. 170, 38-42.

HIDAKA, H., INAGAKI, M., KAWAMOTO, S. and SASAKI, Y. (1984)
Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. Biochemistry 23, 5036-5041.

HIDAKA, H., TANAKA, T., ONODA, K., HAGIWARA, M., WATANABE, M., OHTA, H., HO, Y., TSURODOME, M. and YOSHIDA, T. (1988)
Cell type-specific expression of PKC isozymes in the rat cerebellum. J. Biol. Chem. 263, 4523-4526.

HIRAYAMA, M., SILBERBERG, D.H., LISAK, R.P. and PLEASURE, D. (1983)
Long-term culture of oligodendrocytes isolated from rat corpus callosum by percoll density gradient. J. Neuropath. Exp. Neurol. 42, 16-28.

HOKIN, M.R. and HOKIN, L.E. (1953)
Enzyme secretion and the incorporation of ^{32}P into phospholipids of pancreas slices. J. Biol. Chem. 263, 967-977.

HOLLINGSWORTH, E.B., UKENA, D. and DALY, J.W. (1986)
The protein kinase C activator phorbol-12-myristate-13-acetate enhances cyclic AMP accumulation in pheochromocytoma cells. Febs. Letts. 196, 131-134.

HONEGGER, P. (1986)
PKC-activating tumour promoters enhance the differentiation of astrocytes in aggregating fetal brain cell cultures.
J. Neurochem. 46, 1561-1566.

HONEGGER, P. (1989)

Aggregating brain cell cultures: a model to study myelination and demyelination. In: Abstracts of NATO conference on "Cellular and Molecular Biology of Myelination", September 1989, Osnabruck, FRG, Plenum Press, New York. In press.

HOSADA, K., SAITO, N., KOSE, A., HO, A., TSUJINO, T., OGITA, K., KIKKAWA, U., ONO, Y., IGARASHI, K., NISHIZUKA, Y. and TANAKA, C. (1989)

Immunocytochemical localisation of the β_1 subspecies of protein kinase C in rat brain. Proc. Natl. Acad. Sci. USA. 86, 1393-1397.

HOUSEY, G.M. JOHNSON, M.D., HSIAO, W.L.W., O'BRIAN, C.A., MURPHY, J.P., KIRSCHMEIER, P. and WEINSTEIN, I.B. (1988)

Overproduction of PKC causes disordered growth control in rat fibroblasts. Cell. 52, 343-354.

HUANG, K-P., NAKABAYASHI, H. and HUANG, F.L. (1986)

Isozymic forms of rat brain Ca^{2+}-activated and phospholipid-dependent protein kinase. Proc. Natl. Acad. Sci. USA. 83, 8535-8539.

HUANG, F.L., YOSHIDA, Y., CUNHA-MELO, J.R., BEAVEN, M.A. and HUANG, K-P. (1989)

Differential downregulation of protein kinase C isozymes. 264, 4238-4243.

HUANG, F.L., YOSHIDA, Y., NAKABAYASHI, H. and HUANG, K-P. (1987a)

Differential distribution of protein kinase C isozymes in the various regions of brain. J. Biol. Chem. 262, 15714-15720.

HUANG, F.L., YOSHIDA, Y., NAKABAYASHI, H., KNOPF, J.L., YOUNG, W.S.III. and HUANG, K-P. (1987b)

Immunocytochemical identification of PKC isozymes as products of discrete genes. Biochem. Biophys. Res. Commun. 149, 946-952.

HUANG, F.L., YOSHIDA, Y., NAKABAYASHI, H., YOUNG, W.S. III and HUANG, K-P. (1988)

Immunocytochemical localisation of protein kinase C isozymes in rat brain. J. Neurosci. 8, 4734-4744.

HUGHES, S., LILLIEN, L.E., RAFF, M.C., ROHRER, H. and SENDTNER, M. (1988)

Ciliary neurotrophic factor induces type-2 astrocyte differentiation in culture. Nature 335, 70-73.

INOUE, M., KISHIMOTO, A., TAKAI, Y. and NISHIZUKA, Y. (1977)

Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissue. J. Biol. Chem. 252, 7610-7616.

INGEBRITSEN, T.S. and COHEN, P. (1983)

Protein phosphatases: properties and role in cellular regulation. Science 221, 331-338.

INGEBRITSEN, T.S., STEWART, A.A. and COHEN, P. (1983)

The protein phosphatases involved in cellular regulation. Eur. J. Biochem. 132, 297-307.

IRVINE, R.F. (1982)

How is the level of free arachidonic acid controlled in mammalian cells? Biochem J. 204, 3-16.

IRVINE, R.F. and MOOR, R.M. (1986)

Micro-injection of inositol 1,3,4,5-tetrakisphosphate activates sea urchin eggs by a mechanism dependent on external Ca^{2+}. Biochem. J. 240, 917-920.

IRVINE, R.F., MOOR, R.M., POLLOCK, W.K., SMITH, P.M. and WREGGETT, K.A. (1988)

Inositol phosphates: proliferation, metabolism and function. Phil. Trans. R. Soc. Lond. B 320, 281-298.

ITOYAMA, Y., STERNBERGER, N.H., WEBSTER, H. de F., QUARLES, R.H., COHEN, S.R. and RICHARDSON E.P. (1980)

Immunocytochemical observations of the distribution of myelin associated glycoprotein and myelin basic protein in multiple sclerosis lesions. Ann. Neurol. 7, 167-177.

IWASA, Y., TAKAI, Y., KIKKAWA, U. and NISHIZUKA, Y. (1980)

Phosphorylation of calf thymus H1 histone by calcium-activated, phospholipid-dependent protein kinase. Biochem. Biophys. Res. Commun. 96, 180-187.

JACOBS, S., SAHYOUN, N.E., SALTIEL, A.R. and CUATRECASAS, P. (1983)

Phorbol esters stimulate the phosphorylation of receptors for insulin and somatamedin C. Proc. Natl. Acad. Sci. USA. 80, 6211-6213.

JACOBSEN, G.R. and SAIER, M.H. Jr. (1984)

Biological membranes: structure and assembly. In: Biochemistry, pp. 573-620. Ed. Zubay, G. Addison-Wesley, Massachusetts.

JOHNSSON, A., HELDIN, C-H., WASTESON, A., WESTERMARK, B., DEUEL, T.F., HUANG, J.S., SEEBURG, D.H., GRAY, E., ULLRICH, A., SCRACE, G., STROOBANT, P. and WATERFIELD, M.D. (1984)

The C-*sis* gene encodes a precursor of the B chain of platelet-derived growth factor. EMBO. J. 3, 921-928.

JOSEPH, S.K. (1985)

Receptor-stimulated phosphoinositide metabolism: a role for GTP-binding proteins? Trends Biochem. Sci. 10, 297-298.

KAIBUCHI, K., TAKAI, Y., SAWAMURA, M., HOSHIJIMA, M., FUJIKURA, T. and NISHIZUKA, Y. (1983)

Synergistic functions of protein phosphorylation and calcium mobilisation in platelet activation. J. Biol. Chem. 258, 701-6704.

KAIBUCHI, K., TAKAI, Y. and NISHIZUKA, Y. (1981)

Cooperative roles of various membrane phospholipids in the activation of calcium-activated, phospholipid-dependent protein kinase. J. Biol. Chem. 256, 7146-7149.

KAIBUCHI, K., TAKAI, Y. and NISHIZUKA, Y. (1985)

Protein kinase C and calcium ion in mitogenic response of macrophage-depleted human peripheral lymphocytes. J. Biol. Chem. 260, 1366-1369.

KANOH, H., YAMADA, K., SAKANE, F. and IMAIZUMI, T. (1989)

Phosphorylation of diacylglycerol kinase *in vitro* by protein kinase C. Biochem. J. 258, 455-462.

KAPLAN, D.R., WHITMAN, M., SCHAFFHAUSEN, B., PALLAS, D.C., WHITE, M., CANTLEY, L. and ROBERTS, T.M. (1987).

Common elements in growth factor stimulation and oncogenic transformation: 85kDa phosphoprotein and phosphatidylinositol kinase activity. Cell 50, 1021-1029.

KASAI, N. and YU, R.K. (1983)

The monoclonal antibody A2B5 is specific to ganglioside G_{Q1C}. Brain Res. 277, 155-158.

KASSIS, S., ZAREMBA, T., PATEL, J. and FISHMAN, P.H. (1985)

Phorbol esters and β-adrenergic agonists mediate desensitisation of adenylate cyclase in rat glioma C6 cells by distinct mechanisms. J. Biol. Chem. 260, 8911-8917.

KATAKAMI, Y., KAIBUCHI, K., SAWAMURA, M., TAKAI, Y. and NISHIZUKA, Y. (1984)

Synergistic action of protein kinase C and calcium for histamine release from rat peritoneal mast cells. Biochem. Biophys. Res. Commun. 121, 573-578.

KATOH, N. and KUO, J.F. (1982)

Subcellular distribution of phospholipid-sensitive calcium-dependent protein kinase in guinea pig heart, spleen and cerebral cortex, and inhibition of the enzyme by Triton X-100. Biochem. Biophys. Res. Commun. 106, 590-595.

KAZLAUSKAS, A., BOWEN-POPE, D., SEIFERT, R., HART, C.E. and COOPER, J.A. (1988)

Different effects of homo- and heterodimers of platelet-derived growth factor A and B chains on human and mouse fibroblasts. EMBO. J. 7, 3727-3735.

KENNEDY, P.G.E. (1982)

Neural cell markers and their applications to neurology. J. Neuroimmunol. 2, 35-53.

KHANNA, N.C., TOKUDA, M. and WAISMAN, D.M. (1986)

Phosphorylation of lipocortins in vitro by protein kinase C. Biochem. Biophys. Res. Commun. 141, 547-554.

KIDO, H., FUKUSEN, N., ISHIDOH, K. and KATUNUMA, N. (1986)

Diacylglycerol amplified the induction in vivo of tyrosine aminotransferase and ornithine decarboxylase by glucocorticoid. Biochem. Biophys. Res. Commun. 138, 275-282.

KIKKAWA, U., KITANO, T., SAITO, S., FUJIWARA, H., NAKANISHI, J., KISHIMOTO, A., TANIYAMA, K., TANAKA, C. and NISHIZUKA, Y. (1986)

Possible roles of protein kinase C in signal transduction in nervous tissues. Prog. Brain Res. 69, 29-35.

KIKKAWA, U. and NISHIZUKA, Y. (1986)

The role of protein kinase C in transmembrane signalling. Ann. Rev. Cell Biol. 2, 149-178.

KIKKAWA, U., ONO, Y., OGITA, K., FUJII, T., ASAOKA, Y., SEKIGUCHI, K., KOSAKA, Y., IGARISHI, K. and NISHIZUKA, Y. (1987)

Identification of the structures of multiple subspecies of protein kinase C expressed in brain. Febs. Letts. 217, 227-231.

KIKKAWA, U., TAKAI, Y., MINAKUCHI, R., INOHARA, S. and NISHIZUKA, Y. (1982)

Calcium-activated, phospholipid-dependent protein kinase from rat brain - subcellular distribution, purification and properties. J. Biol. Chem. 257, 13341-13348.

KIKKAWA, U., TAKAI, Y., TANAKA, Y., MIYAKE, R. and NISHIZUKA, Y. (1983)

Protein kinase C as a possible receptor protein of tumour-promoting phorbol esters. J. Biol. Chem. 258, 11442-11445.

KIMURA, K., KUBO, S., SAKURADA, K., ABE, K. and KATOH, N. (1987)

PKC phosphorylation of protamine is Ca^{2+}-independent, but the addition of DNA renders it Ca^{2+}-dependent. Biochim. Biophys. Acta. 929, 203-207.

KIRSCH, D., OBERMAIER, B. and HARING, H.U. (1985)

Phorbol esters enhance basal D-glucose transport but inhibit insulin stimulation of D-glucose transport and insulin binding in isolated rat adipocytes. Biochem. Biophys. Res. Commun. 128, 824-832.

KISHIMOTO, A., KAJIKAWA, N., SHIOTA, M. and NISHIZUKA, Y. (1983)

Proteolytic activation of calcium-activated, phospholipid-dependent protein kinase by calcium-dependent neutral protease. J. Biol. Chem. 258, 1156-1164.

KISHIMOTO, A., MIKAWA, K., HASHIMOTO, K., YASUDA, T., TANAKA, S-I., TOMINAGA, M., KURODA, T. and NISHIZUKA, Y. (1989)

Limited proteolysis of protein kinase C subspecies by calcium-dependent neutral protease (calpain). J. Biol. Chem. 264, 4088-4092.

KISHIMOTO, A., TAKAI, Y., MORI, T., KIKKAWA, U. and NISHIZUKA, Y. (1980)

Activation of calcium and phospholipid-dependent protein kinase by DAG; its possible relation to PI turnover. J. Biol. Chem. 255, 2273-2276.

KNIGHT, D.E. and BAKER, P.F. (1983)

The phorbol ester TPA increases the affinity of excytosis for calcium in "leaky" adrenal medullary cells. Febs. Letts. 160, 98-100.

KNOPF, J.L., LEE, M-H., SULTZMAN, L.A., KRIZ, R.W., LOOMIS, C.R., HEWICK, R.M. and BELL, R.M. (1986)

Cloning and expression of multiple PKC cDNAs. Cell 46, 491-502.

KOJIMA, I., LIPPES, H., KIJIMA, K. and RASMUSSEN, H. (1983)

Aldosterone secretion: effect of phorbol ester and A23187. Biochem. Biophys. Res. Commun. 116, 555-562.

KOLESNICK, R.N. and GERSHENGORN, M.C. (1985)

Arachidonic acid inhibits thyrotropin-releasing hormone-induced elevation of cytoplasmic free calcium in GH_3 pituitary cells. J. Biol. Chem. 260, 707-713.

KOPER, J.W., LOPES-CARDOZO, M., ROMIJN, H.J. and van GOLDE, L.M.B. (1984)

Culture of rat cerebral oligodendrocytes in a serum-free, chemically defined medium. J. Neurosci. Meth. 10, 157-169.

KOSAKA, Y., OGITA, K., ASE, K., NOMURA, H., KIKKAWA, U. and NISHIZUKA, Y. (1988)

The heterogeneity of protein kinase C in various rat tissues. Biochem. Biophys. Res. Commun. 151, 973-981.

KRAFT, A.S. and ANDERSON, W.B. (1987)

Phorbol esters increase the amount of Ca^{2+}, phospholipid-dependent protein kinase associated with plasma membrane. Nature 301, 621-623.

KREBS, E.G. and BEAVO, J.A. (1979)

Phosphorylation-dephosphorylation of enzymes. Ann. Rev. Biochem. 48, 923-959.

KU, Y., KISHIMOTO, A., TAKAI, Y., OGAWA, Y., KUMURA, S. and NISHIZUKA, Y. (1981)

A new possible regulatory system for protein phosphorylation in human peripheral lymphocytes. J. Immunol. 127, 1375-1379.

KUO, J.F., ANDERSSON, R.G.G., WISE, B.C., MACKERLOVA, L., SALMONSSON, I., BRACKETT, N.L., KATOH, N., SHOJI, M. and WRENN, R.W. (1980)

Calcium-dependent protein kinase: widespread occurrence in various tissues and phyla of the animal kingdom and comparison of effects of phospholipids, calmodulin and trifluoperazine. Proc. Natl. Acad. Sci. USA. 77, 7039-7043.

LABARCA, R., JANOWSKY, A., PATEL, J. and PAUL, S.M. (1984)

Phorbol ester inhibits agonist-induced [^{3}H] inositol-1-phosphate accumulation in rat hippocampal slices. Biochem. Biophys. Res. Commun. 123, 703-709.

LAEMMLI, U.K. (1970)

Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Le PEUCH, C.J., BALLESTER, R. and ROSEN, O. (1983)

Purified rat brain calcium- and phospholipid-dependent protein kinase phosphorylates ribosomal protein S6. Proc. Natl. Acad. Sci. USA. 80, 6858-6862.

LEVINE, H. III., SAHYOUN, N., McCONNELL, R., BRONSON, D. and CUATRECASAS, P. (1984)

Specificity of a phosphatase for phospholipid, Ca^{2+}-dependent protein kinase-phosphorylated histone H1 resides in the catalytic subunit. Biochem. Biophys. Res. Commun. 118, 278-283.

LILLIEN, L.E., SENDTNER, M., ROHRER, H., HUGHES, S. and RAFF, M.C. (1988)

Type-2 astrocyte development in rat brain cultures is initiated by a CNTF-like protein produced by type-1 astrocytes. Neuron 1, 485-494.

LIM, R. (1985)

Glia maturation factor and other factors acting on glia. In: Growth and maturation factors. Ed. Guroff, G. Volume 3 pp.119-147. Wiley, New York.

LINNINGTON, C., WEBB, M. and WOODHAMS, P.L. (1984)

A novel myelin-associated glycoprotein defined by a mouse monoclonal antibody (JN1 00202). J. Neuroimmunol. 6, 387-396.

LOWRY, O.H., ROSEBROUGH, N.T., FARR, A.L. and RANDALL, R.J. (1951)

Protein measurement with folin phenol reagent. J. Biol. Chem. 193, 265-275.

MACFARLANE, D.E. (1986)

Phorbol diester-induced phosphorylation of nuclear matrix proteins in HL60 promyelocytes: possible role in differentiation studied by cationic detergent gel electrophoresis systems. J. Biol. Chem. 261, 6947-6953.

MAJERUS, P.W., CONNOLLY, T.M., DECKMYN, H., ROSS, T.S., BROSS, T.E., ISHII, H., BANSAL, V.S. and WILSON, D.B. (1986)

The metabolism of phosphoinositide-derived messenger molecules. Science 234, 1519-1526.

MARTIN, T.F.J. (1983)

Thyrotropin-releasing hormone rapidly activates the phosphodiester hydrolysis of polyphosphoinositides in GH_3 pituitary cells. J. Biol. Chem. 258, 14816-14822.

MARTIN, T.F. and KOWALCHYK, J.A. (1984)

Evidence for the role of calcium and diacylglycerol as dual second messengers in thyrotropin-releasing hormone action: involvement of diacylglycerol. Endocrinology 115, 1517-1526.

MASMOUDI, A., LABOURDETTE, G., MERSEL, M., HUANG, F.L., HUANG, K-P., VINCENDON, G. and MALVIYA, A.N. (1989)

Protein kinase C located in rat liver nuclei: partial purification and biochemical and immunochemical characterisation. J. Biol. Chem. 264, 1171-1179.

MAY, W.S., JACOBS, S. and CUATRECASAS, P. (1984)

Association of phorbol ester-induced hyperphosphorylation and reversible regulation of transferrin membrane receptors in HL-60 cells. Proc. Natl. Acad. Sci. USA. 81, 2016-2020.

McCARTHY, K.D. and de VELLIS, J. (1980)

Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. J. Cell Biol. 85, 890-902.

McDONALD, J.R., GROSCHEL-STEWART, U. and WALSH, M.P. (1987)

Properties and distribution of the protein inhibitor (Mr 17000) of PKC. Biochem. J. 242, 695-705.

McMORRIS, F.A. (1989)

Regulation of oligodendrocyte development by insulin-like growth factor and cyclic AMP. In: Abstracts of NATO conference on "Cellular and Molecular Biology of Myelination", September 1989, Osnabruck, FRG, Plenum Press, New York. In press.

McPHAIL, L.C., CLAYTON, C.C. and SNYDERMAN, R. (1984)

A potential second messenger role for unsaturated fatty acids: activation of calcium-dependent protein kinase. Science 224, 622-625.

MEISLER, M.H. and LANGAN, T.A. (1969)

Characterisation of a phosphatase specific for phosphorylated histone and protamine. J.Biol.Chem. 244, 4961-4968.

MERCOLA, M., MELTON, D.A. and STILES, C.D. (1988)

Platelet-derived growth factor A chain is maternally encoded in Xenopus embryos. Science 241, 1223-1225.

MICHELL, R.H. (1975)

Inositol lipids and cell surface receptor function. Biochim. Biophys. Acta. 415, 81-147.

MICHELL, R.H., ALLAN, D. and FINEAN, J.B. (1976)

Significance of minor glycerolipids in membrane structure and function. Adv. Exp. Biol. Med. 72, 3-13.

MILLER, R.J. (1986)

Protein kinase C: a key regulator of neuronal excitability? Trends Neurol. Sci. 9, 538-541.

MILLER, R.H., DAVID, S., PATEL, R., ABNEY, E.R. and RAFF, M.C. (1985)

A quantitative immunohistochemical study of macroglial cell development in the rat optic nerve: in vivo evidence for two distinct astrocyte lineages. Dev. Biol. 111, 35-41.

MILLER, R.H. and RAFF, M.C. (1984)

Fibrous and protoplasmic astrocytes are biochemically and developmentally distinct. J. Neurosci. 4, 585-592.

MIYAKE, R., TANAKA, Y., TSUDA, T., KAIBUCHI, K., KIKKAWA, U. and NISHIZUKA, Y. (1984)

Activation of protein kinase C by non-phorbol tumour promoter, mezerein. Biochem. Biophys. Res. Commun. 121, 649-656.

MOBLEY, A. and TAI, H.H. (1985)

Synergistic stimulation of thromboxane biosynthesis by calcium ionophore and phorbol ester or thrombin in human platelets. Biochem. Biophys. Res. Commun. 130, 717-723.

MORI, T., TAKAI, Y., MINAKUCHI, R., YU, B. and NISHIZUKA, Y. (1980)

Inhibitory action of chlorpromazine, dibucaine, and other phospholipid-interacting drugs on calcium-activated, phospholipid-dependent protein kinase. J. Biol. Chem. 255, 8378-8380.

MORRIS, C. and ROZENGURT, E. (1988)

Purification of a phosphoprotein from rat brain closely related to the 80kDa substrate of protein kinase C identified in Swiss 3T3 fibroblasts. Febs. Letts. 231, 311-316.

MURAKAMI, K. and ROUTTENBERG, A. (1985)

Direct activation of purified protein kinase C by unsaturated fatty acids (oleate and arachidonate) in the absence of phospholipids and Ca^{2+}. Febs. Letts. 192, 189-193.

MURAKAMI, K., WHITELY, M.K. and ROUTTENBERG, A. (1987)

Regulation of protein kinase C activity by cooperative interaction of Zn^{2+} and Ca^{2+}. J.Biol.Chem. 262, 13902-13906.

MURDOCH, G.F., WATERMAN, M., EVANS, R.M. and ROSENFELD, M.G. (1985)

Molecular mechanisms of phorbol ester, thyrotropin-releasing hormone, and growth factor stimulation of prolactin gene transcription. J. Biol. Chem. 260, 11852-11858.

MURPHY, J.A., CHAPMAN, J.A., SUCKLING, A.J. and RUMSBY, M.G. (1988)

Protein kinase C activity in soluble fractions from glial cells in primary culture and subcultures. Neurosci. Letts. 85, 255-260.

MURPHY, S., McCABE, N., MORROW, C. and PEARCE, B. (1987)

Phorbol ester stimulates proliferation of astrocytes in primary culture. Dev. Brain Res. 31, 133-135.

NABIKA, T., NARA, Y., YAMORI, Y., LOVENBERG, W. and ENDO, J. (1985)
Angiotensin II and phorbol ester enhance isoproterenol- and vasoactive intestinal peptide (VIP)-induced cyclic AMP accumulation in vascular smooth muscle cells. Biochem.Biophys.Res.Commun. 131, 30-36.

NAIRN, A.C., HEMMINGS, H.C.Jr. and GREENGARD, P. (1985)
Protein kinases in the brain. Ann. Rev. Biochem. 54, 931-976.

NATHANIEL, E.J.H. and NATHANIEL, D.R. (1981)
The Reactive Astrocyte. In: Advances in Cellular Neurobiology Vol. 2, pp. 249-301. Eds. Federoff, S. and Hertz, L. Academic Press Inc., London.

NEARY, J.T., NAITO, S., De WEER, A. and ALKON, D.L. (1986a)
Ca^{2+}/diacylglycerol-activated, phospholipid-dependent protein kinase in the Hermissenda CNS. J. Neurochem. 47, 1405-1411.

NEARY, J.T., NORENBERG, L-O.B. and NORENBERG, M.D. (1986)
Calcium-activated, phospholipid-dependent protein kinase and protein substrates in primary cultures of astrocytes. Brain Res. 385, 420-424.

NEARY, J.T., NORENBERG, L-O.B. and NORENBERG, M.D. (1988)
PKC in primary cultures: cytoplasmic localisation and translocation by a phorbol ester. J. Neurochem. 50, 1179-1184.

NISHIZAWA, Y., KURIHARA, T. and TAKAHASHI, Y. (1981)
Immunohistochemical localisation of 2',3'-cyclic nucleotide 3'-phosphodiesterase in the central nervous system. Brain Res. 212, 219-222.

NISHIZUKA, Y. (1983)
Phospholipid degradation and signal translation for protein phosphorylation. Trends Biochem. Sci. 8, 13-16.

NISHIZUKA, Y. (1986)

Studies and perspectives of protein kinase C. Science 233, 305-312.

NISHIZUKA, Y. (1988)

The molecular heterogeneity of protein kinase C and its implications for cellular regulation. Nature 334, 661-665.

NISTER, M., HAMMACHER, A., MELLSTROM, K., SIEGBAHN, A., RONNSTRAND, L., WESTERMARK, B. and HELDIN, C-H. (1988a)

A glioma-derived PDGF A chain homodimer has different functional activities from a PDGF AB heterodimer purified from human platelets. Cell 52, 791-799.

NISTER, N., LIEBERMANN, T., BETSHOLTZ, C., PETTERSSON, M., CLAESSON-WELSH, L., HELDIN, C-H., SCHLESSINGER, J. and WESTERMARK, B. (1988b)

Expression of messenger RNA's for platelet-derived growth factor and transforming growth factor-alpha and their receptors in human malignant glioma cell lines. Cancer Res. 48, 3910-3918.

NOBLE, M. and MURRAY, K. (1984)

Purified astrocytes promote the in vitro division of a bipotential glial progenitor cell. EMBO. J. 3, 2243-2247.

NOBLE, M., MURRAY, K., STROOBANT, P., WATERFIELD, M.D. and RIDDLE, P. (1988)

PDGF promotes division and motility and inhibits premature differentiation of the O-2A progenitor cell. Nature 333, 560-562.

NORENBERG, M.D. and MARTINEZ-HERNANDEZ, A. (1979)

Fine structural localisation of glutamine synthetase in astrocytes of rat brain. Brain Res. 161, 303-310.

NORTON, W.T. and AUTILIO, L.A. (1966)

The lipid composition of purified bovine brain myelin. J. Neurochem. 13, 213-222.

NORTON, W.T. and PODUSLO, S.E. (1970)

NORTON, W.T. and PODUSLO, S.E. (1970)

Neuronal soma and whole neuroglia of rat brain: a new isolation technique. Science 167, 1144-1146.

OGAWA, H., SATO, Y., TAKESHITA, I., TATEISHI, J. and KITAMURA, K. (1985)

Transient expression of glial fibrillary acidic protein in developing oligodendrocytes in vitro. Dev. Brain Res. 18, 133-141.

OHMURA, E. and FRIESEN, H.G. (1985)

12-O-tetradecanoylphorbol-13-acetate stimulates rat growth hormone (GH) release through different pathways from that of human pancreatic GH-releasing factor. Endocrinology 116, 185-191.

OISHI, K., RAYNOR, R.L., CHARP, P.A. and KUO, J.F. (1989)

Regulation of PKC by lysophospholipids - potential role in signal transduction. J. Biol. Chem. 263, 6865-6871.

ONO, Y., FUJII, T., OGITA, K., KIKKAWA, U., IGARISHI, K. and NISHIZUKA, Y. (1988)

The structure, expression and properties of additional members of the PKC family. J. Biol. Chem. 263, 6927-6932.

ONO, Y., KIKKAWA, U., OGITA, K., FUJII, T., KUROKAWA, T., ASAOKA, Y., SEKIGUCHI, K., ASE, K., IGARASHI, K. and NISHIZUKA, Y. (1987)

Expression and properties of two types of PKC: alternative splicing from a single gene. Science 236, 1160-1120.

ONO, Y., KUROKAWA, T., FUJII, T., KAWAHARA, K., IGARASHI, K., KIKKAWA, U., OGITA, K. and NISHIZUKA, Y. (1986)

Two types of complementary DNA's of rat brain PKC. Febs. Letts. 206, 347-352.

ORELLANA, S.A., SOLSKI, P.A. and BROWN, J.H. (1985)
Phorbol ester inhibits phosphoinositide hydrolysis and calcium mobilisation in cultured astrocytoma cells. J. Biol. Chem. 260, 5236-5239.

OTANI, S., MATSUI, I., KURAMOTO, A. and MORISAWA, S. (1985)
Induction of ornithine decarboxylase in guinea pig lymphocytes. Synergistic effect of diacylglycerol and calcium. Eur. J. Biochem. 147, 27-31.

PAPINI, E., GRZESKOWIAK, M., BELLAVITE, P. and ROSSI, F. (1985)
Protein kinase C phosphorylates a component of NADPH oxidase of neutrophils. Febs. Letts. 190, 204-208.

PARKER, P.J., COUSSENS, S.L., TOTTY, N., RHEE, L., YOUNG, S., CHEN, E., STABEL, S., WATERFIELD, M.D. and ULLRICH, A. (1986)
The complete primary structure of PKC - the major phorbol ester receptor. Science 233, 853-858.

PARKER, P.J., STABEL, S. and WATERFIELD, M.D. (1984)
Purification to homogeneity of protein kinase C from bovine brain - identity with the phorbol ester receptor. EMBO J. 3, 953-959.

PEARCE, B., MORROW, C. and MURPHY, S. (1986)
Receptor-mediated phospholipid hydrolysis in astrocytes. Eur. J. Pharmacol. 121, 231-243.

PEARCE, B., MORROW, C. and MURPHY, S. (1988)
A role for protein kinase C in astrocyte glycogen metabolism. Neurosci.Letts. 90, 191-196.

PEARCE, B., MURPHY, S., JEREMY, J., MORROW, C. and DANDONA, P. (1989)
ATP-evoked Ca^{2+} mobilisation and prostanoid release from astrocytes: P_2-purinergic receptors linked to phosphoinositide hydrolysis. J. Neurochem. 52, 971-977.

PELECH, S.L., PADDON, H.B. and VANCE, D.E. (1984)

Phorbol esters stimulate phosphatidylcholine biosynthesis by translocation of CTP: phosphocholine cytidyltransferase from cytosol to microsomes. Biochim. Biophys. Acta. 795, 447-451.

PELECH, S.L. and VANCE, D.E. (1989)

Signal transduction via phosphatidylcholine cycles. Trends Biochem. Sci. 14, 28-30.

PELOSIN, J-M., VILGRAIN, I. and CHAMBAZ, E.M. (1987)

A single form of PKC is expressed in bovine adrenocortical tissue, as compared to four chromatographically resolved isozymes in rat brain. Biochem. Biophys. Res. Commun. 147, 382-391.

PHILLIPS, W.A., FUJIKI, T., ROSSI, M.W., KORCHAK, H.M. and JOHNSTON, R.B.Jr. (1989)

Influence of calcium on the subcellular distribution of protein kinase C in human neutrophils. J. Biol. Chem. 264, 8361-8365.

POZZAN, T., GATTI, G., DOZIO, N., VINCENTINI, L.M. and MELDOLESI, J. (1984)

Ca^{2+}-dependent and -independent release of neurotransmitters from PC12 cells: a role for protein kinase C activation? J. Cell Biol. 99, 628-638.

PRUSS, R.M., BARTLETT, P.F., GAVRILOVIC, J., LISAK, R.P. and RATTRAY, S. (1982)

Mitogens for glial cells: a comparison of the response of cultured astrocytes, oligodendrocytes and Schwann cells. Dev. Brain Res. 254, 19-35.

RAFF, M.C. (1989)

Glial cell diversification in the rat optic nerve. Science 243, 1450-1455.

RAFF, M.C., ABNEY, E.R. and FOK-SEANG, J. (1985)
Reconstitution of a developmental clock in vitro: a critical role for astrocytes in the timing of oligodendrocyte differentiation. Cell 42, 61-69.

RAFF, M.C., ABNEY, E.R. and MILLER, R.H. (1984)
Two glial cell lineages diverge prenatally in rat optic nerve. Dev. Biol. 106, 53-60.

RAFF, M.C., LILLIEN, L.E., RICHARDSON, W.D., BURNE, J.F. and NOBLE, M.D. (1988)
Platelet-derived growth factor from astrocytes drives the clock that times oligodendrocyte development in culture. Nature 333, 562-565.

RAFF, M.C., MILLER, R.H. and NOBLE, M. (1983)
A glial progenitor cell that develops in vitro into an astrocyte or oligodendrocyte depending on culture medium. Nature 303, 390-396.

RAFF, M.C., MIRSKY, R., FIELDS, K.L., LISAK, R.P., DORFMAN, S.H., SILBERBERG, D.H., GREGSON, N.A., LIEBOWITZ, S. and KENNEDY, M.C. (1978)
Galactocerebroside is a specific cell-surface antigenic marker for oligodendrocytes in culture. Nature 274, 813-816.

RAFF, M.C., TEMPLE, S. and ffRENCH-CONSTANT, C. (1987)
Glial cell development and function in the rat optic nerve. Prog. Brain Res. 71, 435-438.

RAIBLE, D.W. and McMORRIS, F.A. (1989)
Cyclic AMP regulates the rate of differentiation of oligodendrocytes without changing the lineage commitment of their progenitors. Dev. Biol. 133, 437-446.

RANDO, R.R. and YOUNG, N. (1984)
The stereospecific activation of protein kinase C. Biochem. Biophys. Res. Commun. 122, 818-823.

RAPPOLEE, D.A., BRENNER, C.A., SCHULTZ, R., MARK, D. and WEBB, Z. (1988)

Developmental expression of PDGF, TGF-α, and TGF-β in preimplantation mouse embryos. Science 241, 1823-1825.

RASMUSSEN, H., FORDER, J. and KOJIMA, I. (1984)

TPA-induced contraction of isolated rabbit vascular muscle. Biochem. Biophys. Res. Commun. 122, 776-784.

RICHARDSON, W. (1989)

The role of platelet-derived growth factor in glial development. In: Abstracts of NATO conference on "Cellular and Molecular Biology of Myelination", September 1989, Osnabruck, FRG, Plenum Press, New York. In press.

RICHARDSON, W.D., PRINGLE, N., MOSLEY, M.J., WESTERMARK, B. and DUBOIS-DALCQ, M. (1988)

A role for PDGF in normal gliogenesis in the central nervous system. Cell 53, 309-319.

RITCHIE, T., COLE, R., KIM, H-S., de VELLIS, J. and NOBLE, E.P. (1987).

Inositol phospholipid hydrolysis in cultured astrocytes and oligodendrocytes. Life Sci. 41, 31-39.

RITTENHOUSE, S.E. and SASSON, J.P. (1985)

Mass changes in myoinositol trisphosphate in human platelets stimulated by thrombin. J. Biol. Chem. 260, 8657-8660.

ROACH, P.J. and GOLDMAN, M. (1983)

Modification of glycogen synthase activity in isolated rat hepatocytes by tumour-promoting phorbol esters: evidence for differential regulation of glycogen synthase and phosphorylase. Proc. Natl. Acad. Sci. USA. 80, 7170-7172.

RODRIGUEZ-PENA, A. and ROZENGURT, E. (1984)

Disappearance of Ca^{2+}-sensitive, phospholipid-dependent protein kinase activity in phorbol ester-treated 3T3 cells. Biochem. Biophys. Res. Commun. 120, 1053-1059.

ROGHANI, M., DaSILVA, C., GUVELLI, D. and CASTAGNA, M. (1987)

Benzene and toluene activate protein kinase C. Carcinogen 8, 1105-1107.

ROSS, R., GLOMSET, J.A., KARIYA, B. and HARKER, L. (1974)

A platelet-dependent serum factor that stimulates the proliferation of arterial smooth muscle cells in vitro. Proc. Natl. Acad. Sci. USA. 71, 1207-1210.

ROSS, R., RAINES, E.W. and BOWEN-POPE, D.F. (1986)

The biology of platelet-derived growth factor. Cell 46, 155-169.

ROZENGURT, E., RODRIGUEZ-PENA, A., COOMBS, M. and SINNETT-SMITH, J. (1984)

Diacylglycerol stimulates DNA synthesis and cell division in mouse 3T3 cells: role of Ca^{2+}-sensitive phospholipid-dependent protein kinase. Proc. Natl. Acad. Sci. USA. 81, 5748-5752.

ROZENGURT, E., RODRIGUEZ-PENA, A. and SMITH, K.A. (1983)

Phorbol esters, phospholipase C, and growth factors rapidly stimulate the phosphorylation of a Mr 80,000 protein in intact quiescent 3T3 cells. Proc. Natl. Acad. Sci. USA. 80, 7244-7248.

RUBIN, K., TINGSTROM, A., HANSSON, G.K., LARSSON, E., RONNSTRAND, L., KLARESKOG, L., CLAESSON-WELSH, L., HELDIN, C-H., FELLSTROM, B. and TERRACIO, L. (1988)

Induction of B-type receptors for platelet-derived growth factor in vascular inflammation: possible implications for development of vascular proliferative lesions. Lancet (i), 1353-1356.

RUMSBY, M.G., CHAPMAN, J.A., MURPHY, J.A. and SUCKLING, A.J. (1988)
Stimulation of protein phosphorylation in mixed glial cell primary cultures and subcultures by the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA). Neurosci. Letts. 89, 251-257.

SAHYOUN, N., LEVINE, H. III., McCONNELL, R., BRONSON, D. and CUATRECASAS, P. (1983)
A specific phosphoprotein phosphatase acts on histone H1 phosphorylated by PKC. Proc. Natl. Acad. Sci. USA. 80, 6760-6764.

SAITO, N., KOSE, A., HO, A., HOSADA, K., MORI, M., HUATA, M., OGITA, K., KIKKAWA, U., ONO, Y., IGARISHI, K., NISHIZUKA, Y. and TANAKA, C. (1989)
Immunocytochemical localisation of β_{II} subspecies of protein kinase C in rat brain. Proc. Natl. Acad. Sci. USA. 86, 3409-3413.

SAKAMOTO, C., MATOZAKI, T., NAGAO, M. and BABA, S. (1985)
Combined effect of phorbol ester and A23187 or dibutyryl cyclic AMP on pepsinogen secretion from isolated gastric gland. Biochem. Biophys. Res. Commun. 131, 314-319.

SANETO, R.P. and DE VELLIS, J. (1985)
Characterisation of cultured rat oligodendrocytes proliferating in a serum-free, chemically defined medium. Proc. Natl. Acad. Sci. USA. 82, 3509-3513.

SCHONTAL, A., HERRLICH, P., RAHMSDORF, H.J. and PONTA, H. (1988)
Requirement for fos gene expression in the transcriptional activation of collagenase by other oncogenes and phorbol esters. Cell. 54, 325-334.

SCHROTER, J. and ALTHAUS, H.H. (1987)
The phorbol ester TPA dramatically accelerates oligodendroglial process regeneration. Naturwissenschaften 74, 393-394.

SEKAR, M.C. and HOKIN, L.E. (1986)

The role of phosphoinositides in signal transduction. J. Membrane Biol. 89, 193-210.

SEKIGUCHI, K., TSUKUDA, M., ASE, K., KIKKAWA, U. and NISHIZUKA, Y. (1988)

Mode of activation and kinetic properties of three distinct forms of protein kinase C from rat brain. J. Biochem. 103, 759-765.

SEKIGUCHI, K., TSUKUDA, M., OGITA, K., KIKKAWA, U. and NISHIZUKA, Y. (1987)

Three distinct forms of rat brain PKC: differential response to unsaturated fatty acids. Biochem. Biophys. Res. Commun. 145, 797-802.

SERHAN, C.N., BROEKMAN, M.J., KORCHAK, H.M., SMOLEN, J.E., MARCUS, A.J. and WEISSMANN, G. (1983)

Changes in phosphatidylinositol and phosphatidic acid in stimulated human neutrophils. Biochem. Biophys. Acta. 762, 420-428.

SHACKELFORD, D.A. and TROWBRIDGE, I.S. (1984)

Induction of expression and phosphorylation of the human interleukin-2 receptor by a phorbol ester. J. Biol. Chem. 259, 11706-11712.

SHAFIT-ZAGARDO, B., KUME-IWAKA, A. and GOLDMAN, J.E. (1988)

Astrocytes regulate GFAP mRNA levels by cyclic AMP and protein kinase C-dependent mechanisms. Glia 1, 346-354.

SHEARMAN, M.S., NAOR, Z., KIKKAWA, U. and NISHIZUKA, Y. (1987)

Differential expression of multiple protein kinase C subspecies in rat central nervous tissue. Biochem. Biophys. Res. Commun. 147, 911-919.

SHIMOKADO, K., RAINES, E.W., MADTES, D.K., BARRETT, T.B., BENDITT, E.P. and ROSS, R. (1985)

A significant part of macrophage-derived growth factor consists of at least two forms of PDGF. Cell 43, 277-286.

SHOJI, M., GIRARD, P.R., MAZZEI, G.J., VOGLER, W.R. and KUO, J.F. (1986)

Immunocytochemical evidence for phorbol ester-induced protein kinase C translocation in HL-60 cells. Biochem. Biophys. Res. Commun. 135, 1144-1149.

SKOFF, R.P., PRICE, D.L. and STOCKS, A. (1976)

Electron microscopic autoradiographic studies of gliogenesis in rat optic nerve. II. Time of origin. J. Comp. Neurol. 169, 313-334.

SNYDER, D.S., RAINE, C.S., FAROOQ, M. and NORTON, W.T. (1980)

The bulk isolation of oligodendrocytes from whole rat forebrain: a new procedure using physiologic media. J. Neurochem. 34, 1614-1621.

SOMMER, I. and SCHACHNER, M. (1981)

Monoclonal antibodies (01 to 04) to oligodendrocyte cell surfaces - an immunocytological study in the central nervous system. Dev. Biol. 83, 311-327.

SPEIZER, L.A., WATSON, M.J., KANTER, J.R. and BRUNTON, L.L. (1989)

Inhibition of phorbol ester binding and protein kinase C activity by heavy metals. J. Biol. Chem. 264, 5581-5585.

STERNBERGER, N.H., ITOYAMA, Y., KIES, M.W. and WEBSTER, H. de F. (1978)

Immunocytochemical method to identify basic protein in myelin-forming oligodendrocytes of newborn rat CNS. J. Neurocytol. 7, 251-263.

STREB, H., IRVINE, R.F., BERRIDGE, M.J. and SCHULZ, I. (1983)
Release of Ca^{2+} from a non-mitochondrial intracellular store in pancreatic acinar cells by inositol 1,4,5-trisphosphate. Nature 306, 67-69.

STREIT, O.J., GRAEBER, M.B. and KREUTZBERG, G.W. (1988)
Functional plasticity of microglia: a review. Glia 1, 301-307.

STROOBANT, P. and WATERFIELD, M.D. (1984)
Purification and properties of porcine platelet-derived growth factor. EMBO J. 3, 2963-2967.

SYKES, J.E.C. and LOPES-CARDOZO, M. (1988)
The effect of serum on lipid synthesis and on the expression of oligodendrocyte marker-enzymes in oligodendrocyte-enriched glial cells in culture. Neurochem. Int. 12, 467-474.

SZWERGOLD, B.S., GRAHAM, R.A. and BROWN, T.R. (1987)
Observation of inositol pentakis- and hexakisphosphates in mammalian tissues by ^{31}P-NMR. Biochem. Biophys. Res. Commun. 149, 874-881.

TAKAI, Y., KIKKAWA, U., KAIBUCHI, K. and NISHIZUKA, Y. (1984)
Membrane phospholipid metabolism and signal transduction for protein phosphorylation. Adv. Cyc. Nuc. Prot. Phosp. Res. 18, 119-158.

TAKAI, Y., KISHIMOTO, A., INOUE, M. and NISHIZUKA, Y. (1977)
Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. 1. Purification and characterisation of an active enzyme from bovine cerebellum. J. Biol. Chem. 252, 7603-7609.

TAKAI, Y., KISHIMOTO, A., IWASA, Y., KAWAHARA, Y., MORI, T. and NISHIZUKA, Y. (1979a)
Calcium-dependent activation of a multifunctional protein kinase by membrane phospholipids. J. Biol. Chem. 254, 3692-3695.

TAKAI, Y., KISHIMOTO, A., KIKKAWA, U., MORI, T. and NISHIZUKA, Y. (1979b)

Unsaturated diacylglycerol as a possible messenger for the activation of calcium-activated, phospholipid-dependent protein kinase system. Biochem. Biophys. Res. Commun. 91, 1218-1224.

TAMAOKI, T., NOMOTO, H., TAKAHASHI, I., KATO, Y., MORIMOTO, M. and TOMITA, F. (1986)

Staurosporine, a potent inhibitor of phospholipid/Ca^{2+}-dependent protein kinase. Biochem. Biophys. Res. Commun. 135, 397-402.

TAMURA, T., FRIIS, R.R. and BAUER, H. (1984)

$pp60^{c-src}$ is a substrate for phosphorylation when cells are stimulated to enter cycle. Febs. Letts. 177, 151-156.

TANAKA, C., FUJIWARA, H. and FUJII, Y. (1986)

Acetylcholine release from guinea pig caudate slices evoked by phorbol ester and calcium. Febs. Letts. 195, 129-134.

TANAKA, C., TANIYAMA, K. and KUSUNOKI, M. (1984)

A phorbol ester and A23187 act synergistically to release acetylcholine from the guinea pig ileum. Febs. Letts. 175, 165-169.

TEMPLE, S. and RAFF, M.C. (1986)

Clonal analysis of oligodendrocyte development in culture: evidence for a clock that counts cell divisions. Cell 44, 773-779.

TENNEKOON, G.I., KISHIMOTO, Y., SINGH, I., NONAKA, G. and BOURRE, J-M. (1980)

The differentiation of oligodendrocytes in the rat optic nerve. Dev. Biol. 79, 149-158.

TERRACIO, L., RONNSTRAND, L., TINGSTROM, A., RUBIN, K., CLAESSON-WELSH, L., FUNA, K. and HELDIN, C-H. (1987)
Induction of platelet-derived growth factor receptor expression in smooth muscle cells and fibroblasts upon tissue culturing. J. Cell. Biol. 107, 1947-1957.

THOMAS, T.P., GOPALAKRISHNA, R. and ANDERSON, W.B. (1987)
Hormone- and tumour promoter-induced activation or membrane association of PKC in intact cells. In: Methods in Enzymology, Volume 141, Eds. Conn, P.M. and Means, A.R. pp. 399-411. Academic Press Inc., London.

TIAMAGAWA, T., NIKI, H. and NIKI, A. (1985)
Insulin release independent of a rise in cytosolic free Ca^{2+} by forskolin and phorbol ester. Febs. Letts. 183, 430-432.

TRAN, P.L., CASTAGNA, M., SALA, M., VASSENT, G., HOROWITZ, A.D., SCHACHTER, D. and WEINSTEIN, I.B. (1983)
Differential effect of tumour promoters on phorbol ester receptor binding and membrane fluorescence anisotropy in C3H 10T1/2 cells. Eur. J. Biochem. 130, 155-160.

TRUNEH, A., ALBERT, F., GOLDSTEIN, P. and SCHMITT-VERHULST, A-M. (1985)
Early steps of lymphocyte activation bypassed by synergy between calcium ionophores and phorbol ester. Nature 313, 318-320.

TURNER, R.S., CHOU, C-H.J., KIBLER, R.F. and KUO, J.F. (1982)
Basic protein in brain myelin is phosphorylated by endogenous phospholipid-sensitive Ca^{2+}-dependent protein kinase. J. Neurochem. 39, 1397-1404.

TURNER, R.S., CHOU, C-H.J., MAZZEI, G.J., DEMBURE, P. and KUO, J.F. (1984)
Phospholipid-sensitive Ca^{2+}-dependent protein kinase preferentially phosphorylates serine-115 of bovine myelin basic protein. J. Neurochem. 43, 1257-1264.

TURNER, R.S. and KUO, J.F (1986)
Phospholipid-sensitive Ca^{2+}-dependent protein kinase (PKC): the enzyme, substrates and regulation. In: Phospholipids and Cellular Regulation. Ed. Kuo, J.F. pp. 75-110. Academic Press Inc., London.

URATSUJI, Y., NAKANISHI, H., TAKEYAMA, Y., KISHIMOTO, A. and NISHIZUKA, Y. (1985)
Activation of cellular PKC and mode of inhibitory action of phospholipid-interactory compounds. Biochem. Biophys. Res. Commun. 130, 654-661.

VACCARINO, F., GUIDOTTI, A. and COSTA, E. (1987)
Ganglioside inhibition of glutamate-mediated PKC translocation in primary cultures of cerebellar neurones. Proc. Natl. Acad. Sci. USA. 84, 8707-8711.

VALLEJO, M., JACKSON, T., LIGHTMAN, S. and HANLEY, M.R. (1987)
Occurrence and extracellular actions of inositol pentakis- and hexakisphosphate in mammalian brain. Nature 330, 656-658.

van de WERVE, G., PROIETTO, J. and JEANRENAUD, B. (1985)
Tumour-promoting phorbol esters increase basal and inhibit insulin-stimulated lipogenesis in rat adipocytes without decreasing insulin binding. Biochem. J. 225, 523-527.

VARTANIAN, T., SPRINKLE, T.J., DAWSON, G. and SZUCHET, S. (1988)
Oligodendrocyte substratum adhesion modulates expression of adenylate cyclase-linked receptors. Proc. Natl. Acad. Sci. USA 85, 939-943.

VARTANIAN, T., SZUCHET, S., DAWSON, G. and CAMPAGNONI, A.T. (1986)
Oligodendrocyte adhesion activates protein kinase C-mediated phosphorylation of myelin basic protein. Science 234, 1395-1398.

WALKER, A.G., CHAPMAN, J.A., BRUCE, C.B. and RUMSBY, M.G. (1985)

Immunocytochemical characterisation of cell cultures grown from dissociated 1-2 - day postnatal rat cerebral tissue - a developmental study. J. Neuroimmunol. *I*, 1-20.

WALTER, U. (1989)

Molecular aspects of cyclic nucleotide-dependent protein kinases and their function in gene expression. In: Abstracts of NATO conference on "Cellular and Molecular Biology of Myelination", September 1989, Osnabruck, FRG, Plenum Press, New York. In press.

WARRINGA, R.A.J., HOEBEN, R.C., KOPER, J.W., SYKES, J.E.C., van GOLDE, L.M.G. and LOPES-CARDOZO, M. (1987)

Hydrocortisone stimulates the development of oligodendrocytes in primary glial cultures and affects glucose metabolism and lipid synthesis in these cultures. Dev. Brain Res. 34, 79-86.

WEINBERG, R.A. (1989)

Positive and negative controls on cell growth. Biochemistry 28, 8263-8269.

WERTH, D.K., NIEDEL, J.E. and PASTAN, I. (1983)

Vinculin, a cytoskeletal substrate of protein kinase C. J. Biol. 258, 11423-11426.

WHITING, J.A. and BARITT, G.J. (1982)

On the mechanism by which hormones induce the release of Ca^{2+} from mitochondria in the liver cell. Biochem. J. 206, 121-129.

WIGHTMAN, P.D. and RAETZ, C.R.H. (1984)

The activation of protein kinase C by biologically active lipid moieties of lipopolysaccharide. J. Biol. Chem. 259, 10048-10052.

WILLIAMS, L.T., ESCOBEDO, J.A., KEATING, M.T. and COUGHLIN, S.R. (1989)
Signal transduction by the platelet-derived growth factor receptor. In: Cold Spring Harbor Symposium on Quantitative Biology. Volume LIII. Molecular Biology of Signal Transduction. pp.455-466. Cold Spring Harbor, New York.

WILLIAMS, L.T. (1989)
Signal transduction by the platelet-derived growth factor receptor. Science 243, 1564-1570.

WILLIAMSON, J.R. (1985)
Role of inositol lipid breakdown in the generation of intracellular signals. State of the Art Lecture. Blood Pressure Council. Suppl. II: Hypertension, 8, 140-156.

WINDEBANK, A.J. (1986)
Specific inhibition of myelination by lead *in vitro*: comparison with Arsenic, Thallium and Mercury. Exp.Neurol. 94, 203-212.

WISE, B.C., RAYNOR, R.L. and KUO, J.F. (1982)
Phospholipid-sensitive Ca^{2+}-dependent protein kinase from heart. I. Purification and general properties. J. Biol. Chem. 257, 8481-8488.

WITTERS, L.A. and BLACKSHEAR, P.J. (1987)
Protein kinase C-mediated phosphorylation in intact cells. In: Methods in Enzymology, Volume 141, Eds. Conn, P.M. and Means, A.R. pp. 412-424. Academic Press Inc., London.

WITTERS, L.A., VATER, C.A. and LIENHARD, G.E. (1985)
Phosphorylation of the glucose transporter *in vitro* and *in vivo* by protein kinase C. Nature 315, 777-778.

WOLF, M., SAHYOUN, N., LEVINE, H.III. and CUATRECASAS, P. (1984)
PKC: rapid enzyme purification and substrate-dependence of the DAG effect. Biochem. Biophys. Res. Commun. 122, 1268-1275.

WOLFMAN, A. and MACARA, I.G. (1987)

Elevated levels of diacylglycerol and decreased phorbol ester sensitivity in ras-transformed fibroblasts. Nature 325, 359-361.

WOLFMAN, A., WINGROVE, T.G., BLACKSHEAR, P.J. and MACARA, I.G. (1987)

Downregulation of protein kinase C and of an endogenous 80kDa substrate in transformed fibroblasts. J. Biol. Chem. 262, 16546-16552.

WOLSWIJK, G. and NOBLE, M. (1989)

Identification of an adult-specific glial progenitor cell. Development 105, 387-400.

WOODGETT, J.R., HUNTER, T. and GOULD, K.L. (1987)

Protein kinase C and its role in cell growth. In: Cell Membranes, Methods and Reviews, Volume 3, pp.215-340 . Eds. Elson, E., Frazier, W. and Glaser, L. Plenum Press, New York.

WOOGE, C.H. and CONN, P.M. (1987)

Measurement of synergistic effects of PKC activators and Ca^{2+} ionophore in pituitary cell cultures. In: Methods in Enzymology Volume 141, 429-435. Eds. Conn, P.M. and Means, A.R. Academic Press Inc., London.

WOOTEN, M.W. and WRENN, R.W. (1988)

Linoleic acid is a potent activator of PKC type III-α isoform in pancreatic acinar cells; its role in amylase secretion. Biochem. Biophys. Res. Commun. 153, 67-73.

YAMADA, K. and KANOH, H. (1988)

Occurrence of immunoreactive 80kDa and non-immunoreactive diacylglycerol kinases in different pig tissues. Biochem. J. 255, 601-608.

YARDEN, Y., ESCOBEDO, J.A., KUANG, W-J, YANG-FENG,, T.L., DANIEL, T.O., TREMBLE, P.M., CHEN, E.Y., ANDO, M.E., HARKINS, R.N., FRANCKE, U., FRIED, V.A., ULLRICH, A. and WILLIAMS, L.T. (1986) Structure of the receptor for platelet-derived growth factor helps define a family of closely related growth factor receptors. Nature 323, 226-232.

YONG, V.W., SEKIGUCHI, S., KIM, M.W. and KIM, S.U. (1988) Phorbol ester enhances morphological differentiation of oligodendrocytes in culture. J.Neurosci.Res. 19, 187-194.

YOSHIDA, Y., HUANG, F.L. NAKABAYASHI, H. and HUANG, K-P. (1988) Tissue distribution and developmental expression of protein kinase C isozymes. J. Biol. Chem. 263, 9868-9873.

ZAWALICH, W., BROWN, C. and RASMUSSEN, H. (1983) Insulin secretion: combined effects of phorbol esters and A23187. Biochem. Biophys. Res. Commun. 117, 448-455.

ZURGIL, N. and ZISAPEL, N. Phorbol ester and calcium act synergistically to enhance neurotransmitter release by brain neurons in culture. Febs. Letts. 185, 257-261.

DIFFERENTIATION AND FUNCTIONS OF GLIAL CELLS

Proceedings of a Satellite Meeting of the International Society for Neurochemistry Held in Rome, Italy, April 19-21, 1989

Editor

Giulio Levi

Neurobiology Section
Laboratory of Pathophysiology
Istituto Superiore di Sanità
Rome, Italy

WILEY-LISS, NEW YORK

Address all Inquiries to the Publisher
Alan R. Liss, Inc., 41 East 11th Street, New York, NY 10003

Copyright © 1990 Alan R. Liss, Inc.

Printed in United States of America

Under the conditions stated below the owner of copyright for this book hereby grants permission to users to make photocopy reproductions of any part or all of its contents for personal or internal organizational use, or for personal or internal use of specific clients. This consent is given on the condition that the copier pay the stated per-copy fee through the Copyright Clearance Center, Incorporated, 27 Congress Street, Salem, MA 01970, as listed in the most current issue of "Permissions to Photocopy" (Publisher's Fee List, distributed by CCC, Inc.), for copying beyond that permitted by sections 107 or 108 of the US Copyright Law. This consent does not extend to other kinds of copying, such as copying for general distribution, for advertising or promotional purposes, for creating new collective works, or for resale.

Library of Congress Cataloging-in-Publication Data

Differentiation and functions of glial cells : proceedings of a satellite meeting of the International Society for Neurochemistry held in Rome, Italy, April 19-21, 1989 / editor, Giulio Levi.
p. cm. -- (Neurology and neurobiology ; v. 55)
Proceedings of the Symposium on "Differentiation and Functions of Glial Cells," a satellite of the 12th Meeting of the International Society for Neurochemistry, held at the Istituto Superiore di Sanità in Rome on April 19-21, 1989.
Includes bibliographical references.
ISBN 0-471-56701-9.
1. Neroglia--Differentiation--Congresses. 2. Neuroglia--Physiology--Congresses. I. Levi, Giulio, 1937- . II. Symposium on "Differentiation and Functions of Glial Cells" (1989 : Istituto Superiore di Sanità) III. International Society of Neurochemistry. Meeting (12th : 1989 : Albufeira, Portugal) IV. Series.
[DNLM: 1. Cell Differentiation--congresses. 2. Neuroglia--pathology--congresses. 3. Neuroglia--physiology--congresses. W1 NE337B v. 55 / WL 102 D569 1989]
QP363.2.D54 1989
599'.0188--dc20
DNLM/DLC
for Library of Congress 89-24865
CIP

PROTEIN KINASE C IN PRIMARY GLIAL CELL CULTURES AND SUB-CULTURES: 80kDa PROTEIN PHOSPHORYLATION, THE EFFECT OF PLATELET-DERIVED GROWTH FACTOR AND THE TURNOVER OF PHOSPHATIDYLCHOLINE.

Martin G. Rumsby, John A. Murphy, Rebecca Sayner, Shaun McNulty and Anthony J. Suckling

Department of Biology, University of York, York, YO1 5DD, England.

INTRODUCTION

Signal transduction events linked through the phosphoinositide (PI) pathway (Berridge, 1984) induce the activation of protein kinase C (PkC). This kinase requires calcium ions and phosphatidylserine for activity (Takai et al.,1979) and is associated with the control of key cell events such as growth, differentiation and secretion (Nishizuka, 1986). Diacylglycerol formed during stimulation of the PI pathway increases the affinity of PkC for calcium ions resulting in the activation of the kinase at physiological calcium concentrations (Takai et al., 1979). A number of PkC subspecies having different characteristics have now been identified (Nishizuka, 1988). PkC can be activated independently of the PI pathway by the tumour-promoting phorbol esters such as 12-O-tetradecanoylphorbol-13-acetate (TPA) which act as structural analogues of diacylglycerol (Castagna et al.,1982). In certain cell lines activation of PkC stimulates the phosphorylation of an 80kDa acidic polypeptide of unknown function (Blackshear et al.,1986; Rozengurt et al.,1983) which, in fibroblasts at least, is different from autophosphorylated PkC (Blackshear et al.,1986). PkC is most active in the CNS where its role in neurones has been widely examined (Kikkawa et al.,1986; Miller,1986). Less information is available on the activity and role of PkC in glia though the PI pathway is active in such cells (e.g. Pearce et al.,1986; Ritchie et al.,1987) and the kinase has been localised in cells resembling oligodendrocytes by immunocytochemical methods (Girard et al.,1985).

Certain mitogens such as platelet-derived growth factor (PDGF) which stimulate the PI pathway (Blackshear et al., 1985) will enhance PkC activity. PDGF from Type 1 astrocytes is now known to play a major role in glial cell development (Raff, 1989); some of the effects of this growth factor on glia may therefore be mediated by activation of PkC. PkC may also be involved in the control of lipid turnover in glia since phorbol esters such as TPA, which activate the kinase, stimulate fatty acid synthesis in hepatocytes (Vaartjes and de Haas, 1985) and phospholipid turnover in a range of cultured cells (Pelech et al.,1984). Here we summarise our data on PkC in primary glial cell cultures and subcultures derived from rat brain cerebral tissue.

ACTIVITY OF PkC IN GLIAL CELLS

To establish the presence of PkC in the glial compartment of the CNS we have taken the direct approach of measuring the activity of the enzyme in 100,000g cytosolic and particulate fractions prepared from primary glial cultures and subcultures at different stages of development. Methods for the preparation of our glial cultures derived from cerebra of 1-2 day rat pups are based on the original work of McCarthy and de Vellis (1980) and have been described elsewhere (Walker et al., 1985i,1985ii). For assay of PkC, cell cultures were rinsed well and then cells scraped out and homogenised in 50mM Tris-HCl, pH 7.5 containing 5mM dithiothreitol and 2mM EGTA. Centrifugation at 100,000g for 60 minutes at 4°C gave cytosolic and particulate fractions for assay. PkC activity was measured by following the transfer of the γ - group of [32P]ATP to Type IIIS histone(Sigma) for 10 minutes at 30°C with shaking, using the method described by Kikkawa et al.(1983). Ca^{2+}-independent kinase activity was measured in the presence of 100μM EGTA omitting calcium chloride and lipid from the system, Ca^{2+} - dependent kinase activity measured with calcium chloride present omitting EGTA and lipid and PkC activity measured with calcium chloride and lipid present but omitting EGTA. All assays were in quadruplicate and reactions were stopped by the addition of ice-cold 25% TCA. After standing on ice TCA-precipitable material was recovered on glass-fibre filters which were dried prior to scintillation counting. We have expressed kinase activities (mean +/- S.D.) as pmol 32P incorporated/10 min/mg protein at 30°C.

As we have reported (Murphy et al.,1988) and show in Fig. 1, basal calcium-independent kinase activity and also calcium-

dependent and calcium + phospholipid-dependent kinase activities are all detected in cytosolic fractions prepared from the same batch of primary glial cultures at two stages of growth after preparation. Primary cultures at 7 days of growth consist of a layer of Type 1 astrocytes on which are growing progenitor glial cells and a smaller number of Type 2 astrocytes and oligodendrocytes. By 12 days after preparation the proportion of these smaller process-bearing cells has increased and clumps of these cells are beginning to form on the Type 1 astrocyte layer. As the data show, the specific activities of PkC corrected for basal calcium-independent kinase activity are 100 and 243 pmols 32P incorporated/10 min/mg protein respectively. Though the data suggest that PkC activity increases with development of the cultures confirmation of this relationship is difficult because of variation between different culture preparations. PkC activity can also be measured in 100,000g cytosolic fractions from subcultures of glial cells lacking Type 1 astrocytes (Murphy et al.,1988).

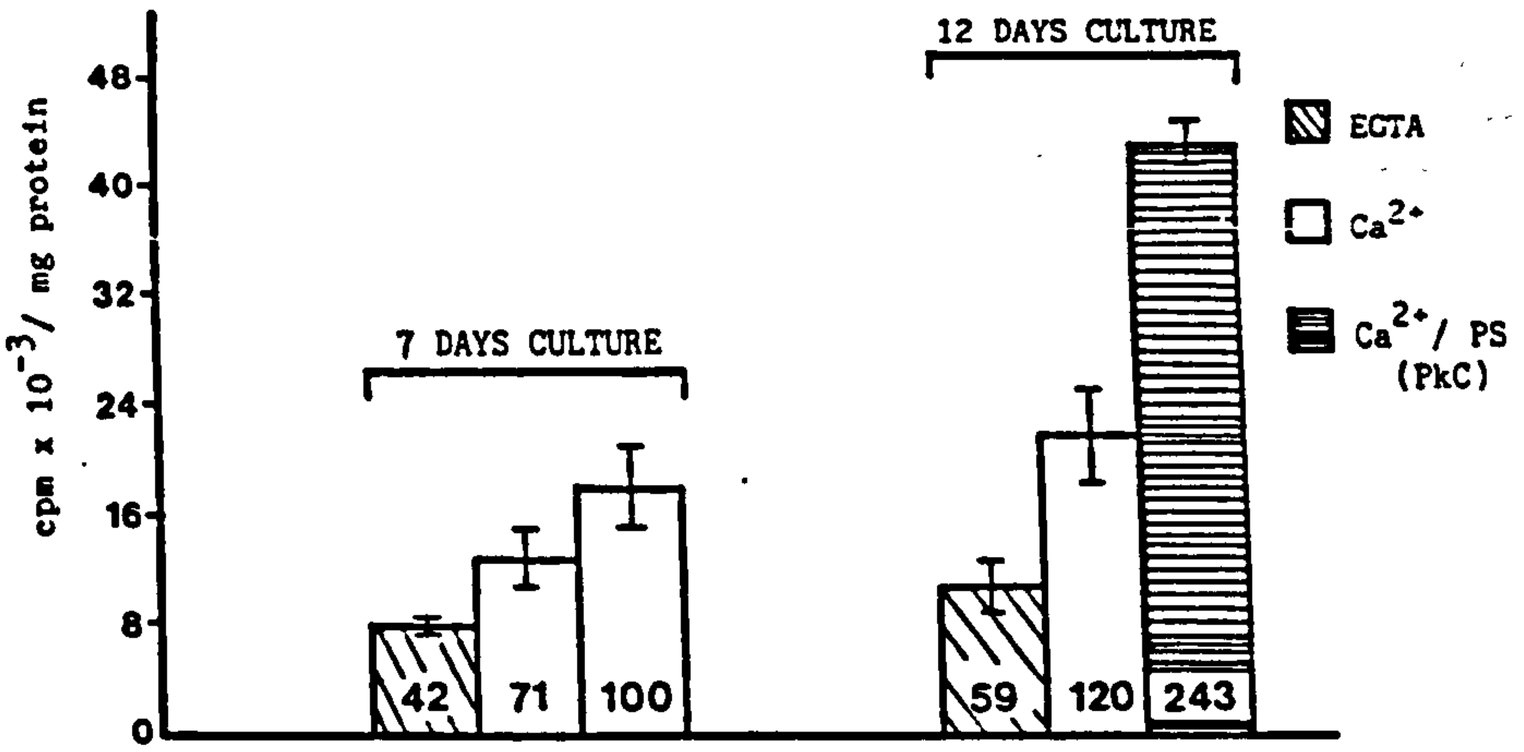


Fig. 1. Protein kinase C activity in primary glial cells at two ages in culture. Kinase activity is expressed as counts/mg protein on the vertical axis. Numbers inside bars are specific activity; calcium-dependent and calcium + phospholipid-dependent activities are corrected for basal calcium-independent kinase activity. Bar lines are means +/- S.D.(n=4).

Direct measurement of PkC activity in crude 100,000g cytosolic fractions from cell homogenates is complicated by the presence of certain endogenous inhibitors such as phosphatases (Sahyoun et al.,1983) and contaminating phospholipid (Kikkawa et al.,1983). It is better to assay cytosolic fractions

purified by DE-52 anion exchange column chromatography according to the method of Kikkawa et al.(1983) to remove inhibitors. Glial cytosolic fractions purified by this method show much higher specific activities compared with unfractionated cytosolic preparations. For example, PkC specific activities in DE-52 purified 100,000g glial cytosolic fractions are between 5-20 times higher than the level measured in non-purified samples. The role played by the various endogenous inhibitors of PkC in the control of PkC-linked pathways has not been defined.

As shown in table 1 PkC in cytosolic fractions from glia is activated most effectively by phosphatidylserine in the presence of calcium ions and diacylglycerol in keeping with earlier observations (Takai et al.,1979). Other phospholipids we have tested are less effective at activating glial PkC. The γ subspecies of PkC can be activated by arachidonic acid in the absence of phosphatidylserine and diacylglycerol (Naor et al.,1988) while α and β subspecies require higher arachidonate concentrations with calcium. As shown in Table 1 the PkC activity associated with glia is only slightly activated by arachidonate at a concentration of 40µg/ml (130µM). We are presently defining the arachidonate specificity of glial PkC to gain some indication of which subspecies are present in glial cells. Hydroxyapatite column chromatography has also been used to resolve PkC subspecies (Huang et al., 1986). Saito et al.(1989) have reported recently using immunocytochemical methods that the β_{II} subspecies is not present in glia.

Table 1. In vitro effects of different lipids on the activation of PkC in DE-52 purified glial cell cytosolic fractions. Lipids were all at 40µg/ml. Results are expressed against phosphatidylserine* as 100% in the presence of sn-1,2 diolein (0.8µg/ml) and calcium (100µM)

phosphatidylserine	100
phosphatidylcholine	16
phosphatidylinositol	46
phosphatidylethanolamine	35
sphingomyelin	19
arachidonic acid	11

* specific activity:1580pmoles 32P incorp/10 min/mg prot.

Primary glial cultures grown in defined medium lacking foetal calf serum in place of the usual DMEM + 10% FCS give markedly higher PkC specific activities in 100,000g cytosolic fractions (Murphy, J.A. & Rumsby, M.G., unpublished). Such culture methods should increase the numbers of galacto-cerebroside-positive cells present (Espinosa de los Monteros et al.,1988) and the result suggests that PkC is active in oligodendrocytes. When primary glial cultures are shaken (McCarthy and de Vellis, 1980) to remove top layer cells for subcultures we can measure PkC activity in both the residual Type 1 astrocytes and in the shaken-off cells which are largely O-2A progenitor cells, Type 2 astrocytes and oligodendrocytes. Our data indicates that PkC is active in all classes of glial cells. This activity is not confined to the cytosol since Triton X-100 extracts of membrane pellets from glial cultures show a higher PkC specific activity than that in the cytosol (Murphy et al.,1988). This is in keeping with other observations that in the CNS active PkC is detected in two localisations in cells - cytosolic and membrane-associated (Kikkawa et al., 1982). PkC may also have a nuclear localisation (Masmoudi et al.,1989) but we have not examined this possibility in glia yet.

80kDa POLYPEPTIDE PHOSPHORYLATION

PkC activation by growth factors through the PI path-way, or directly by phorbol esters, in a variety of cell lines results in enhanced protein phosphorylation, especially of an 80kDa component (Witters and Blackshear, 1987). We have found the same effect in glial cell cultures and subcultures. When glial cells, preincubated with 32P-orthophosphate to label ATP pools, are treated with 25nM TPA for 15 minutes and poly-peptides then recovered and separated by gradient SDS-PAGE a clear stimulation of phosphorylation associated with an 80kDa component is observed (Rumsby et al.,1988). The effect is concentration dependent from 1nM to 100nM TPA, 0.1nM TPA being without effect. TPA concentrations above 100nM decrease the phosphorylation effect perhaps because of some distortion of membrane structure by the phorbol ester. Controls with a non-active phorbol ester,such as 4α phorbol, do not show the phosphorylation effect. There is a 2-3 fold stimulation of 80kDa polypeptide phosphorylation by TPA activation of PkC. Maximum phosphorylation of the 80kDa component in primary glial cultures was after 15 minutes treatment with TPA; sub-cultures showed faster phosphorylation of the same 80kDa band the effect being detected after even 1 minute and maximal after

5 minutes. Blackshear et al.(1986) have shown in fibroblasts that the phosphorylated 80kDa polypeptide is distinct from autophosphorylated PkC. This is being examined in our glial system where we have found that the 80kDa component is in the cytosol. In platelets a 40kDa polypeptide is phosphorylated by TPA stimulation of PkC (Witters and Blackshear, 1987); no such polypeptide is detected in the glial system. In fibroblasts PDGF stimulates phosphorylation of an 80kDa polypeptide (Blackshear et al.,1985). Since PDGF has a role in glial differentiation we might therefore expect this growth factor to act on glial cell primary cultures and subcultures to enhance 80kDa polypeptide phosphorylation. Preliminary results with recombinant PDGF-BB suggest that some stimulation of 80kDa phosphorylation occurs. However, the effect is not as marked as is seen with TPA. PDGF receptors on glia are mainly specific for PDGF-AA (Heldin and Westermark, 1989) and thus the weak phosphorylation response we observe with PDGF-BB may be because the less specific growth factor is being used. This line of investigation is being studied further to define the biochemical pathways involved in PDGF effects on glia. The role of the cytoplasmic 80kDa polypeptide which is phosphorylated in response to PkC activation is being characterised at present.

PROTEIN KINASE C ENHANCEMENT OF PHOSPHATIDYLCHOLINE TURNOVER

We have noted that when PkC in glial cells is activated with TPA there is a marked stimulation of phospholipid turnover (Rumsby et al.,1988), especially in relation to phosphatidylcholine (PC). The effect of TPA on PC turnover in a variety of cell lines has been widely studied (reviewed by Pelech and Vance, 1984) while the possible involvement of PC cycles in signal transduction processes has been summarised (Pelech and Vance, 1989). It is not surprising, therefore, that PC turnover in primary glial cell cultures is increased when PkC is activated with TPA. Enhanced PC turnover due to PkC activation by TPA or growth factors is accompanied by a release to the extracellular medium of choline and phosphorylcholine (e.g. Besterman et al.,1986; Kolesnick and Paley, 1987; Mufson et al.,1981; Muir and Murray, 1987). We have now made similar observations with BHK cells,C6 glioma cells and with our primary glial cell cultures and subcultures. Glial cells grown in six-well multiplates are rinsed and then incubated with fresh medium (DMEM + 0.1% FCS) with 5 µCi 3H choline for 24 hours to label choline-containing lipids. Culture medium is then removed and the cells rinsed three

times with fresh medium to remove extracellular labelled choline. Fresh medium is added together with agonist or TPA. Aliquots of the medium are then removed at various times, centrifuged to sediment any membrane contamination and radioactivity released to the medium monitored. Release of labelled choline metabolites to the extracellular medium is expressed as % above control with no additions. The effect of an inactive phorbol ester such as 4 ∝ phorbol is always studied.

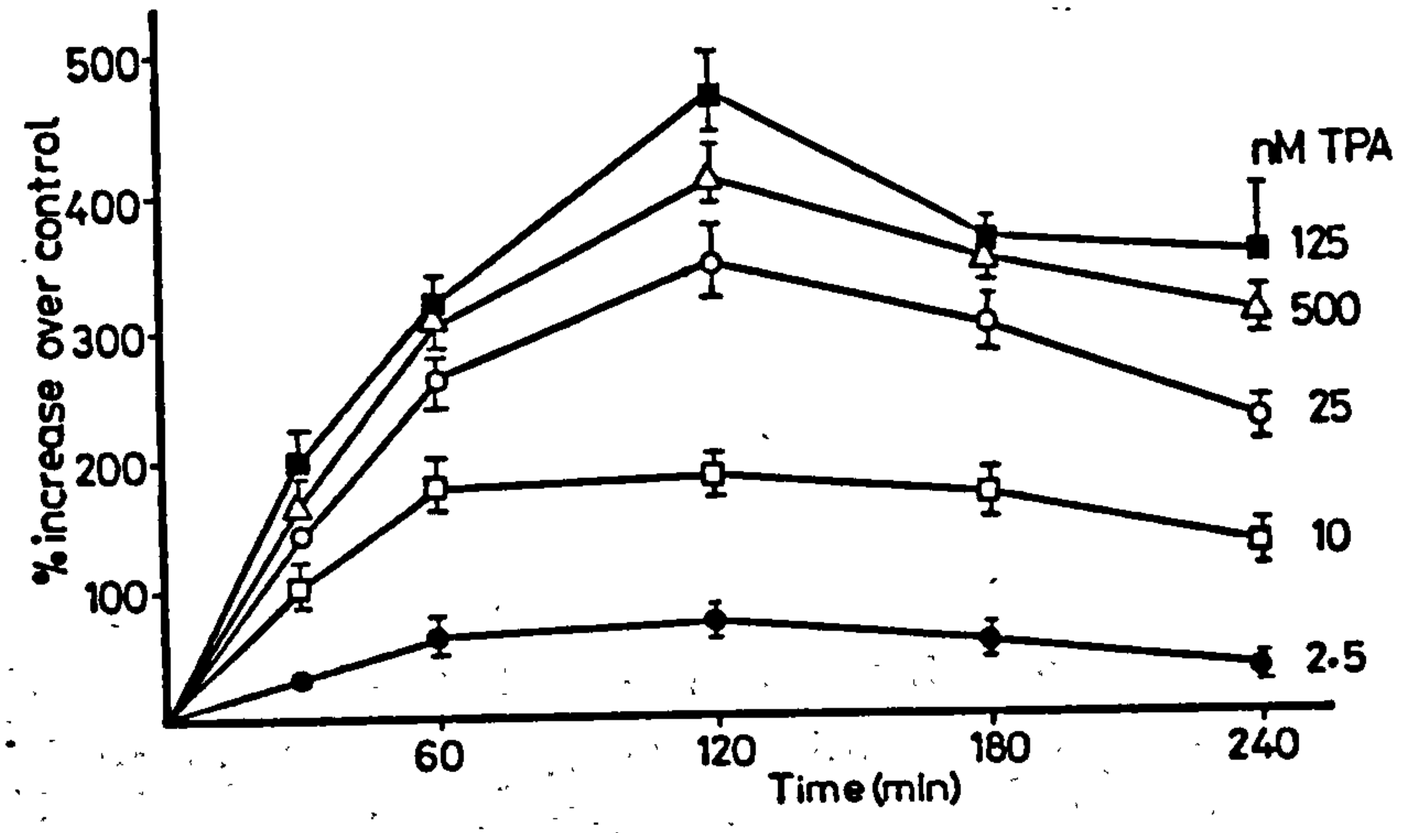


Fig. 2. Effect of TPA on the release of choline metabolites from C6 cells to the extracellular medium.Error bars show S.D. where data are in triplicate from three separate cultures of cells.

The data in Fig. 2 indicate that when TPA at different concentrations is added to the C6 glioma cell line there is a hydrolysis of labelled phosphatidylcholine and a release to the extracellular medium of choline metabolites above control levels. The effect is not detected with 0.1nM TPA and is maximal at about 125nM; concentrations of TPA above this figure are slightly inhibitory. For all TPA concentrations the maximal effect is seen at 120 minutes after stimulation. Besterman et al.(1986) noted that both choline and phosphoryl-choline appear in the extracellular medium. We have not yet made these measurements with glial cells. As shown in Fig.3 TPA elicits the same release of choline metabolites to the extracellular medium from primary glial cells and from sub-cultures lacking Type 1 astrocytes. The TPA effect seen in Fig. 3 is not as marked as that observed with C6 cells (Fig.

inhibited by staurosporine suggesting that PkC controls the turnover process. It has to be noted, however, that staurosporine is not a specific inhibitor of PkC; it inhibits the activity of PkA as well (Woodgett et al.,1987).

SUMMARY

Our results of direct measurement of protein kinase C activity reveal that this key enzyme is present in glial cells in primary culture and in subculture. It is not yet clear which subspecies of PkC is/are present in different glial cell classes. PkC activity is both cytosolic and membrane-associated; an inhibitor present in normal cytosol can be removed by purification of crude cytosolic fractions by DE-52 anion exchange chromatography to achieve maximal PkC activity. Astrocytes and oligodendrocytes both have PkC activity. PkC stimulation results in the phosphorylation of an 80kDa polypeptide in the cytosol of glial cells. The identity of this polypeptide is not yet known. Platelet-derived growth factor, through its stimulation of PkC, may also enhance the phosphorylation of the same 80kDa polypeptide. Activation of PkC in glia enhances phosphatidylcholine turnover leading to a release into the medium of choline metabolites. A phospholipase D activated by PkC may be responsible for the observed phosphatidylcholine turnover.

ACKNOWLEDGEMENTS

We thank The Wellcome Trust (JAM), the Science and Engineering Research Council (SM) and the Multiple Sclerosis Research Fund Ltd. for financial support. Clare Harris contributed to some of the work mentioned above as part of her final year undergraduate project.

REFERENCES

Berridge, M J (1984) Inositol triphosphate and diacylglycerol as second messengers. Biochem J 220: 345-360.

Besterman JM, Duronio V, Cuatrecasas P (1986). Rapid formation of diacylglycerol from phosphatidylcholine:a pathway for the generation of a second messenger. Proc Natl Acad Sci USA 83: 6785-6789.

Bijleveld C, Geelen MJH, Houweling M, Vaartjes WJ (1988). Dissimilar effects of 1-oleoyl-2-acetylglycerol and phorbol 12-myristate 13-acetate on fatty acid synthesis in isolated rat-liver cells. Biochem Biophys Res Comm 151: 193-200.

2); in C6 cells 25nM TPA has over twice the effect on choline metabolite release as is observed with primary cultures. The effect of TPA on subcultures is lower still. Our preliminary observations with 1-oleoyl-2-acetyl-sn-glycerol (OAG) shown in Fig. 3 suggest that this synthetic analogue decreases PC turnover slightly compared with the stimulatory effect of TPA. OAG and other short-chain diacylglycerols such as diC6 and diC8 all activate PkC (Davis et al.,1985; Kaibuchi et al., 1983). However, several authors have noted that these more water-soluble diacylglycerols have different effects from TPA on the turnover of PC (e.g. Muir and Murray, 1986; Kolesnick and Paley, 1987). Bijleveld et al.(1988) have suggested that OAG is metabolised by cells via a deacylation pathway in which the oleoyl-CoA formed may inhibit PC turnover.

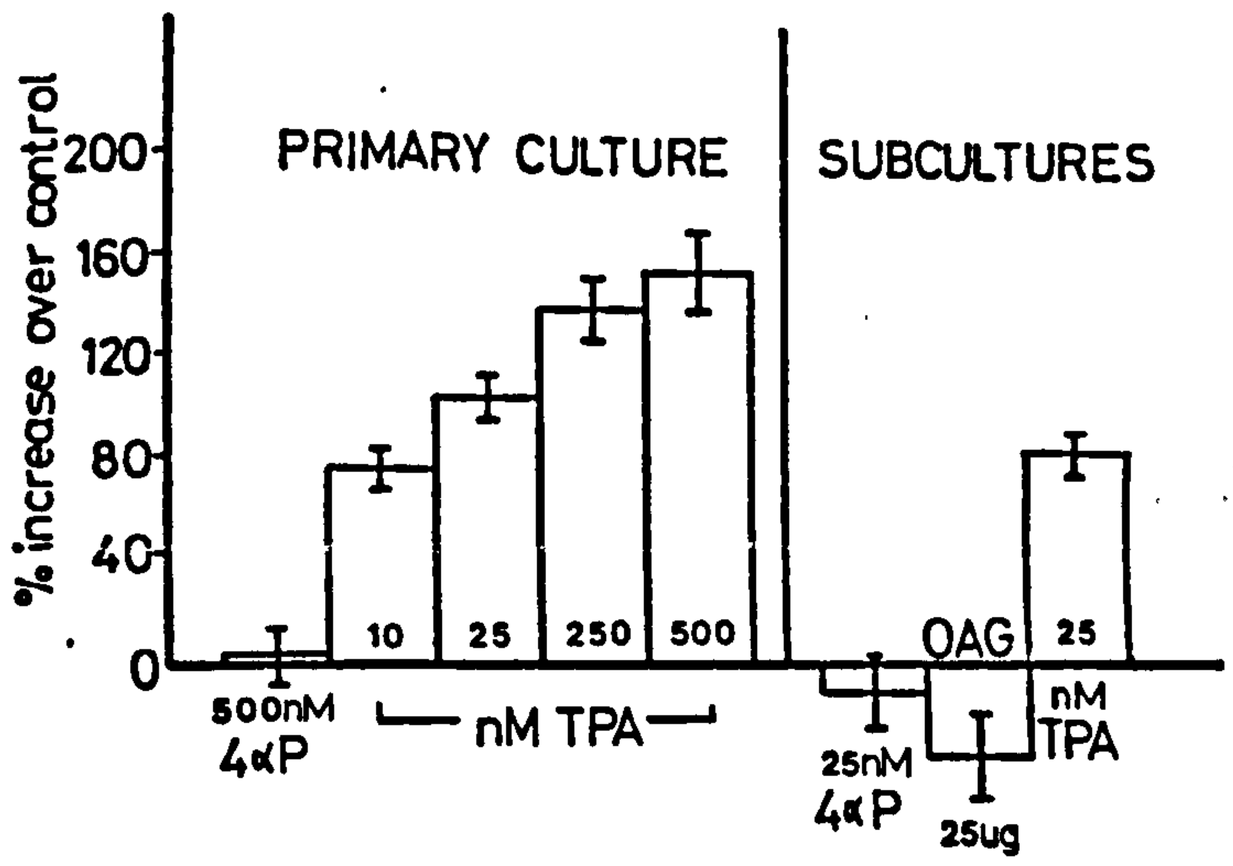


Fig. 3. Choline metabolite release after 120 minutes from primary glial cells and subcultures treated with TPA and a synthetic diacylglycerol. Error bars show S.D. where data are in triplicate from three separate cultures of cells.

These studies show that glial cells in primary culture respond similarly to several cell lines in the way TPA stimulation of PkC causes turnover of PC,and a release to the cell and to the extracellular medium of choline metabolites. Whether this is due to the activation of a PC-specific phospholipase C or D in the glial cells is not yet known but in REF52 cells Cabot et al.(1988) found phospholipase D to be responsible for the PC turnover. The same workers have found more recently (Cabot et al.,1989) that the TPA-activated phospholipase D pathway causing PC turnover is almost fully

Blackshear PJ, Witters LA, Girard PR, Kuo JF, Quamo SN (1985). Growth factor-stimulated protein phosphorylation in 3T3-L1 cells. J Biol Chem 260: 13304-13315.

Blackshear PJ, Wen L, Glynn BP, Witters, LA (1986). Protein kinase C-stimulated phosphorylation in vitro of a Mr80,000 protein phosphorylated in response to phorbol esters and growth factors in intact fibroblasts. J Biol Chem 261: 1459-1469.

Cabot MC, Welsh CJ, Cao H-t, Chabbott H (1988).The phosphatidylcholine pathway of diacylglycerol formation stimulated by phorbol diesters occurs via phospholipase D activation. FEBS Lett 233: 153-157.

Cabot MC, Welsh CJ, Zhang Z-c, Cao H-t (1989). Evidence for a protein kinase C-directed mechanism in the phorbol diester-induced phospholipase D pathway of diacylglycerol generation from phosphatidylcholine. FEBS Lett 245: 85-90.

Castagna M, Takai Y, Kaibuchi K, Sano K, Kikkawa U, Nishizuka Y (1982). Direct activation of calcium-activated phospholipid-dependent protein kinase by tumour-promoting phorbol esters. J Biol Chem 257: 7847-7851.

Davis RJ, Ganong BR, Bell RM, Czech MP (1985). sn-1,2-Dioctanoylglycerol. A cell-permeable diacylglycerol that mimics phorbol diester action on the epidermal growth factor receptor and mitogenesis. J Biol Chem 260: 1562-1566.

Espinosa de los Monteros A, Roussel G, Neskovic NM, Nussbaum JL (1988). A chemically defined medium for the culture of mature oligodendrocytes. J Neurosci Res 19: 202-211.

Girard PR, Mazzei GJ, Wood JG,Kuo JF (1985). Polyclonal antibodies to phospholipid/Ca^{2+}-dependent protein kinase and immunocytochemical localisation of the enzyme in rat brain. Proc natl Acad Sci USA 82: 3030-3034.

Heldin C-H, Westermark B (1989). Platelet-derived growth factor: three isoforms and two receptor types. Trends Genet. 5: 108-111.

Huang KP, Nakabayashi H, Huang FL (1986). Isozymic forms of rat brain Ca^{2+}-activated and phospholipid-dependent protein kinase. Proc Natl Acad Sci USA 83: 8535-8539.

Kaibuchi K, Takai Y, Sawamura M, Hoshijima M, Fujikura T, Nishizuka Y (1983). Synergistic functions of protein phosphorylation and calcium mobilisation in platelet activation. J Biol Chem 258: 6701-6704.

Kikkawa U, Takai Y, Minakuchi R,Inohara S, Nishizuka Y (1982). Calcium-activated, phospholipid-dependent protein kinase from rat brain:subcellular distribution, purification and properties. J Biol Chem 257: 13341-13348.

Kikkawa U, Minakuchi R, Takai Y, Nishizuka Y (1983). Calcium-

activated, phospholipid-dependent protein kinase (protein kinase C) from rat brain. In Corbin JD, Hardman JG (eds): "Methods in Enzymology," New York: Academic, 99 pp288-298.

Kikkawa U, Kitano T, Saito N, Fujiwara H, Nakanishi H, Kishimoto A, Taniyama K, Tanaka C, Nishizuka Y (1986). Possible roles of protein kinase C in signal transduction in nervous tissues. Prog Brain Res 69: 29-35.

Kolesnick RN , Paley AE (1987). 1,2-Diacylglycerols and phorbol esters stimulate phosphatidylcholine metabolism in GH_3 pituitary cells. J Biol Chem 262: 9204-9210.

Masmoudi A, Labourette G, Mersel M, Huang FL, Huang K-P, Vincendon G, Malviya AN (1989). Protein kinase C in rat liver nuclei: partial purification and biochemical and immunochemical characterisation. J Biol Chem 264: 1172-1179.

McCarthy KD, de Vellis J (1980). Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. J Cell Biol 85: 890-902.

Miller RJ (1986) Protein kinase C: a key regulator of neuronal excitability? Trends Neurosci 9:538-541.

Murphy JA, Chapman JA, Suckling AJ, Rumsby MG (1988). Protein kinase C activity in soluble fractions from glial cells in primary culture and subcultures. Neurosci Lett 85: 255-260.

Mufson RA, Okin E, Weinstein IB (1981) Phorbol esters stimulate the rapid release of choline from prelabelled cells. Carcinogenesis 2: 1095-1102.

Muir JG, Murray AW (1986). Mimicry of phorbol ester responses by diacylglycerols. Differential effects on phosphatidylcholine biosynthesis, cell-cell communication and epidermal growth factor binding. Biochim Biophys Acta 885: 176-184.

Muir JG, Murray AW (1987). Bombesin and phorbol ester stimulate phosphatidylcholine hydrolysis by phospholipase C: evidence for a role of protein kinase C. J Cell Physiol 130: 382-391.

Naor Z, Shearman MS, Kishimoto A, Nishizuka Y (1988). Calcium-independent activation of hypothalamic Type 1 protein kinase C by unsaturated fatty acids. Mol Endocrinol 2: 1043-1048.

Nishizuka Y (1986). Studies and perspectives of protein kinase C. Science 233: 305-312.

Nishizuka Y (1988). The molecular heterogeneity of protein kinase C and its implications for cellular regulation. Nature 334: 661-665.

Pearce B, Morrow C, Murphy S (1986). Receptor-mediated phospholipid hydrolysis in astrocytes. Eur J Pharmacol 121: 231-243.

Pelech SL, Paddon HB, Vance DE (1984). Phorbol esters stimu-

late phosphatidylcholine biosynthesis by translocation of CTP:phosphocholine cytidylyltransferase from cytosol to microsomes. Biochim Biophya Acta 795: 447-451.

Pelech SL, Vance DE (1984). Regulation of phosphatidylcholine biosynthesis. Biochim Biophys Acta 779: 217-251.

Pelech SL, Vance DE (1989). Signal transduction via phosphatidylcholine cycles. Trends Biochem Sci. 14: 28-30.

Raff, MC (1989). Glial cell diversification in the rat optic nerve. Science 243: 1450-1455.

Ritchie T, Cole R, Hun-Soo K, de Vellis J, Noble EP (1987). Inositol lipid hydrolysis in cultured astrocytes and oligodendrocytes. Life Sci. 41: 31-39.

Rozengurt E, Rodriguez-Pena M, Smith KA (1983). Phorbol esters, phospholipase C and growth factors rapidly stimulate the phosphorylation of a Mr80,000 protein in intact quiescent 3T3 cells. Proc Natl Acad Sci USA 80:7244-7248.

Rumsby MG, Chapman JA, Murphy JA, Suckling AJ (1988). Stimulation of protein phosphorylation in mixed glial cell primary cultures and subcultures by the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA). Neursci Lett 89: 251-257.

Sahyoun N, Levine H, McConnell R, Bronson D, Cuatrecases P (1983). A specific phosphoprotein phosphastase acts on histone H1 phosphorylated by protein kianse C. Proc Natl Acad Sci USA 80: 6760- 6764.

Saito N, Kose A, Ito A, Hosoda K, Mori M, Hirata M, Ogita K, Kikkawa U,Ono Y, Igrashi K, Nishizuka Y, Tanaka C (1989). Immunocytochemical localisation of β_{II} subspecies of protein kinase C in rat brain. Proc Natl Acad Sci USA 86: 3409-3413.

Takai Y, Kishimoto A, Kikkawa U, Mori T, Nishizuka Y (1979). Unsaturated diacylglycerol as a possible messenger for the activation of calcium-activated phospholipid-dependent protein kinase system. Biochem Biophys Res Commun 91: 1218 -1224.

Vaartjes WJ, de Haas CGM (1985). Acute effects of tumour-promoting phorbol esters on hepatic intermediary metabolism. Biochem Biophys Res Commun 129:721-726.

Walker AG, Chapman JA, Bruce CB, Rumsby MG (1985i).Immunocytochemical characterisation of cell cultures grown from dissociated 1-2 day post-natal rat cerebral tissue. J. Neuroimmunol 7: 1-20.

Walker AG, Chapman JA, Rumsby MG (1985ii). Immunocytochemical demonstration of glial-neuronal interactions and myelinogenesis in subcultures of rat brain cells. J Neuroimmunol 9: 159-177.

Witters LA, Blackshear PJ (1987). Protein kinase C-mediated phosphorylation in intact cells. In Conn PM, Means AR (eds): "Methods in Enzymology," Orlando: Academic, 141:pp 412-424.

Woodgett JR, Hunter T, Gould KL (1987). Protein kinase C and its role in cell growth. In Elson E, Frazier W, Glaser L (eds): "Cell membranes, Methods and Reviews," New York: Plenum, 3: pp 215-314.

the membrane are insensitive to such changes (Stoltz & Donner, 1985). The change in filterability could reflect small changes in the mean corpuscular volume resulting from damage to the membrane lipids via membrane-bound iron.

We would like to thank the S.E.R.C. for financial support (S.-A.J.) and Neville Green for expert assistance with the atomic absorption spectroscopy studies.

Barry, M., Flynn, D. M., Letsky, E. A. & Risdon, R. A. (1974) *Br. Med. J.* **2**, 16–20
Bauminger, E. R., Cohen, S. G., Offer, S. & Rachmilewitz, E. A. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 939–943
Baysal, E. & Rice-Evans, C. (1987) *Free Radical Res. Commun.* **3**, 227–232
Beutler, E., Duron, O. & Kelly, B. M. (1963) *J. Lab. Clin. Med.* **61**, 882–888
Bomford, A. & Williams, R. (1976) *Q. J. Med.* **45**, 611–616
Jones, J. G., Holland, B. M., Humphrys, J. & Wardrop, C. A. (1985) *Br. J. Haematol.* **59**,, 541–546
Isaacson, C., Seftel, H., Keeley, K. & Bothwell, T. H. (1961) *J. Lab. Clin. Med.* **58**, 845–853
Rachmilewitz, E. A. (1985) *Clin. Haematol.* **14**, 163–182
Rice-Evans, C. (1987) in *Free Radicals, Oxidant Stress and Drug Action* (Rice-Evans, C., ed.), pp. 307–330, Richelieu Press, London
Rice-Evans, C. & Baysal, E. (1987) *Biochem. J.* **244**, 191–196
Rice-Evans, C., Baysal, E., Kontoghiorghes, G., Flynn, D. & Hoffbrand, A. V. (1985) *Free Radical Res. Commun.* **1**, 55–62
Rice-Evans, C., Baysal, E., Flynn, D. M. & Kontoghiorghes, G. (1986) *Biochem. Soc. Trans.* **14**, 368–369
Shaklai, N., Shviro, Y., Rabizadeh, E. & Kirschner-Zilber, I. (1981) *Biochim. Biophys. Acta* **821**, 355–366
Stoltz, J. F. & Donner, M. (1985) *Clin. Haematol.* **5**, 813–848

Received 2 December 1987

Purification, characterization and cDNA cloning of bovine brain diacylglycerol lipase

AKHLAQ A. FAROOQUI,* SHIYUAN CHENG,*
KOTTIL RAMMOHAN,†
PAPACHAN KOLATTUKUDY‡ and
LLOYD A. HORROCKS*

**Department of Physiological Chemistry and †Department of Neurology, College of Medicine, and ‡Biotechnology Center, The Ohio State University, Columbus, OH 43210, U.S.A.*

Diacylglycerol lipase (EC 3.1.1.34), the enzyme catalysing the hydrolysis of 1,2-diacyl-*sn*-glycerol into free fatty acids and 2-acyl-*sn*-glycerol, is found in the plasma membrane and microsomal fraction from adult bovine brain. The enzyme was assayed using a thioester substrate analogue of 1,2-diacylglycerol, *rac*-1-*S*,2-*O*-didecanoyl-1-mercapto-2,3-propanediol (Farooqui *et al.*, 1984, Farooqui & Horrocks, 1987). Diacylglycerol lipase activity of bovine brain microsomes and plasma membranes was solubilized with 0.25% Triton X-100 and purified 433- and 476-fold, respectively, using multiple-column chromatographic procedures. The final enzyme preparations were homogeneous as judged by polyacrylamide-gel electrophoresis. Polyclonal antibodies were raised against microsomal diacylglycerol lipase in rabbits. The antiserum strongly inhibited both diacylglycerol lipases in a concentration-dependent manner and showed a strong cross-reaction with plasma membrane diacylglycerol lipase.

Both diacylglycerol lipases were strongly inhibited by heparin and could be completely separated from monoacylglycerol lipase and lysophospholipase activities by heparin–Sepharose chromatography. The retention of microsomal and plasma membrane diacylglycerol lipases on a concanavalin-A–Sepharose column and their elution with methyl α-D-mannoside indicated the glycoprotein nature of these enzymes. The molecular masses of the diacylglycerol lipases were 27 000 and 52 000 daltons, respectively. With *rac*-1-*S*,2-*O*-didecanoyl-1-mercapto-2,3-propanediol, the K_m and $V_{max.}$ values of microsomal and plasma membrane diacylglycerol lipases were 30 and 12 μM and 180 and 200 nmol/min per mg of protein, respectively.

These enzymes were markedly inhibited by free fatty acids. Palmitate was the strongest inhibitor, followed by arachidonate and linoleate. Addition of fatty acid free bovine serum albumin resulted in a reversal of the fatty acid inhibition. C-MT peptide, a chemically synthesized peptide known to inhibit phospholipases A_2, C and D, strongly inhibited both diacylglycerol lipases in a concentration-dependent manner.

The amino acid composition and the sequence of amino acids 2–26, 28 and 30 of the microsomal diacylglycerol lipase have been determined. The *N*-terminal portion of this enzyme contained a large proportion of hydrophobic amino acids. The amino acid in position 1 was not identified, probably because it is attached to a sugar residue. Based on the amino acid sequence an oligonucleotide probe (20-mer) was synthesized and used for screening a λgt11 bovine brain cDNA library (Clone Tech, Palo Alto, CA, U.S.A.). This screening has resulted in isolation of four cDNA clones. Further studies on cDNA cloning of diacylglycerol lipases are in progress in this laboratory.

Diacylglycerol lipases are very active in brain. They can control the transient levels of diacylglycerol. The latter acts as a second messenger and stimulates the activities of protein kinase C, phospholipase A_2, tyrosine aminotransferase and ornithine decarboxylase (Nishizuka, 1984; Dawson *et al.*, 1984; Kido *et al.*, 1986), and inhibits the activities of Na^+,K^+-ATPase and glycogen synthetase (Goldberg *et al.*, 1985; Bouscarel & Exton, 1986). Under normal conditions the action of diacylglycerol lipases on diacylglycerols provides free fatty acids that may be used for the synthesis of prostaglandins *in vivo*, but in pathological situations (ischaemia, spinal cord trauma, cancer and viral infections) these enzymes may be involved in the massive release of free fatty acids and prostaglandins (Abe *et al.*, 1987; Farooqui *et al.*, 1987) which may cause serious tissue damage.

Supported by research grants NS-08291 and NS-10165 from the National Institutes of Health and NATO Research Grant 0080/87.

Abe, K., Kogure, K., Yamamoto, H., Imazawa, M. & Miyamoto, K. (1987) *J. Neurochem.* **48**, 503–509
Bouscarel, B. & Exton, J. H. (1986) *Biochim. Biophys. Acta* **888**, 126–134
Dawson, R. M. C., Irvine, R. F., Bray, J. & Quinn, B. J. (1984) *Biochem. Biophys. Res. Commun.* **125**, 836–842
Farooqui, A. A. & Horrocks, L. A. (1987) In *Neuromethods*, Vol. 7, *Lipids and related compounds* (Boulton, A. A., Baker, G. B. & Horrocks, L. A., eds.), Humana Press, NJ, in the press
Farooqui, A. A., Taylor, W. A., Pendley II, C. E., Cox, J. W. & Horrocks, L. A. (1984) *J. Lipid Res.* **25**, 1555–1562
Farooqui, A. A., Taylor, W. A. & Horrocks, L. A. (1987) *Neurochem. Pathol.* in the press
Goldberg, W. J., Dorman, R. V., Dabrowiecki, Z. & Horrocks, L. A. (1985) *Neurochem. Pathol.* **3**, 237–248
Kido, H., Fukusen, N., Ishidoh, K. & Katunuma, N. (1986) *Biochim. Biophys. Res. Commun.* **138**, 275–282
Nishizuka, Y. (1984) *Nature (London)* **308**, 693–698

Received 20 October 1987

Protein kinase C in astrocyte- and oligodendrocyte-enriched brain cell primary cultures: their phorbol ester response

J. A. MURPHY, J. A. CHAPMAN and M. G. RUMSBY
Department of Biology, University of York, York YO1 5DD, U.K.

Since its discovery in 1977 (Inoue *et al.*, 1977), protein kinase C (PKC), a calcium- and phospholipid-dependent protein kinase, has attracted much interest. The enzyme is activated during agonist binding and the receptor-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate (Berridge, 1984; Sekar & Hokin, 1986), since the diacylglycerol so produced increases the affinity of PKC for Ca^{2+}, allowing for activation at physiological Ca^{2+} levels (Kishimoto *et al.*, 1980). PKC is very active in neurones of the central nervous system (CNS) (Burgess *et al.*, 1986). It has also been measured in primary astrocyte cultures (Neary *et al.*, 1986) and identified in cells resembling oligodendrocytes (Girard *et al.*, 1985). Murphy, J. *et al.* (1987) have reported on the activity of PKC present in primary glial cell cultures and subcultures derived from rat brain cerebra (Walker *et al.*, 1985*a*,*b*).

For PKC assay, cultures were treated as before (Murphy, J. *et al.*, 1987). Cells were rinsed, scraped out and homogenized, and centrifuged at 100 000 *g* for 60 min at 4°C to give soluble and particulate fractions. The particulate pellet was treated according to Parker *et al.* (1987) to determine membrane-bound PKC activity. Kinase activity in both fractions was measured by following the transfer of the γ-phosphate group of ^{32}P-ATP to type IIIS histone for 10 min at 30°C with shaking in a complete reaction mixture described by Kikkawa *et al.* (1983). The specific activity of PKC (mean ± S.D.) was expressed as pmol ^{32}P incorporated/10 min per mg of protein at 30°C, and calculated by subtracting basal (calcium-independent) kinase values from the results obtained.

The specific activity of PKC in supernatants from glial cells in 12-day culture was 184 ± 10 pmol ^{32}P incorporated/10 min per mg of protein. This was considerably greater than the PKC values measured in supernatants of glial cells cultured for 18 and 28 days, i.e. 51 ± 20 and 53 ± 8 pmol ^{32}P incorporated/10 min per mg of protein, respectively. Supernatants from 28-day glial cell subcultures had a PKC activity of 75 ± 15 pmol ^{32}P incorporated/10 min per mg of protein.

Accurate measurement of PKC activity in crude supernatant extracts may be complicated by the presence of factors such as endogenous inhibitors, and an initial purification step using DE-52 anion-exchange chromatography has been advised (Kikkawa *et al.*, 1983). Using the protocol described by Anderson *et al.* (1985), we have verified that glial cell supernatants contain considerable PKC activity. After DE-52 column purification there was a 20-fold increase in the specific activity of PKC in supernatants of 18-day primary glial cell cultures and an approximately 6-fold increase in supernatants of 28-day glial cell cultures.

In 12-day primary cultures, membrane-bound PKC was found to have a specific activity of 485 ± 120 pmol ^{32}P incorporated/10 min per mg of protein, approximately 2.5 times the level found in the cytosolic fraction. In 28-day primary cultures, the specific activities of PKC were about the same in both membrane-bound and soluble fractions. As membrane-bound PKC is believed to be the active form of the enzyme, the higher ratio of membrane-bound PKC to cytosolic PKC activity in 12-day glial cells compared to 28-day glial cells may indicate a role for the enzyme in cellular signal transduction at this earlier stage of development. We have examined the effect of 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) on PKC activity in glial cell cultures and have shown translocation of PKC activity from cytosol to membrane, confirming observations in other cell types (for example, Kraft & Anderson, 1983).

We have obtained direct evidence that PKC is active in rat glial cells. It is unlikely that the activity we have detected in the cultures is due solely to the presence of contaminating neurones (see Murphy, J. *et al.*, 1987). Although we have not directly revealed which individual glial cell classes contain PKC, previous evidence suggests it to be present in both oligodendrocytes and astrocytes (Girard *et al.*, 1985; Neary *et al.*, 1986; Murphy S. *et al.*, 1987). Recently, we have found PKC activity in protoplasmic astrocytes to be approximately 50% that of whole glial cultures. A monoclonal antibody against PKC (Amersham International plc) is now being used to investigate the localization of PKC in the different cell classes in our glial cell cultures.

Tumour-promoting phorbol esters such as TPA produce some of their actions on cells by activating PKC which acts as a receptor for these agents (Ashendel, 1985). When cells are treated with TPA, PKC is activated stimulating the phosphorylation of a protein of approximately 80 000 Da molecular mass (Blackshear *et al.*, 1985, 1986). These authors have also shown that TPA stimulates phosphorylation of an 80 000 Da protein in three types of neuronal cells in culture. We have examined the effect *in vivo* of TPA on our mixed glial cells. Growth medium [Dulbecco's modified Eagle's medium (DMEM) ± 10% (v/v) fetal calf serum (FCS)] was removed from 14-day cultures growing in 6 cm Petri dishes and was replaced for 24 h with DMEM + 0.5% (v/v) FCS. Cells were then transferred to phosphate-free medium and 100 μCi of [^{32}P]orthophosphate added for 90 min at 37°C to allow the phosphate pools to equilibrate. TPA, 4-α-phorbol (both at 25 nM concentration) or dimethylsulphoxide (DMSO) was then added to cultures which were further incubated for 15, 30 or 60 min. Dishes of cells were rinsed well and cells were solubilized directly into SDS/polyacrylamide-gel electrophoresis sample buffer. Equal volumes of sample were applied to 6–30% (w/v) gradient gels which were run overnight. Protein bands on gels were stained with Coomassie Blue R and were destained, dried and exposed to Hyperfilm-β-max (Amersham International plc) overnight. It was observed that TPA stimulated the incorporation of ^{32}P-phosphate into only one main band which had an approximate molecular mass of 80 000 Da in agreement with the findings of Blackshear *et al.* (1985, 1986) and others with a variety of mammalian and avian cells; 4-α-phorbol and DMSO did not stimulate phosphorylation of this band. It has been shown *in vitro* that an 80 000 Da molecular mass protein is a substrate for PKC (Blackshear *et al.*, 1986), but that this component is distinct from PKC. TPA is known to activate phospholipid synthesis in cultured myoblasts (Grove & Schimmel, 1982) and in Swiss 3T3 cells (Muir & Murray, 1987) for example; the phorbol ester may stimulate phosphatidylcholine biosynthesis by activating CTP:phosphocholine cytidylyltransferase stimulating the translocation of the enzyme from cytosol to endoplasmic reticulum (Pelech *et al.*, 1984). We have noted that phospholipid biosynthesis, in particular that of phosphatidylcholine, is stimulated when our cultured glial cells are exposed to TPA.

Abbreviations used: PKC, protein kinase C; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; DMSO, dimethylsulphoxide.

Anderson, W. B., Estival, A., Tapiovaara, J. & Gopalakrishna, R. (1985) in *Advances in Cyclic Nucleotide and Protein Phosphoryla-*

tion Research (Cooper, D. M. F. & Seamon, K. B., eds.), vol. 19, pp. 287–306, Raven Press, New York.
Ashendel, C. L. (1985) *Biochim. Biophys. Acta* **822**, 219–242
Berridge, M. J. (1984) *Biochem. J.* **220**, 345–360
Blackshear, P. J., Witters, L. A., Girard, P. R., Kuo, J. R. & Quamo, S. N. (1985) *J. Biol. Chem.* **260**, 13304–13315
Blackshear, P. J., Wen, L., Glynn, B. P. & Witters, L. A. (1986) *J. Biol. Chem.* **261**, 1459–1469
Burgess, S. K., Sahyoun, N., Blanchard, S. G., LeVine, H., Chang, K-J. & Cuatrecasas, P. (1986) *J. Cell Biol.* **102**, 312–319
Girard, P. R., Mazzei, G. J., Wood, J. G. & Kuo, J. F. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 3030–3034
Grove, R. I. & Schimmel, S. D. (1982) *Biochim. Biophys. Acta* **711**, 272–280
Inoue, M., Kishimoto, A., Kikkawa, U., Mori, T. & Nishizuka, Y. (1977) *J. Biol. Chem.* **252**, 7610–7616
Kikkawa, U., Minakuchi, R., Takai, Y. & Nishizuka, Y. (1983) *Methods Enzymol.* **99**, 288–298
Kishimoto, A., Takai, Y., Mori, T., Kikkawa, U. & Nishizuka, Y. (1980) *J. Biol. Chem.* **255**, 2273–2276
Kraft, A. S. & Anderson, W. B. (1983) *Nature (London)* **301**, 621–623
Muir, J. G. & Murray, A. W. (1987) *J. Cell Physiol.* **130**, 382–391
Murphy, J. A., Chapman, J. A., Suckling, A. J. & Rumsby, M. G. (1987) *Neurosci. Lett.* in the press
Murphy, S., McCabe, N., Morrow, C. & Pearce, B. (1987) *Dev. Brain Res.* **31**, 133–135
Neary, J. T., Norenberg, L-O. B. & Norenberg, M. D. (1986) *Brain Res.* **385**, 420–424
Parker, J., Daniel, L. W. & Waite, M. (1987) *J. Biol. Chem.* **262**, 5385–5393
Pelech, S. L., Paddon, H. B. & Vance, D. E. (1984) *Biochim. Biophys. Acta* **795**, 447–451
Sekar, M. C. & Hokin, L. E. (1986) *J. Membr. Biol.* **89**, 193–210
Walker, A. G., Chapman, J. A., Bruce, C. B. & Rumsby, M. G. (1985*a*) *J. Neuroimmunol.* **7**, 1–20
Walker, A. G., Chapman, J. A. & Rumsby, M. G. (1985*b*) *J. Neuroimmunol.* **9**, 159–177

Received 30 November 1987

Influence of nerve cells on the development of astrocytes

ANTHONY HUNT, MASAO HAYASHI, PATRICIA SEATON and AMBRISH J. PATEL*
M.R.C. Developmental Neurobiology Unit, Institute of Neurology, 1 Wakefield Street, London WC1N 1PJ, U.K.

The central nervous system consists of a complex array of many different neuronal and neuroglial cell types whose precise interrelationships during development are vital for its functioning. Recently we have reported that during development the major increase in astrocyte-enriched proteins, glutamine synthetase (GS) and glial fibrillary acidic protein (GFAP), is associated with differentiation rather than proliferation of astroglial cells (Patel *et al.*, 1983*a*; Weir *et al.*, 1984). Further, marked differences have been observed in the regional distribution of these proteins, the GS to GFAP ratio varying about fivefold (Patel *et al.*, 1985). The observations provided direct evidence for the presence of more than one type of astroglial cell, in terms of their biochemical composition in the brain. It is now possible to obtain cultures greatly enriched in metabolically competent astrocytes in relatively large quantities (see Patel & Hunt, 1985). The biochemical properties and differentiation of the cells, derived from either neonatal forebrain or cerebellum, are very similar to those of astroglial cells *in situ* (Patel & Hunt, 1985; Patel, 1986). These include a greater amount and an earlier development of GS in forebrain than in cerebellar astrocytes. Also, in astrocytes cultured from fetal brain, no appreciable increase is observed in GS activity (Hansson, 1986), indicating that the differentiation of these cells is severely limited. Here, we show that one of the factors contributing to the differences in the properties of astrocytes may relate to a requirement of astroglial cells, during differentiation, for trophic substances produced by nerve cells.

Primary cultures, enriched in astroglial cells, were derived from either newborn forebrain or 8-day-old cerebellum of rats as described by Patel & Hunt (1985). At 7 days *in vitro* the culture medium was changed to chemically defined medium (Hayashi & Patel, 1987). Three days later some of the culture dishes were co-cultured with dissociated cells derived from the 17-day-old embryonic forebrain. In another experiment, some of these astrocyte culture dishes were grown in a conditioned medium derived from 7-day-old primary cultures enriched in nerve cells (Hayashi & Patel, 1987). One week later the cells were washed and homogenized in 10 mM-imidazole buffer, pH 7.2. Whole homogenate was used for the estimation of GS using a sensitive radioisotopic assay (Patel *et al.*, 1983*a*). Enzyme activity was expressed as nmol of glutamine formed/h per 35 mm culture dish. Astrocytes were stained immunocytochemically using a rabbit antiserum to GFAP and were classified as polygonal or stellate (Patel & Hunt, 1985).

The primary astroglial cell cultures exhibited mainly a polygonal epitheloid cell morphology (see Patel & Hunt, 1985). In contrast, on co-culturing with nerve cells, a marked increase was observed in the proportion of stellate-shaped astroglial cells (Thangnipon *et al.*, 1983; Nagata *et al.*, 1986). The co-culturing of astrocytes with nerve cells also increased the GS activity, much more than the sum of the values for both astrocyte and neuron cultures (Table 1). The observed

Table 1. *Influence of nerve cells on the development of astroglial cell marker enzyme GS activity in vitro*

Primary cultures of astrocytes were derived from either newborn forebrain or 8-day-old cerebellum (Patel & Hunt, 1985), while those of nerve cells were derived from the 17-day-old embryonic forebrain of rats (Hayashi & Patel, 1987). The whole homogenate of the cells was used for the estimation of GS activity (Patel *et al.*, 1983*a*). The values are means ± S.E.M. for three experiments.

	GS activity (nmol/h per dish)
Effect of co-culture with neurons	
Forebrain astrocytes	215 ± 18
Forebrain neurons	16 ± 4
Forebrain astrocytes co-cultured with forebrain neurons	616 ± 48
Effect of neuronal conditioned medium	
Forebrain astrocytes grown in control medium	201 ± 12
Forebrain astrocytes grown in neuronal conditioned medium	412 ± 16
Cerebellar astrocytes grown in control medium	72 ± 6
Cerebellar astrocytes grown in neuronal conditioned medium	223 ± 15

Abbreviations used: GS, glutamine synthetase; GFAP, glial fibrillary acidic protein.

* To whom correspondence should be addressed.

augmentation of GS activity may be due to an increase per astroglial cell, since it has been reported that the presence of nerve cells inhibits proliferation of astrocytes (Hatten, 1985; Nagata *et al.*, 1986). Furthermore, the activity of GS in forebrain astrocytes was about 2.5-fold greater than in the cerebellar astrocytes (Table 1). This is consistent with our previous findings that the biological properties of forebrain astrocytes differ markedly from those of cerebellar astrocytes (Patel & Hunt, 1985; Patel, 1986). When the astroglial cells were grown in the conditioned medium obtained from forebrain nerve cell cultures, the activity of GS was markedly increased in both forebrain and cerebellar astrocytes (Table 1). However, the magnitude of the effect was lower in the former than in the latter, indicating marked differences in the quantitative responses to the trophic factor of astrocytes derived from either the forebrain or the cerebellum. Similar regional differences were also observed in the induction of GS by glucocorticoids, both *in vivo* and *in vitro* (Patel *et al.*, 1983*b*; Patel & Hunt, 1985).

Our findings that neuronal cells induce astrocytes to become stellate-shaped and to enhance GS activity *in vitro* may suggest that during brain development neurons play a role in the differentiation of astrocytes. The results would also indicate that the marked regional variations in the properties of astrocytes may relate to the differences in the quantitative responses to the trophic substance of astroglial cells during maturation in different areas of the brain. However, this does not exclude the possibility that varying amounts of trophic factor may be produced by different types of nerve cell.

Hansson, E. (1986) *Dev. Brain Res.* **24**, 203–209
Hatten, M. E. (1985) *J. Cell Biol.* **100**, 384–396
Hayashi, M. & Patel, A. J. (1987) *Dev. Brain Res.* **36**, 109–120
Nagata, I., Keilhauer, G. & Schachner, M. (1986) *Dev. Brain Res.* **24**, 217–232
Patel, A. J. (1986) *Adv. Biosci.* **61**, 87–96
Patel, A. J. & Hunt, A. (1985) *Dev. Brain Res.* **18**, 175–184
Patel, A. J., Hunt, A. & Tahourdin, C. S. M. (1983*a*) *Dev. Brain Res.* **8**, 31–37
Patel, A. J., Hunt, A. & Tahourdin, C. S. M. (1983*b*) *Dev. Brain Res.* **10**, 83–91
Patel, A. J., Weir, M. D., Hunt, A., Tahourdin, C. S. M. & Thomas, D. G. T. (1985) *Brain Res.* **331**, 1–9
Thangnipon, W., Kingsbury, A., Webb, M. & Balazs, R. (1983) *Dev. Brain Res.* **11**, 177–189
Weir, M. D., Patel, A. J., Hunt, A. & Thomas, D. G. T. (1984) *Dev. Brain Res.* **15**, 147–154

Received 20 October 1987

Production by astrocytes of a trophic factor for cholinergic neurons

PATRICIA SEATON, ANTHONY HUNT and AMBRISH J. PATEL*

M.R.C. Developmental Neurobiology Unit, Institute of Neurology, 1 Wakefield Street, London WC1N 1PJ, U.K.

In the central nervous system, astrocytes have been implicated in the control of migration and elongation of neurons during development *in vivo*, and in the survival and emission of neuritic outgrowths of nerve cells *in vitro* (Rakic, 1972; Lindsay, 1979; Rudge *et al.*, 1985; Hatten & Mason, 1986; Lindner *et al.*, 1986). However, rather little is known about the influence of astrocytes on the development of a defined population of nerve cells. Recently, we have reported (Hayashi & Patel, 1987) the procedures for obtaining, from the septal-diagonal band region of embryonic rat brain, neuronal cultures relatively enriched in cholinergic cells (see also, Hefti *et al.*, 1985). In initial experiments during the development of this culture system, when the dissociated cells were plated at low density in a medium containing 10% (v/v) fetal calf serum (Patel *et al.*, 1982), a close association was detected between astrocytes and nerve cells, including cholinergic neurons (Sensenbrenner & Mandel, 1974; Denis-Donini *et al.*, 1984). Furthermore, we attempted to remove contaminating non-neuronal cells from these cultures grown in serum-containing medium, by treatment with different doses of cytosine arabinoside for various lengths of time. In some of these experiments, the metabolic status of astrocytes and cholinergic neurons were monitored in terms of the activity of glutamine synthetase and of choline acetyltransferase (ChAT), respectively. In our hands, the concentration of 10 μM-cytosine arabinoside normally used (Hefti *et al.*, 1985) killed both glial and neuronal cells. However, under the conditions which removed astroglial cells partially but in varying proportions, a significant correlation was observed between ChAT and glutamine synthetase activities. Here, we extend these observations and show that a trophic factor important for the development of cholinergic cells is produced by astrocytes.

Primary cultures, relatively enriched in cholinergic cells, were derived from the septal-diagonal band region of 17-day-old embryonic rat brain, as described by Hayashi & Patel (1987). Other primary cultures, enriched in astrocytes, were derived from newborn forebrain (Patel & Hunt, 1985) and enriched in granule cells, were derived from 8-day-old rat cerebellum (Patel *et al.*, 1982; Kingsbury *et al.*, 1985). The two last-mentioned cultures were maintained in a chemically defined medium (Hayashi & Patel, 1987) for at least a few days, before they were used to obtain conditioned medium specific to the cell type. In co-culture experiments, the medium of 7-day-old astrocyte cultures was changed to a chemically defined medium, and three days later the dissociated subcortical cells, derived from the 17 day-old-rat embryos, were plated on top of this sheet of astroglial cells. The cholinergic cells, cultured for ten days *in vitro* under different experimental conditions, were washed and homogenized in 50 mM-sodium phosphate buffer, pH 7.4. Whole homogenate was used for the estimation of ChAT activity and protein (Patel *et al.*, 1987). Enzyme activity was expressed as amount of product formed/h per 60 mm dish or per mg of protein. The characterization of various cell types present in the culture was made by immunocytochemistry or by histochemistry (see Hayashi & Patel, 1987).

The co-culturing of subcortical cholinergic neurons with astrocytes enhanced the expression of ChAT activity, and in comparison with the cells cultured in the absence of astrocytes the increase was about threefold (Table 1). As the astroglial cells were devoid of ChAT activity and neurons do not divide *in vitro*, the observed augmentation of ChAT activity would represent the increase per cholinergic cell. Furthermore, in the cultures of dissociated cells derived from the cholinergic regions of embryonic brain and grown in the astrocyte-conditioned medium, the activity of ChAT was 100% greater than in the cell cultures grown in the control

Abbreviations used: ChAT, choline acetyltransferase; NGF, nerve growth factor.

*To whom correspondence should be addressed.

Neuroscience Letters, 89 (1988) 251–257
Elsevier Scientific Publishers Ireland Ltd.

NSL 05371

Stimulation of protein phosphorylation in mixed glial cell primary cultures and subcultures by the phorbol ester 12-*o*-tetradecanoylphorbol-13-acetate (TPA)

Martin G. Rumsby, Jill A. Chapman, John A. Murphy and Anthony J. Suckling

Department of Biology, University of York, York (U.K.)

(Received 2 February 1988; Revised version received 4 March 1988; Accepted 5 March 1988)

Key words: Glial primary culture; Glial subculture; Phorbol ester; Protein kinase C; Polypeptide 80,000-Da phosphorylation

Glial cell primary cultures consisting of protoplasmic and fibrous astrocytes, oligodendrocytes and progenitor glial cells incubated in medium containing 0.5% foetal calf serum and treated with 25 nM 12-*o*-tetradecanoylphorbol-13-acetate (TPA) for periods between 15 and 60 min showed a stimulation of protein phosphorylation which was most prominent in a polypeptide with a molecular weight of about 80,000 Da. Glial subcultures consisting mainly of Type 2 astrocytes, oligodendrocytes and progenitor glia showed a similar TPA stimulation of 80,000 Da protein phosphorylation detectable within 1 min of phorbol ester addition. TPA treatment of primary glial cultures led to an enhancement of phospholipid turnover but exposure of primary glial cultures to concentrations of TPA up to 250 nM caused no morphological change in protoplasmic astrocytes. 4-Phorbol (4-PH) or dimethylsulfoxide (DMSO) was without effect on protein phosphorylation or lipid turnover in glial cultures.

Protein kinase C (PKC), an enzyme requiring calcium and phospholipid for activity and implicated in secretion, proliferation and differentiation [19], is activated during agonist binding and the receptor-mediated hydrolysis of phosphatidylinositol 4,5-biphosphate (PtdIns4,5P2). The diacylglycerol produced during this signal transduction pathway increases the affinity of PkC for Ca^{2+} [14] and may also activate PkC in the absence of elevated Ca^{2+} levels [9, 10]. While PkC has been well characterised in neurones in the central nervous system [15] less attention has been paid to the enzyme in the glial compartment. However, recent evidence suggests that PkC is important in astrocyte biochemistry [13, 17], and is perhaps also active in oligodendrocytes. For example, certain agonists including noradrenaline have been found to stimulate the receptor-linked PtdIns4,5P2 pathway in astrocytes in culture [21, 25] while

Correspondence: M.G. Rumsby, Department of Biology, University of York, York Y01 5DD, U.K.

0304-3940/88/$ 03.50 © 1988 Elsevier Scientific Publishers Ireland Ltd.

carbachol was effective with cultured oligodendrocytes [25]. PkC has been localised immunocytochemically in cells resembling oligodendrocytes [12]. Additionally, PkC activity has been measured directly in astrocyte cultures [18] and in mixed astrocyte and oligodendrocyte primary cultures and subcultures [16].

The tumour-promoting phorbol esters such as 12-*o*-tetradecanoylphorbol-13-acetate (TPA) initially act on cells by activating PkC, their main cellular receptor [3, 8]. Phorbol esters mimic diacylglycerols in structure and activate PkC without increasing the turnover of inositol lipids. Phorbol esters stimulate many biochemical pathways and induce morphological changes in different cells [2], but, though activation of PkC is the initial event, subsequent biochemical mechanisms are not clear. As with some growth factors phorbol esters have been found to stimulate phosphorylation of certain polypeptides [27] though the function of this increased phosphorylation is not yet known. Among other effects phorbol esters increase phospholipid synthesis [2]. We have previously measured PkC activity in our mixed glial primary cultures [16] and are now using phorbol esters to study the biochemistry of PkC activation in astrocytes and oligodendrocytes. We report here that phorbol ester treatment of glial primary cultures and subcultures stimulates protein phosphorylation and phospholipid turnover.

Rat glial primary cultures were set up as we have described elsewhere [28] and were used after 14 days in culture. At this stage protoplasmic astrocytes form a confluent monolayer on which grow smaller multiprocessed phase-bright cells, chiefly fibrous astrocytes, oligodendrocytes and progenitor glia [28]. We have found that phosphorylation effects are enhanced if cells are cultured in Dulbecco's modified Eagle's medium (DMEM) containing 0.5% foetal calf serum (FCS) for 24 h prior to stimulation. This change is without obvious effect on the morphology of cells in the cultures but would clearly reduce the rate of proliferation of growing cells as noted by Murphy et al. [17]. However, at the 14 day stage, protoplasmic astrocytes in our cultures are fully confluent. Accordingly at 14 days culture fluid was replaced with fresh DMEM containing 0.5% FCS. Twenty-four h later this medium was replaced with phosphate-free DMEM+0.5% FCS and then 100 μCi [^{32}P]orthophosphate (PBS 13, Amersham) was added: flasks were incubated at 37°C for 120 min to equilibrate phosphate pools. TPA and 4-phorbol (4-PH), in dimethylsulfoxide (DMSO), were diluted in sterile distilled water and were added to glial cultures to give a final concentration of 25 nM, with controls of DMSO. After 15, 30 and 60 min further incubation at 37°C cells were rinsed and then solubilised directly in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer. Resolution of glial polypeptides by sodium dodecyl sulphate–polyacrylamide gel SDS–PAGE (applying equal sample volumes) to 6–30% gels followed by autoradiography (-max film, Amersham) revealed (Fig. 1) that TPA caused a marked stimulation of phosphorylation in one polypeptide (Fig. 1, arrow) over the time period studied and less significantly in several minor polypeptides. This stimulation effect was not observed in DMSO and 4-PH controls but was observed with cultures grown in the normal growth medium (DMEM/10% FCS). By reference to standards run simultaneously on the gradient gel and running samples on 12.5% homogeneous gels it was found that the

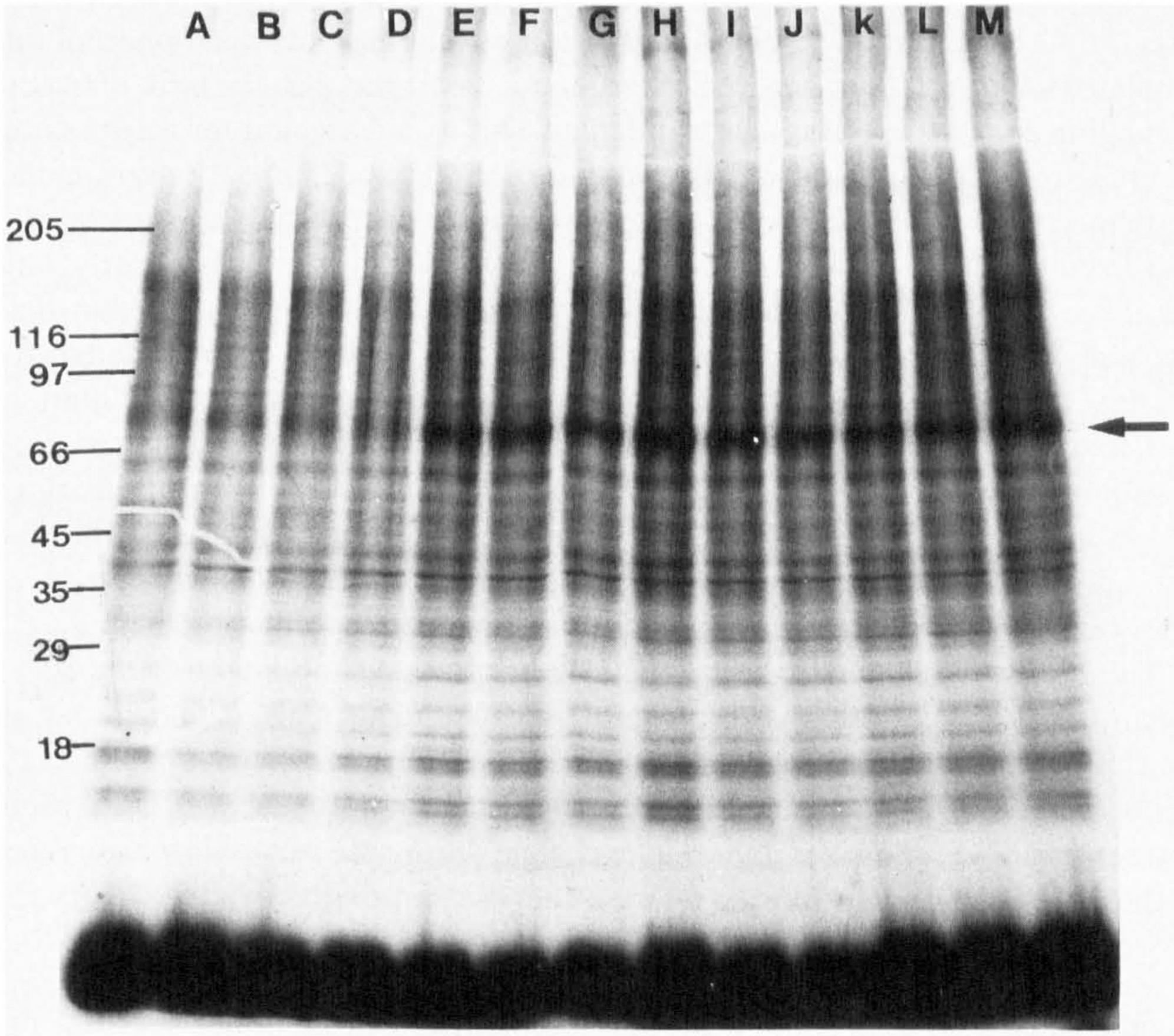


Fig. 1. Autoradiograph showing stimulation of protein phosphorylation in primary glial cultures by tetradecanoylphorbol 13-acetate (TPA). Each lane is a separate 14 day primary culture. Positions of molecular weight markers (kDa) are on the left: major polypeptide of approx. 80,000 Da showing enhanced phosphorylation is indicated (arrow). Lanes A, B: DMSO control; C, D: 25 nM 4-phorbol, 15 min; E–M: 25 nM TPA for 15 min (E–G); 30 min (H–J) and 60 min (K–M). Other details are in the text.

polypeptide showing marked phosphorylation was of approximately 80,000 Da molecular weight. Minor polypeptides, in the 18,000–28,000 range, showed slight enhancement of phosphorylation over the time period studied. We confirmed the stimulation of phosphorylation in the 80,000 band by cutting out the appropriate region of the dry gel, rehydrating it in water, extracting protein from the slice in central nervous system (CNS) tissue solubiliser (Amersham) followed by scintillation counting. In 3 separate cultures treated with TPA for 15 min counts of ^{32}P incorporated in the excised 80,000 polypeptide region were 904, 861 and 723 compared with 581 and 490 in DMSO controls and 521 and 545 in 4-PH controls. This 1.6-fold stimulation factor at 15 min had decreased to 1.3-fold in cultures stimulated with TPA for 60 min. Rozengurt et al. [26] noted that 200 nM phorbol 12,13-dibutyrate (PBt2) very rapidly stimulated phosphorylation of an 80 kDa polypeptide in Swiss 3T3 cells, a 3.9-fold maximal stimulation occurring 1–2 min after phorbol ester addition. Our finding of a 1.6-fold stimulation of protein phosphorylation by TPA at 15 min in the primary glial cultures is lower. This is perhaps because: (1) we used a 10-fold

lower TPA concentration to be just at the top of the range at which phorbol esters stimulate PkC [8]. (2) even our 15 min incubation point was past the peak of maximal stimulation and (3) the cell system is different. This would account for why the stimulation factor drops to 1.3 after TPA addition for 60 min. Phorbol ester-stimulated phosphorylation of an 80,000 Da polypeptide has been reported in a variety of cell types in culture [1, 5, 6, 27], is prominent in brain tissue [6] and is particularly noticeable in synaptosomes [30]. Blackshear and colleagues have determined that the 80 kDa polypeptide which shows enhanced phosphorylation is distinguishable from PkC [6]. In reviewing PkC-mediated phosphorylation in intact cells Witters and Blackshear [27] have suggested that phosphorylation of the 80 kDa polypeptide is a useful marker of PkC activation. Our results show therefore that in primary glial cells, as in several other cell types, TPA stimulates protein phosphorylation through PkC activation. This further confirms the activity of PkC in glia as we have previously measured [16] again accounting for the prominence of this protein in the CNS [6]. The biochemical significance of the stimulated phosphorylation of the 80 kDa protein is not yet known [5, 27]; preliminary experiments suggest that in our glial cells this polypeptide is a cytosolic component (Rumsby, M.G. and Chapman, J.A., unpublished observations) in agreement with findings in fibroblasts [5]. Since protein substrates for PkC phosphorylation may change during the cell cycle we are repeating this study with 7 day cultures when astrocytes are rapidly dividing. Neary et al. [18] using 23,000 g or 100,000 g cytosolic preparations from astrocyte cultures found the in vitro phosphorylation by PkC for several endogenous cytoplasmic polypeptides but not an 80 kDa component. No comparison can be drawn between PkC-stimulated phosphorylation in their cytosolic preparations which involve the disruption of cell compartments and the present work with growing cells in which all compartments are intact. Further, we stimulated PkC with TPA. During the present work we noted that exposure of our 14 day primary glial cultures to TPA concentrations as high as 250 nM for up to 48 h caused no morphological change in the protoplasmic astrocyte layer confirming observations of others [11]. However, TPA has been found to increase certain biochemical reactions in astrocytes including thymidine incorporation [17] and glutamine synthetase activity [13]. Further we found in this study that 25 nM TPA stimulated phospholipid turnover in the primary cultures as judged by increased incorporation of ^{32}P into total phospholipid extracts which at 15, 30 and 60 min exposure to TPA were increased 1.5–2.0 fold over DMSO and 4-PH controls (results not shown). When phospholipids were resolved in thin-layer chromatography this increase was largely associated with phosphatidylcholine. TPA is known to increase phospholipid turnover in cells [22] and has been shown to stimulate the activity of the key regulatory enzyme of de novo phospholipid synthesis, cytidine triphosphate, phosphocholine cytidylyltransferase [23]. These preliminary lipid observations are being examined more closely in view of the recent observations of Besterman et al. [4] that phorbol esters and growth factors rapidly stimulate diacylglycerol formation from phosphatidylcholine in a variety of cell types.

In platelets TPA stimulates phosphorylation of a 40,000 Da polypeptide, thought to be a 5′-monophosphoesterase [27]; with glial cultures (Fig. 1) we did not notice

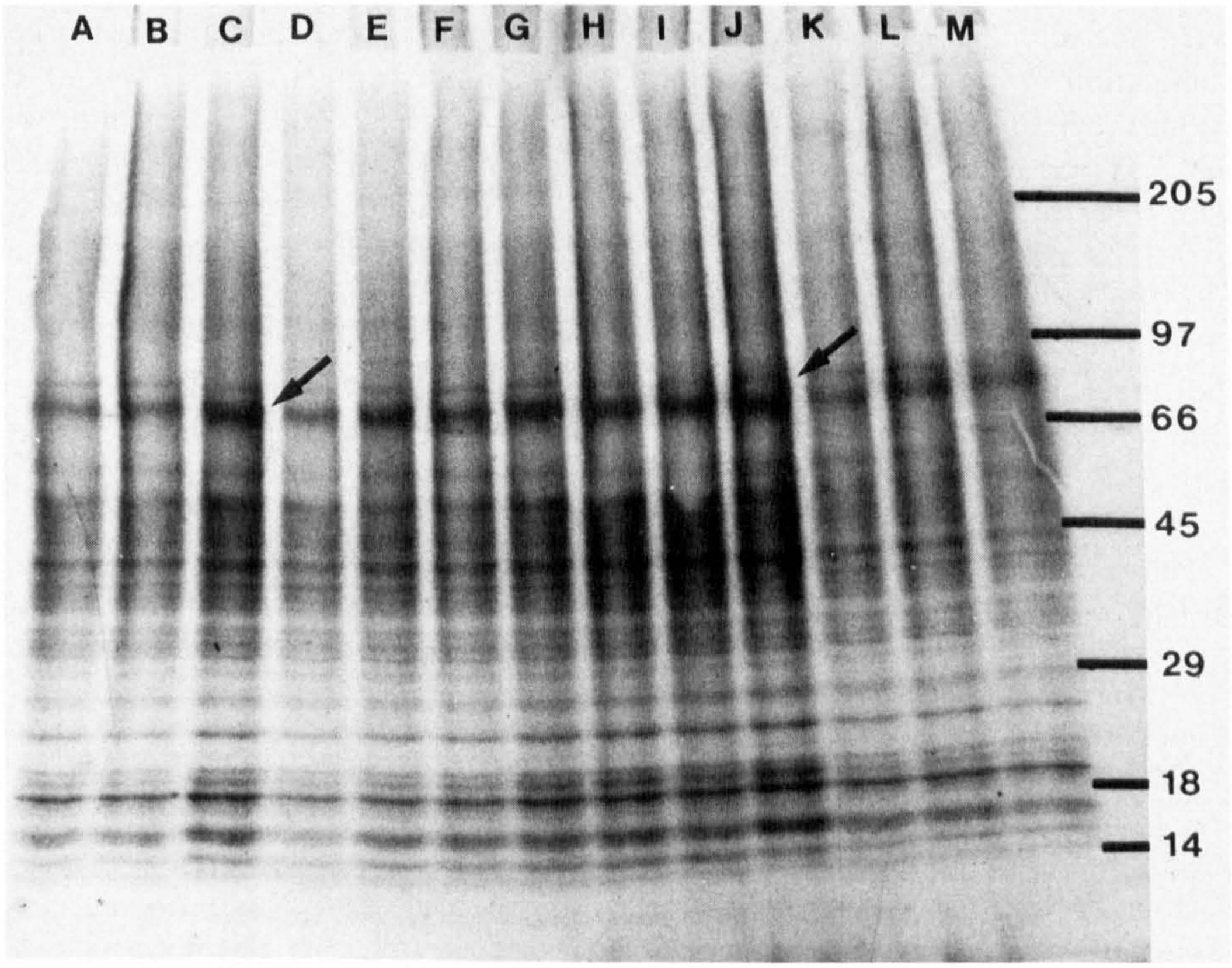


Fig. 2. Autoradiograph showing time course of TPA stimulation of protein phosphorylation in glial cell subcultures. Each lane is a separate glial subculture treated as described in the text. Positions of molecular weight markers (kDa) are on the right; major polypeptide of approx. 80,000 Da showing enhanced phosphorylation is indicated (arrows). Lanes A, K, L, M: 25 nM 4-phorbol 1, 2.5, 5, 15 min; other lanes: 25 nM TPA; B, C: 1 min; D, E: 2.5 min; F, G: 5 min; H, I: 15 min; J: 30 min.

significant stimulation of polypeptide phosphorylation in this region of SDS gels. It is reported [5] that platelet-derived growth factor (PDGF) stimulates phosphorylation of polypeptides in fibroblasts, acting through two mechanisms, a PkC-dependent pathway phosphorylating an 80 kDa component and through a PkC-independent route to phosphorylate 22 kDa and 31 kDa polypeptides. This finding is important in view of the fact that PDGF, secreted by Type 1 astrocytes, may control progenitor glial cell differentiation [20, 24]. Further, PDGF is an active chemo-attractant for rat brain astrocytes [7]. Since it is known that in fibroblasts phorbol esters and PDGF both stimulate 80 kDa protein phosphorylation through PkC activation [5] our present observations with TPA suggest that an initial effect of PDGF on glial cells may be to stimulate phosphorylation of the 80 kDa protein through PkC activation. This is presently being tested and it will be relevant to discover whether different classes of glial cells, especially progenitor glia, respond to PDGF in the same way. It is not yet clear whether all glial cell classes in our primary cultures give the protein phosphorylation effect seen above. When glial subcultures [29] containing Type 2 astrocytes, oligodendrocytes and progenitor glia but virtually devoid of Type 1 astrocytes

were treated with 25 nM TPA using the above experimental conditions a marked stimulation in phosphorylation of the 80,000 Da polypeptide (Fig. 2) was noted within 1 min of TPA application. Phosphorylation of the 80,000 Da polypeptide was not maximal at this point unlike the situation with Swiss 3T3 cells [26]. This work with subcultures reveals that the stimulation of protein phosphorylation induced by TPA is certainly associated with Type 2 astrocytes, oligodendrocytes and progenitor glia. It is now necessary to separate these glial classes, and to examine protoplasmic (Type 1) astrocytes, to assess the PkC-induced protein phosphorylation response of individual glial cell types. We are also comparing these TPA effects on PkC activation with those produced by diacylglycerol, the natural second messenger initiating PkC activation.

This work is supported by grants from The Multiple Sclerosis Society of Great Britain and Northern Ireland and The Wellcome Trust. We thank The Multiple Sclerosis Research Trust for tissue culture facilities.

1 Albert, K.A., Walaas, S.I., Wang, J.K.-T. and Greengard, P., Widespread occurrance or '87kDa', a major specific substrate for protein kinase C, Proc. Natl. Acad. Sci. U.S.A., 83 (1986) 2822–2826.

2 Anderson, W.B. and Salomon, D.S., Calcium, phospholipid-dependent protein kinase C as a cellular receptor for phorbol ester tumor promoters: possible role in modulating cell growth and tumor promotion. In J.F. Kuo (Ed.), Phospholipids and Cellular Regulation, Vol. 2, CRC, Boca raton. FL., 1986, pp. 127–170.

3 Ashendel, C.L., The phorbol ester receptor: a phospholipid regulated protein kinase, Biochim. Biophys. Acta, 822 (1985) 219–242.

4 Besterman, J.M., Duronio, V. and Cuatrecasas, P., Rapid formation of diacylglycerol from phosphatidylcholine: a pathway for the generation of a second messenger, Proc. Natl. Acad. Sci. U.S.A., 83 (1986) 6785–6789.

5 Blackshear, P.J., Witters, L.A., Girard, P.R., Kuc, J.F. and Quamo, S.N., Growth factor-stimulated protein phosphorylation in 3t3-L1 cells, J. Biol. Chem., 260 (1985) 13304–13315.

6 Blackshear, P.J., Wen, L., Glynn, B.P. and Witters, L.A., Protein kinase C-stimulated phosphorylation in vitro of a Mr80,000 protein phosphorylated in response to phorbol esters and growth factors in intact fibroblasts, J. Biol. Chem., 261 (1986) 1459–1469.

7 Bressler, J.P., Grotendorst, G.R., Levitov, C. and Hjelmeland, L.M., Chemotaxis of rat brain astrocytes to platelet-derived growth factor, Brain Res., 344 (1985) 249–254.

8 Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y., Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters, J. Biol. Chem., 257 (1982) 7847–7851.

9 Di Virgilio, F., Lew, D.P. and Pozzan, T., Protein kinase C activation of physiological processes in human neutrophils at vanishingly small cytosolic Ca^{2+} levels, Nature (Lond.), 310 (1984) 691–693.

10 Drust, D.S. and Martin, T.F.J., Protein kinase C translocates from cytosol to membrane upon hormone activation: effects of thyrotropin-releasing hormone in GH3 cells, Biochem. Biophys. Res. Commun., 128 (1985) 531–537.

11 Fawthrop, D.J. and Evans, R.J., The morphological differentiation of cultured astrocytes induced by ionomycin: lack of dependence on protein kinase C activation, Neurosci. Lett., 81 (1987) 257–262.

12 Girard, P.R., Mazzei, G.J., Wood, J.G. and Kuo, J.F., Polyclonal antibodies to phospholipid/Ca^{2+}-dependent protein kinase and immunocytochemical localisation of the enzyme in rat brain, Proc. Natl. Acad. Sci. U.S.A., 82 (1985) 3030–3034.

13 Honegger, P., Protein kinase C-activating tumour promotors enhance the differentiation of astrocytes in aggregating fetal brain cell cultures, J. Neurochem., 46 (1986) 1561–1566.

14 Kishimoto, A., Takai, Y., Mori, T., Kikkawa, U. and Nishizuka, Y., Activation of calcium and phospholipid-dependent protein kinase by diacylglycerol: its possible relation to phosphatidylinositol turnover, J. Biol. Chem., 255 (1980) 2273–2276.
15 Miller, R.J. Protein kinase C: a key regulator of neuronal excitability?, Trends Neurosci., 9 (1986) 538–541.
16 Murphy, J.A., Chapman, J.A., Suckling, A.J. and Rumsby, M.G., Protein kinase C activity in soluble fractions from glial cells in primary culture and subcultures, Neurosci. Lett., in press.
17 Murphy, S., McCabe, N., Morrow, C. and Pearce, B., Phosbol ester stimulates proliferation of astrocytes in primary culture, Dev. Brain Res., 31 (1987) 133–135.
18 Neary, J.T., Norenberg, L.-O.B. and Norenberg, M.D., Calcium-activated, phospholipid-dependent protein kinase and protein substrates in primary cultures of astrocytes, Brain Res., 385 (1986) 420–424.
19 Nishizuka, Y., Studies and perspectives of protein kinase C, Science, 233 (1986) 305–312.
20 Noble, M., Murray, K., Stroobant, P., Waterfield, M.D. and Riddle, A.N.P., Platelet-derived growth factor promotes division and motility and inhibits premature differentiation of the oligodendrocyte-Type 2 astrocyte progenitor cell, Nature (Lond.), submitted.
21 Pearce, B., Morrow, C. and Murphy, S., Receptor-mediated phospholipid hydrolysis in astrocytes, Eur. J. Pharmacol., 121 (1986) 231–243.
22 Pelech, S.L. and Vance, D.E., Regulation of phosphatidylcholine biosynthesis, Biochim. Biophys. Acta, 779 (1984) 217–251.
23 Pelech, S.L., Paddon, H.B. and Vance, D.E., Phorbol esters stimulate phosphatidylcholine biosynthesis by translocation of CTP: phosphocholine cytidylyltransferase from cytosol to microsomes, Biochim. Biophys. Acta, 795 (1984) 447–451.
24 Richardson, W., Pringle, N., Mosley, M.,. Westermark, B. and Dubois-Dalcq. M., A role for platelet-derived growth factor in gliogenesis in the CNS, Cell, in press.
25 Ritchie, T., Cole, R., Hun-Soo, K., de Vellis, J. and Noble, E.P., Inositol phospholipid hydrolysis in cultured astrocytes and oligodendrocytes, Life Sci., 41 (1987) 31–39.
26 Rozengurt, E., Rodriguez-Pena, M. and Smith, K.A., Phorbol esters, phospholipase C and growth factors rapidly stimulate the phosphorylation of a Mr80,000 protein in intact quiescent 3T3 cells, Proc. Natl. Acad. Sci. U.S.A., 80 (1983) 7244–7248.
27 Witters, L.A. and Blackshear, P.J., Protein kinase C-mediated phosphorylation in intact cells, Methods Enzymol., 141 (1987) 412–424.
28 Walker, A.G., Chapman, J.A., Bruce, C.B. and Rumsby, M.G., Immunocytochemical characterisation of cell cultures grown from dissociated 1-2 day post-natal rat cerebral tissue, J. Neuroimmunol., 7 (1985) 1–20.
29 Walker, A.G., Chapman, J.A. and Rumsby, M.G., Immunocytochemical demonstration of glial-neuronal interactions and myelinogenesis in subcultures of rat brain cells, J. Neuroimmunol., 9 (1985) 159–177.
30 Wu, W.C.-S., Walaas, C.I., Nairn, A.C. and Greengard, P., Calcium/phospholipid regulates phosphorylation of a Mr'87k' substrate protein in brain synaptosomes, Proc. Natl. Acad. Sci. U.S.A., 79 (1982) 5249–5253.

Neuroscience Letters, 85 (1988) 255–260
Elsevier Scientific Publishers Ireland Ltd.

NSL 05140

Protein kinase C activity in soluble fractions from glial cells in primary culture and subcultures

John A. Murphy, Jill A. Chapman, Anthony J. Suckling and Martin G. Rumsby

Department of Biology, University of York, York (U.K.)

(Received 2 October 1987; Revised version received 27 October 1987; Accepted 28 October 1987)

Key words: Protein kinase C; Glial cell; Specific activity; Primary culture; DE-52 chromatography; Inhibitor; Membrane-associated–cytosolic compartment

Protein kinase C (calcium + phospholipid-dependent kinase) activity has been measured in soluble 100,000 *g* fractions from mixed glial cells in primary culture; in 12 day cultures the specific activity (mean ± S.D.) was 184 ± 10 pmol ^{32}P incorporated/10 min/mg protein. In glial cell subcultures lacking protoplasmic astrocytes protein kinase C specific activity was lower. An inhibitor of protein kinase C in 100,000 *g* supernatants was removed by chromatography through DE-52 anion exchange resin increasing the specific activity of the calcium + phospholipid-dependent kinase about 20 times. Protein kinase C was also associated with membrane fractions from glial cells; the membrane-associated enzyme had a higher specific activity than in the cytoplasm.

Protein kinase C (PkC), an enzyme associated with a variety of cell responses including secretion, proliferation and differentiation [20], is very active in central nerve tissue (CNS) [7] where its role in neurones has been extensively examined (summarized in refs. 9, 15). PkC is characterized by its requirement for calcium + phospholipid for activation [19]. This enzyme is activated during agonist binding and the receptor-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate [3, 27] since the diacylglycerol produced in this signal transduction pathway increases the affinity of PkC for Ca^{2+}, allowing for PkC activation at physiological Ca^{2+} levels [10]. PkC is a major receptor for the tumour-promoting phorbol esters [2].

Little attention has been paid to the localization and role of PkC in glial cells even though the receptor-linked phosphoinositol pathway can be activated in both astrocytes and oligodendrocytes by certain agonists [22, 23, 25] and myelin basic protein synthesized by oligodendrocytes is a good substrate for PkC [28]. Immunocytochemical approaches suggest that PkC is localized in cells resembling oligodendrocytes in rat brain white matter [5]. Astrocyte primary cultures show measurable PkC activity

Correspondence: M.G. Rumsby, Department of Biology, University of York, York YO1 5DD, U.K.

0304-3940/88/$ 03.50 © 1988 Elsevier Scientific Publishers Ireland Ltd.

[18] while the differentiation and proliferation of this glial cell class are enhanced by phorbol esters [6, 16], all indicating a role for PkC in astrocyte biochemistry. Our interest is in the biochemistry of myelination by oligodendrocytes and here we report the direct measurement of PkC activity in our primary glial cell cultures and subcultures derived from rat brain [29, 30].

We have previously described the preparation and characterization of our glial cell primary cultures which are prepared using cerebra from 1–2 day rat pups [29]. These primary cultures develop a confluent layer of protoplasmic astrocytes on which grow smaller multiprocessed phase bright cells which are largely fibrous astrocytes, oligodendrocytes and progenitor glial cells [24] with a small number of neurones. Subcultures prepared from 9-day-old primary cultures [30] using a modification of the shaking technique of McCarthy and de Vellis [12] lack protoplasmic astrocytes and by 21–28 days contain interlinked clumps of phase bright cells, chiefly fibrous astrocytes, oligodendrocytes, glial progenitor cells and a few neurones [30].

For PkC assay cultures were rinsed and cells then scraped out and homogenized in 50 mM Tris-HCl pH 7.7, 5 mM dithiothreitol and 2 mM EGTA. Homogenates were centrifuged at 100,000 *g* for 60 min at 4°C to give soluble and particulate fractions. Kinase activity in samples was measured by following the transfer of the γ-phosphate group of [^{32}P]ATP to type IIIS calf thymus histone (Sigma) for 10 min at 30°C with shaking. The complete reaction mixture (0.25 ml) for PkC measurement was as described by Kikkawa et al. [8] and contained 50 mM Tris-HCl 7.7, 50 μg histone, 100 μM calcium chloride, 10 mM magnesium acetate, 50 μM ATP, 2 μCi [γ-^{32}P]ATP(3000 Ci/mmol, Amersham), 10 μg phosphatidylserine (P.S.; Lipid Pro-

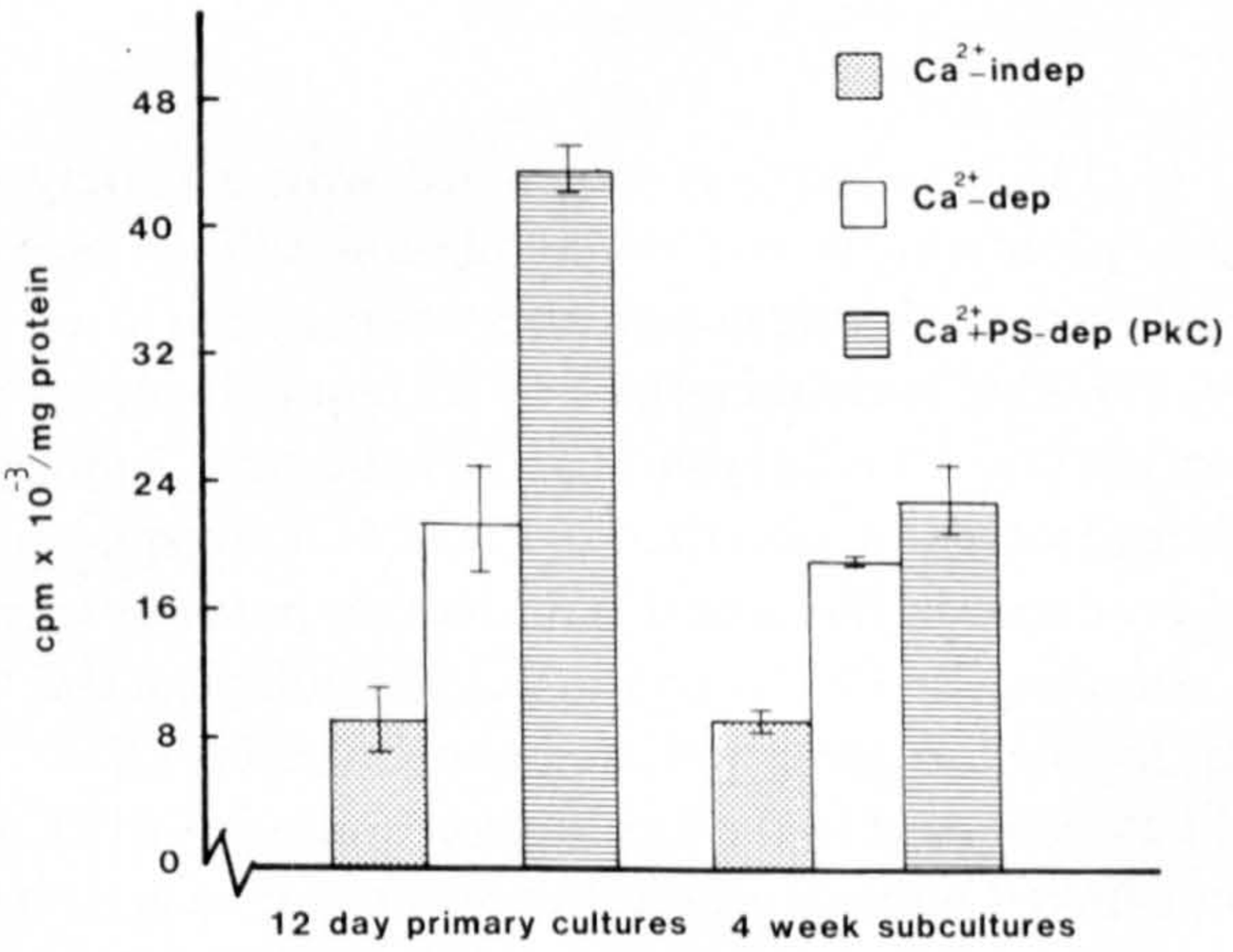


Fig. 1. Incorporation of γ-phosphate group of [γ-^{32}P]ATP into histone by calcium-independent (basal), calcium-dependent, and calcium- + phospholipid-dependent (PkC) kinases in 100,000 *g* supernatants of glial cells in 12 day primary cultures, and in subcultures. Results are means ± S.D. (bar lines) from several separate culture series each assayed in quadruplicate. Specific activities (pmol ^{32}P incorporated/10 min/mg protein) related to these results are 59.5, 120 and 243 for 12 day primary cultures and 53, 107 and 128 for subcultures respectively. Absolute specific activities for calcium-dependent kinases and calcium + phospholipid-dependent kinase are the relevant result less the calcium-independent figure.

ducts) + 0.2 μg diolein (Sigma) and the enzyme sample. PS + diolein were freshly prepared for each assay [8]. Basal kinase activity (Ca^{2+}-independent) was measured in the presence of 100 μM EGTA omitting calcium chloride and lipid from the assay. Ca^{2+}-dependent kinase activity was measured with calcium chloride present omitting EGTA and lipid. PkC activity was assayed with the full system above omitting EGTA. All assays were in quadruplicate and reactions were stopped by the addition of ice-cold 25% trichloroacetic acid (TCA). TCA-precipitable material was recovered on glass-fibre filters (Whatman GF/F, pore size 0.7 μm) which were dried prior to scintillation counting. Kinase specific activities (means $\pm$ S.D.) are expressed as pmol ^{32}P incorporated/10 min/mg protein at 30°C; protein was determined using the method of Lowry et al. [11] with bovine serum albumin as standard.

Basal kinase activity in 100,000 *g* supernatants from 12 day glial cells in primary culture (Fig. 1) more than doubled on addition of calcium due to the variety of calcium-dependent kinases, including calcium/calmodulin kinase, which have now been identified in CNS tissue [17]. Addition of PS in the presence of calcium resulted in a further doubling of phosphorylation per mg protein (Fig. 1) indicating the presence of significant PkC activity in the glial cell supernatant. The specific activity of PkC in supernatants from 12 day glial cells was 184 ± 10 pmol ^{32}P incorporated/10 min/mg protein compared with 60 for calcium-dependent kinases. Calcium + phospholipid-dependent kinase activity was also detected in glial cell supernatants from 18 and 28 day primary cultures but at the much lower specific activities of 51 ± 20 and 53 ± 8 for day 18 and 28 respectively; corresponding calcium-dependent kinase activity was 33 ± 10 and 44 ± 6 pmol ^{32}P incorporated/10 min/mg protein. In supernatants from 28 day glial cell subcultures (Fig. 1) there was a small increase in phosphorylation when PS was included in assays in the presence of calcium indicating a low PkC activ-

TABLE 1

EFFECT OF DE-52 COLUMN PURIFICATION OF Ca^{2+}-INDEPENDENT, Ca^{2+}-DEPENDENT AND Ca^{2+} + PHOSPHOLIPID-DEPENDENT KINASE ACTIVITY IN GLIAL CELL 100,000 *g* SUPERNATANTS

Glial cell fraction	Culture ref.	Specific activity (pmol ^{32}P incorp./10 min/mg protein)		
		Ca^{2+}-ind.	Ca^{2+}-dep.	Ca^{2+} + PS-dep.
Primary culture				
100,000 *g* supernatant	30/61[b]	47.1 ± 1.0	87.2 ± 3.7	112.1 ± 4.0
Column-purified[a]				
100,000 *g* supernatant		168.9 ± 20.5	249.5 ± 25.9	2728.2 ± 30.8
Primary culture				
100,000 *g* supernatant	10/71[c]	92.9 ± 4.2	159.5 ± 9.4	200.4 ± 5.8
Column-purified[a]				
100,000 *g* supernatant		122.4 ± 19.2	127.4 ± 32.8	1151.7 ± 24.9

[a]100,000 *g* Supernatant taken and purified on a DE-52 column as detailed in the Methods section.
[b]18-Day primary culture.
[c]4-Week primary culture.

ity: the specific activity of PkC was 75 ± 15 pmol ^{32}P incorporated/10 min/mg protein compared with the figure of 54 ± 1 for calcium-dependent kinases.

It is known that the detection of PkC activity in crude 100,000 *g* cell soluble fractions is made complicated [8, 26] by the presence of various endogenous inhibitors and a 17 kDa protein inhibitor of PkC has been clearly indentified in central nerve tissue [13, 14]. To confirm the presence of PkC activity in the glial cells therefore we followed the suggestion of Kikkawa et al. [8] and submitted 100,000 *g* crude supernatants from 18 and 28 day glial cell primary cultures to initial purification by DE-52 anion exchange chromatography. Crude supernatants were divided into two portions; one part was held on ice at 4°C while the other was rapidly fractionated on a small DE-52 column as described by Anderson et al. [1]. The unfractionated and fractionated supernatants were then assayed for kinase activities simultaneously (Table I). This simple purification made it very clear that glial supernatants do indeed contain significant calcium + phospholipid-dependent kinase (PkC) activity. Compared with the unfractionated 100,000 *g* supernatant the specific activity of PkC after DE-52 purification is increased by over 20-fold in 18 day cultures and almost 6-fold in 28 day cultures (Table I). We presume that PkC activity is not so readily measured in the crude glial cell supernatants, especially at older culture ages, due to the presence of inhibitory factors. It will be of interest to see if these glial cell supernatants contain the same calcium-independent 17 kDa protein inhibitor of PkC noted already in nerve tissue [13, 14] and what role such an inhibitor plays in PkC regulation in glial cells.

In most tissues PkC is detected largely in the soluble fraction of cells [20]. However, in the CNS, PkC has two localizations, associated with membranes as the active enzyme, and in the inactive form in the cytoplasm [7]. We have confirmed this dual localization for PkC in glial cells. Total membrane pellets from 12 day primary glial cells were treated with Triton X-100 to release the enzyme as described by Parker et al. [21] and the kinase activities assayed. Calcium + phospholipid-dependent kinase activity was released from membranes, the specific activity of this membrane-associated PkC being 485 ± 120 pmol ^{32}P incorporated/10 min/mg protein compared with 180 ± 10 for the enzyme in the cytoplasmic (soluble) fraction. With older primary cultures (28 days) the specific activity of PkC associated with membranes was similar to that in the soluble fraction. Preliminary experiments have also shown that pretreatment of glial cultures with 100 nM 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) for 30 min causes translocation of PkC from the soluble fraction to membranes. This is in keeping with the known role of PkC as a receptor for phorbol esters such as TPA [2] and the fact that phorbol esters mimic the activating role of diacylglycerol. The distribution of PkC between cytoplasm and membranes is probably a reflection of the state of activation of the enzyme in cells. As PkC is closely linked with receptor-mediated inositol phospholipid turnover membrane-associated PkC activity is an indication that agonist-stimulated pathways may be functional in the cells at that stage of growth and development. Our observation that glial cells at 12 days in culture have a higher specific activity ratio of membrane-bound/cytosolic PkC than at 28 days is perhaps an indication that agonist-stimulated events involving

PkC are more active in early stages of glial cell growth.

In this study we have obtained direct evidence that cultured glial cells derived from neonatal rat brain tissue contain a calcium- and PS-activated protein kinase which transfers the γ-phosphate of ATP to a histone acceptor. These are the characteristics of PkC [17, 19, 20]. Thus the findings described above show directly that mixed populations of CNS glia composed of protoplasmic and fibrous astrocytes together with oligodendrocytes contain PkC activity. These findings directly support previous indications suggesting a presence of PkC in cells other than neurones in the CNS [5, 16, 18, 22, 25]. Neurofilament protein-positive cells account for less than 6% of the total cell population in our early cultures [29] and this percentage decreases with culture age. It is therefore unlikely that the PkC activity that we have detected comes exclusively from the small number of neurones present. Further the specific activity of PkC in neuronal cultures is higher [4] than we have detected here. The present study does not yet reveal which individual glial cell classes contain PkC but from the previous evidence [5, 16, 18, 22, 25] it is likely that PkC is active in both astrocytes and oligodendrocytes. Certainly we have noted that calcium + phospholipid-dependent kinase (PkC) activity can be measured in the protoplasmic astrocytes which remain in primary culture after the release by shaking of the smaller glial cells for our subcultures: the specific activity of the protoplasmic astrocytes is about half that of the whole glial cultures before shaking. This value is considerably lower than the figure reported by Neary et al. [18] for PkC activity in 23,000 *g* cytosol fractions from 95%-pure primary astrocyte cultures. However, our culture system and methodology are substantially different. We are now involved in studies to define the role of PkC and its inhibitor in different glial cell classes, especially oligodendrocytes.

This work is supported by grants from The Wellcome Trust and from The Multiple Sclerosis Society of Great Britain and Northern Ireland. We also thank The Multiple Sclerosis Research Trust for facilities. Rachel Daniel provided excellent technical assistance.

1 Anderson, W.B., Estival, A., Tapiovaara, H. and Gopalakrishna, R., Altered subcellular distribution of protein kinase C (a phorbol ester receptor). Possible role in tumour promotion and the regulation of cell growth: relationship to changes in adenylate cyclase activity. In D.M.F. Cooper and K.B. Seamon (Eds.), Advances in Cyclic Nucleotide and Protein Phosphorylation Research, Vol. 19, Raven, New York, 1985, pp. 287–306.

2 Ashendel, C.L., The phorbol ester receptor: a phospholipid regulated protein kinase, Biochim. Biophys. Acta 822 (1985) 219–242.

3 Berridge, M.J., Inositol triphosphate and diacylglycerol as second messengers, Biochem. J., 220 (1984) 345–360.

4 Burgess, S.K., Sahyoun, N., Blanchard, S.G., LeVine, H., Chang, K.-J. and Cuatrecasas, P., Phorbol ester receptors and protein kinase C in primary neuronal cultures: development and stimulation of endogenous phosphorylation, J. Cell Biol., 102 (1986) 312–319.

5 Girard, P.R., Mazzei, G.J., Wood, J.G. and Kuo, J.F., Polyclonal antibodies to phospholipid/Ca^{2+}-dependent protein kinase and immunocytochemical localisation of the enzyme in rat brain, Proc. Natl. Acad. Sci. USA, 82 (1985) 3030–3034.

6 Honegger, P., Protein kinase C-activating tumour promotors enhance the differentiation of astrocytes in aggregating fetal brain cell cultures, J. Neurochem., 46 (1986) 1561–1566.

7 Kikkawa, U., Takai, Y., Minakuchi, R., Inohara, S. and Nishizuka, Y., Calcium-activated, phospholi-

pid-dependent protein kinase from rat brain: subcellular distribution, purification, and properties, J. Biol. Chem., 257 (1982) 13341–13348.
8 Kikkawa, U., Minakuchi, R., Takai, Y. and Nishizuka, Y., Calcium-activated, phospholipid-dependent protein kinase (protein kinase C) from rat brain. In J.D. Corbin and J.G. Hardman (Eds.), Methods in Enzymology, Vol. 99, Academic, New York, 1983, pp. 288–298.
9 Kikkawa, U., Kitano, T., Saito, N., Fujiwara, H., Nakanishi, H., Kishimoto, A., Taniyama, K., Tanaka, C. and Nishizuka, Y., Possible roles of protein kinase C in signal transduction in nervous tissues, Prog. Brain Res., 69 (1986) 29–35.
10 Kishimoto, A., Takai, Y., Mori, T., Kikkawa, U. and Nishizuka, Y., Activation of calcium and phospholipid-dependent protein kinase by diacylglycerol: its possible relation to phosphatidylinositol turnover, J. Biol. Chem., 255 (1980) 2273–2276.
11 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., Protein measurement with the Folin phenol reagent, J. Biol. Chem., 193 (1951) 265–275.
12 McCarthy, K.D. and de Vellis, J., Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue, J. Cell. Biol., 85 (1980) 890–902.
13 McDonald, J.R. and Walsh, M.P., Ca^{2+}-binding proteins from bovine brain including a potent inhibitor of protein kinase C, Biochem. J., 232 (1985) 559–567.
14 McDonald, J.R., Groschel-Stewart, U. and Walsh, M.P., Properties and distribution of the protein inhibitor (Mr 17000) of protein kinase C, Biochem. J., 242 (1987) 695–705.
15 Miller, R.J., Protein kinase C: a key regulator of neuronal excitability?, Trends Neurosci., 9 (1986) 538–541.
16 Murphy, S., McCabe, N., Morrow, C. and Pearce, B., Phorbol ester stimulates proliferation of astrocytes in primary culture, Dev. Brain Res., 31 (1987) 133–135.
17 Nairn, A.C., Hemmings, H.C. and Greengard, P., Protein kinases in the brain, Annu. Rev. Biochem., 54 (1985) 931–976.
18 Neary, J.T., Norenberg, L.-O.B. and Norenberg, M.D., Calcium-activated, phospholipid-dependent protein kinase and protein substrates in primary cultures of astrocytes, Brain Res., 385 (1986) 420–424.
19 Nishizuka, Y., Role of protein kinase C in cell surface signal transduction and tumour promotion, Nature (Lond.), 308 (1984) 693–698.
20 Nishizuka, Y., Studies and perspectives of protein kinase C, Science, 233 (1986) 305–312.
21 Parker, J., Daniel, L.W. and Waite, M., Evidence of protein kinase C involvement in phorbol diester-stimulated arachidonic acid release and prostaglandin synthesis, J. Biol. Chem., 262 (1987) 5385–5393.
22 Pearce, B., Cambray-Deakin, M., Morrow, C., Grimble, J. and Murphy, S., Activation of muscarinic and of α_1-adrenergic receptors on astrocytes results in the accumulation of inositol phosphates, J. Neurochem., 45 (1985) 1534–1540.
23 Pearce, B., Morrow, C. and Murphy, S., Receptor-mediated phospholipid hydrolysis in astrocytes, Eur. J. Pharmacol., 121 (1986) 231–243.
24 Raff, M.C., Miller, R.H. and Noble, M., A glial progenitor cell that develops in vitro into an astrocyte or an oligodendrocyte depending on culture medium, Nature (Lond.), 303 (1983) 390–396.
25 Ritchie, T., Cole, R., Hun-Soo, K., de Vellis, J. and Noble, E.P., Inositol phospholipid hydrolysis in cultured astrocytes and oligodendrocytes, Life Sci., 41 (1987) 31–39.
26 Sahyoun, N., Levine, III, H., McConnell, R., Bronson, D. and Cuatrecasas, P., A specific phosphoprotein phosphatase acts on histone H1 phosphorylated by protein kinase C, Proc. Natl. Acad. Sci. USA, 80 (1983) 6760–6764.
27 Sekar, M.C. and Hokin, L.E., The role of phosphoinositides in signal transduction, J. Membrane Biol., 89 (1986) 193–210.
28 Turner, R.S., Chou, C.-H.J., Mazzei, G.J., Dembure, P. and Kuo, J.F., Phospholipid-sensitive Ca^{2+}-dependent protein kinase preferentially phosphorylates serine-115 of bovine myelin basic protein, J. Neurochem., 43 (1984) 1257–1264.
29 Walker, A.G., Chapman, J.A., Bruce, C.B. and Rumsby, M.G., Immunocytochemical characterisation of cell cultures grown from dissociated 1-2-day post-natal rat cerebral tissue, J. Neuroimmunol., 7 (1985) 1–20.
30 Walker, A.G., Chapman, J.A. and Rumsby, M.G., Immunocytochemical demonstration of glial-neuronal interactions and myelinogenesis in subcultures of rat brain cells, J. Neuroimmunol., 9 (1985) 159–177.