Protein Kinases and Protein Phosphatases in the Central Nervous System-Identification, Characterisation and Functional Correlates

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Abstract

Protein phosphorylation is an important mechanism in the regulation of many brain cell functions. The precise molecular mechanisms can be elucidated by identification and characterisation of the protein kinases and phosphatases involved. Astrocytes play a vital role in the physiology and metabolic activity of the vertebrate brain and the present study sought to explore the roles of some specific protein kinases and phosphatases in CNS astrocytes and rat brain.

The four main protein phosphatases (PP1, PP2A, PP2B, PP2C) were identified in astrocytes and their relative activities measured and compared to other cell types. There was an approximately equal expression of PP1 and PP2A in astrocytes whereas hepatocytes contain more PP2A and human lung mast cells more PP1. Total PP activity increased as astrocytes developed in culture with a peak at day 10. However, there was no change in the relative activities of each PP over this period. Inhibition of PP1 and PP2A by okadaic acid had profound effects on phospholipid associated signal transduction pathways causing stimulation of arachidonic acid release and inhibition of noradrenaline-stimulated inositol phosphate production.

There were subtle differences in the properties of AMP-activated protein kinase (AMPK) from rat brain compared to liver. Brain AMPK had a lower specific activity but was more sensitive to activation by AMP and less sensitive to inhibition by phosphocreatine. Levels of AMPK activity were similar in different brain regions and showed no changes during the first 21 days of postnatal development. The activity of ACC, a major substrate of AMPK, was highest in cortex and lowest in cerebellum and during postnatal development peaked at day 14. The activities of PP2C and PP2A, responsible for dephosphorylation of AMPK and ACC, respectively, did not change over 21 days of postnatal brain development. AMPK activity was identified in astrocytes but ACC activity was undetectable. A possible alternative AMPK target in astrocytes, glial fibrillary acidic protein (GFAP), was investigated as a possible substrate for AMPK.

The activity ratio of cAMP-dependent protein kinase (PKA) was higher in cerebellum than the other brain regions examined and this correlated with a greater proportion of the type I isoform of PKA. Type I PKA from cerebellum exhibited significant cAMP-independent activity which was not observed in type I PKA from rat heart. A tendency for the PKA activity ratio to decrease through 21 days post-natal brain development and through 21 days astrocyte growth in culture, correlated with a tendency for a decrease in the ratio of type I : type II PKA activity.
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‘Lift up your eyes on high, and behold who hath created these things...’

Isaiah 40:26a
Publications arising from this thesis

Some of the results presented in this thesis have been published in the following journals:


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<td>AcCoA</td>
<td>Acetyl Coenzyme A</td>
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<td>Deoxyribonuclease</td>
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<td>Dithiothreitol</td>
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eNOS  Endothelial NO Synthase
EBSS  Earles Balanced Salt Solution
EAE  Experimental Allergic Encephalomyelitis
FPLC  Fast Performance Liquid Chromatography
GABA  Gamma-Amino Butyric Acid
GFAP  Glial Fibrillary Acidic Protein
GLAST  Na^+-dependent Glutamate/Aspartate Transporter
GLT-1  Na^+-dependent Glutamate Transporter-1
GM  Growth Medium
GPAT  sn-Glycerol-3-phosphate Acyltransferase
GS  Glutamine Synthetase
^H-IP  ^H-Inositol Phosphates
HLMC  Human Lung Mast Cells
HMG-CoAR  Hydroxy Methyl-Glutaryl-Coenzyme A Reductase
HSL  Hormone-Sensitive Lipase
I-1  Protein Phosphatase 1 Heat-Stable Inhibitor-1
I-2  Protein Phosphatase 1 Heat-Stable Inhibitor-2
IBMX  Iso-Butyl Methyl Xanthine
iNOS  Inducible Nitric Oxide Synthase
IFN-γ  Interferon-γ
IL-1  Interleukin-1 (-2,-3,-4...)
IP_3  Inositol 1,4,5-Trisphosphate
JNK  c-Jun NH\(^2\)-terminal Kinase
Kd  Dissociation Constant
Ki  Inhibitor Constant
LPS  Lipopolysaccharide
LTP  Long-term Potentiation
MAP2  Microtubule-Associated Protein 2
MAPK  Mitogen-activated Protein Kinase
MEK  MAPK Kinase
MEM  Minimal Essential Medium
MHC  Major Histocompatibility Complex
MiCK  Mitochondrial Creatine Kinase
MLC  Myosin Light Chain
MLCK  MLC Kinase
MM-CK  Cytosolic Creatine Kinase (muscle form)
mRNA  Messenger RNA
MS  Multiple Sclerosis
NA  Noradrenaline
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<td>Nuclear Factor -κB</td>
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<td>NMDA</td>
<td>N-Methyl-D-Aspartate</td>
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<td>NO</td>
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<td>Tumour Necrosis Factor-α</td>
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<td>VIP</td>
<td>Vasoactive Intestinal Peptide</td>
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<td>VSMC</td>
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<td>ZMP</td>
<td>5-Aminoimidazole-4-Carboxamide Ribonucleoside Monophosphate</td>
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CHAPTER 1

INTRODUCTION
1.1 Introduction

Modulation of neuronal activity by extracellular signals is critical for the maintenance and adaptation of brain function. Many hormone and neurotransmitter-stimulated signalling pathways alter the activities of target proteins by reversibly phosphorylating serine and threonine residues. This thesis has studied protein phosphorylation in rat brain during 21 days postnatal development, in regions of adult rat brain, and in astrocytes. The mammalian brain was divided up, for the purposes of this study, into 4 regions. These are the neocortex, which contains the cerebral cortex and hippocampus, the sub cortex, consisting of the striatum, thalamus, hypothalamus and amygdala, the cerebellum, and the ‘pons’, which actually contained the pons, medulla and brainstem.

No region of the brain functions alone, although major functions of each area have been determined. The neocortex coordinates sensory data and motor functions, reasoning, learning and memory. The sub cortex relays information to the cerebral cortex and is the area from which homeostasis is regulated and the endocrine system is controlled. The medulla, brainstem and pons control heart rate, vasoconstriction, breathing and senses. The main role of the cerebellum is to regulate balance, timing and precision of body movements, maintenance of muscle tone and posture. Each region dissected for this study has its own range of functions, although most of these tasks are not exclusive. In spite of the varying roles in different brain areas, the cell types are not localised to any particular area but are present throughout the brain.

The CNS contains two main types of cell, neurons and glial cells. The latter are more numerous and there are four main types; astrocytes, oligodendrocytes, microglia and ependymal cells. Astrocytes are the largest and most numerous glial cell, and play a vital role in the physiology and metabolic activity of the vertebrate brain (Kimelberg and Norenberg, 1989). However, there is much to learn about the mechanisms by which these
cells communicate and about the intracellular signal transduction processes that occur in response to an external signal. Astrocyte signalling was studied with particular reference to the control of intracellular pathways and mediator release by reversible protein phosphorylation.

1.2 Overview of astrocytes as cells of the CNS

The mammalian brain consists mainly of two types of cells; neurons and glial cells. Neurons have attracted far more scientific attention than glial cells, since neurons and neuronal circuits are responsible for information processing in the CNS. However, during the past 25 years or so, methods for identifying and isolating individual cell types from the brain have been developed. The importance of the role played by glial cells has begun to emerge. Neuroglia were so-named from the Greek meaning 'nerve glue' as it was thought they had a passive structural role, filling in the gaps between neurons, in the same way that the fluid in the extracellular space does in other tissues. This view is being replaced by a more dynamic picture in which glia play an active role in the development
and function of the nervous system (Martin, 1992), including intercellular communication within the brain (Cooper et al, 1995).

Figure 1.2 Astrocyte structure and anatomy (Kimelberg and Norenberg, 1989)

Properties of the neuroglia are as follows. Oligodendrocytes wrap themselves around axons to form myelin sheaths in the CNS as schwann cells do in the peripheral nervous system. Microglia are the smallest glial cell and have several branched processes; they are found near to neurons and blood vessels. Microglia are supporting cells that protect the nervous system by destroying invasive microorganisms and other materials that could be harmful, being a special form of macrophage. Ependymal cells are cells that line the central cavities of the brain and spinal cord. They are ciliated and act as a semi-permeable lining between the cavities and normal tissue. The cavities are filled with a cerebro-spinal fluid which cushions the CNS and, due to the beating cilia of the ependymal cells, is constantly circulating. Astrocytes, named for their star-like shape, are the most abundant
type of glial cell. They possess a large number of processes and form elaborate networks with neighbouring cells; for example they attach to neurons at their cell body, nodes of Ranvier and at the synapses, to vascular endothelial cells via the end feet of their processes and to the processes of other astrocytes, with which they form gap junctions (Smith et al, 1992). The capillaries of the brain are completely surrounded by astrocytic end feet, this causes a change in the endothelial cells of the capillary walls so that they form tight junctions rather than being 'leaky' as they are in the peripheral circulation. In this way the astrocytes are responsible for the formation of the blood brain barrier (BBB) which alters the permeability of the brain capillaries, thus restricting the entry of water soluble substances to those for which a transport system exists (reviewed by Goldstein and Betz, 1986)

It is now evident that astrocytes have many roles in the maintenance of neuronal function. Astrocytes form a vital partnership with neurons and are active in many aspects of brain function, particularly in the maintenance of homeostasis of the neuronal environment (Lindsay, 1979, Hertz, 1981). They store energy as glycogen and supply fuel molecules for neuronal activities (Cataldo and Broadwell, 1986a, Swanson and Choi, 1993), and modulate the extracellular distribution of neuro-transmitters by their synthesis, uptake, metabolism and release (Erecinska et al, 1986, Martinez-Hernandez et al, 1977). It is possible that the astrocytes, through their unique position in contact with the synapse, together with their ability to regulate the neuronal environment, are able to influence neuronal activity (Cooper et al, 1995). For example, the astrocyte processes attached to the synapse take up released glutamate and gamma-amino butyric acid (GABA) (Schousboe et al, 1977, Balazs et al, 1970), which are then metabolized to glutamine by the enzyme glutamine synthetase (GS), a protein found only in astrocytes within the CNS (Norenberg, 1979, Norenberg and Martinez-Hernandez, 1979). The glutamine is then conveyed to the neurons where it can be used for synthesis of more of the transmitters (Kimelberg and Norenberg, 1989).
It is not only neurotransmitter levels that are controlled by astrocytes, equally important in the brain is the maintenance of ion concentrations, which must be finely controlled since Na\(^+\) and K\(^+\) are required for generation of the action potential. Astrocytes are therefore intimately involved in the electrical signalling of the brain since they remove K\(^+\) from the synapse once the signal has passed to prevent neurons from becoming chronically depolarized by local accumulation of the ion (Orkand et al, 1966, Orkand, 1986).

Astrocytes participate in cell signalling via the synthesis and release of various neuroactive substances including neurotransmitters, cytokines, growth factors, nitric oxide, eicosanoids, steroids and adenine nucleotides (Martin, 1992). They also contain functional receptors for various mediators known to be released by neurons (Murphy and Pearce, 1987, Bevan, 1990, Kimelberg, 1995). Activation of these receptors leads to the opening of ion channels or the activation of G proteins, leading to the release of second messengers such as cAMP, inositol-1,4,5-trisphosphate (IP\(_3\)) and diacylglycerol (DG). Evidence is emerging that signalling occurs not only between astrocytes, but also from astrocytes to neurons and vice-versa (Giaume and Venance, 1998, Cooper et al, 1995, Parpura et al, 1994).

The development of the brain is an area in which astrocytes actively participate. Cell-cell contact between astrocytes and neurons in the prenatal brain may control the number of certain cell types and their correct proportions (Aschner and LoPachin, 1993).

Possible roles of astrocytes in certain disorders of the CNS

Since astrocytes perform many critical functions in both the adult and the developing brain, disturbances in these cells may have a role in certain disorders of the CNS. For example, injury to the brain initiates the process of reactive gliosis in which astrocytes are rapidly transformed to a reactive state, in which they enlarge, grow more processes...
and release various molecules (Eng et al, 1992, Norton et al, 1992, Eddleston and Mucke, 1993). This leads to the formation of a ‘glial scar’, which is often a characteristic of epilepsy. Disruption of the role of astrocytes in glutamate and GABA metabolism may also be involved in the development of epilepsy (Kimelberg and Norenberg, 1989), Alzheimer's disease, in which astrocytic production of β-amyloid protein may also be implicated (Koh et al, 1990, Mattson et al, 1992), AIDS dementia (Lipton, 1992) and Huntington's disease (Choi, 1988).

Oxidative injury to the brain has been implicated in degenerative diseases, including, epilepsy, trauma, stroke (Wilson, 1997) and Alzheimer's disease (Pappolla et al, 1992). It is a phenomenon that occurs after antioxidant mechanisms are overwhelmed. Astrocytes maintain high intracellular concentrations of certain antioxidants, making these cells particularly resistant to oxidative stress relative to oligodendrocytes and neurons (Thorburne and Juurlink, 1996, Siushansian and Wilson, 1995). Following reactive gliosis, the neuroprotective role of astrocytes may be accentuated because of increases in a number of activities: expression of antioxidant enzymes (Dalton et al, 1995, Manganaro et al, 1995); transport and metabolism of glucose that yields reducing equivalents for antioxidant regeneration and lactate for neuronal metabolism (Walker et al, 1988, McCall et al, 1996); synthesis and recycling, respectively, of the low molecular weight reductants glutathione and vitamin C (Sagara et al, 1996, Siushansian et al, 1997). In the latter process, astrocytes take up oxidized vitamin C (dehydroascorbate, DHAA) through plasma membrane transporters, reduce it to ascorbate, and then release ascorbate into the extracellular fluid, where it may contribute to the antioxidant defence of neurons (Wilson et al, 1997).

Astrocytes are likely to participate in the pathogenesis of multiple sclerosis, thought to result from an attack by the patient's own immune system on myelinated axons. The peripheral immune system is denied access to the brain by the BBB, instead, astrocytes
provide an immune response in the brain, in which they function as antigen-presenting cells and are capable of producing class I and II major histocompatibility complexes (MHC) (Sedgewick et al, 1991). It is therefore vital that research continues into the physiological functions of astrocytes in order to fully understand these and other disease states, since astrocytes are involved in many aspects of brain function.

1.3 The use of primary cultured cells as a model for astrocyte function

While it is clearly more accurate to study a cell type in situ, there are many practical problems associated with this approach. A major advance in the study of astrocytes is the use of primary cell cultures. Using this method a cell population can be obtained consisting typically of 95% astrocytes, with the main contaminants being macrophages and fibroblasts (Juurlink and Hertz, 1985). Much of the current understanding of astrocyte function could not have been obtained without the use of culture, which has become an invaluable tool for the study of these cells (Kimelberg, 1983). However, caution must be used when attempting to extrapolate data obtained using cultures to the situation in vivo (Juurlink and Hertz, 1985, Murphy and Pearce, 1987). Astrocytes in culture are growing in a strange, 2-dimensional environment, and have lost their contact with other cell types. It is not surprising therefore that they appear to exhibit differences to their in vivo counterparts, including receptor expression, mediator release and response to neurotransmitters (Porter and McCarthy, 1997, Eddleston and Mucke, 1993, Kimelberg et al, 1997). A range of factors can influence the properties of cultured astrocytes, such as the area of brain used, species, age of pups, culture medium, serum type, method of preparation and the presence of co-cultured neurons. For this reason, it should be borne in mind that the use of cultured astrocytes gives only a selective view of astrocyte function (Juurlink and Hertz, 1985). However, 'anything a cell is seen to do in culture must be counted among its potentialities' (Murray, 1977).
It is possible to identify astrocytes both by their characteristic morphology and by the use of marker proteins exclusive to astrocytes such as glutamine synthetase (GS), and the glial fibrillary acidic protein (GFAP), an astrocytic structural protein (Bignami and Dahl, 1977). Astrocytes are a heterogeneous class of cells, both \textit{in vitro} and \textit{in vivo}. Two types of astrocyte have been identified \textit{in vitro}, Type 1 are obtained when culturing cells from the neonatal cerebral cortex as in the present study. These cells have a fibroblast-like morphology and are referred to as polygonal or protoplasmic astrocytes. Astrocytes derived from areas of white matter such as the corpus callosum or the optic nerve are a mixture of Type 1 and Type 2, the latter being stellate and also termed fibrous astrocytes (Raff \textit{et al}, 1983a,b). Some differences in properties exist between the two cell types, so this is another factor to be considered when interpreting data obtained in astrocyte cultures from different brain regions. Indeed, there may be many more types of astrocyte existing \textit{in vivo} just as many types of neuron exist, and there may well be important differences between these (Norenberg, 1994, Wilkin \textit{et al}, 1990)

1.4 Signal transduction mechanisms in astrocytes

Astrocytes in the brain exist in a multicellular environment. They need to communicate with surrounding cells in order to regulate their development and organisation, control their growth and division and coordinate their functions, which are largely concerned with the surrounding cells. There are two main mechanisms of communication between cells:

1) Gap junctions or cytoplasmic bridges allowing the passage of small molecules directly into the cytoplasm of adjacent cells. Gap junction channels are a major characteristic of astrocytes, both \textit{in vitro} and \textit{in vivo} (Massa and Mugnaini, 1985, Rohlmann and Wolff, 1996). In this way astrocytes communicate with each other by the rapid transmission of small molecules such as Ca$^{2+}$, glutamine, glutamate and lactate (Giaume and Venance,
1998, Giaume et al, 1997). Opinion has differed over the years as to whether gap junctional communication occurs between astrocytes and neurons (Morales and Duncan, 1975, Legare et al, 2000, Rash et al, 2001), the discrepancy may be explained by Froes et al (1999) who only detected it during embryonic development.

2) Chemical messengers may be released from one cell to act at a receptor on the surface of, or inside a target cell. These messengers may be hormones, neurotransmitters, growth factors or various other mediators. The types of messenger vary in the distance they travel (ie. hormones travel throughout the body in the bloodstream, while neurotransmitters generally act within the synaptic cleft), in their concentration and in their speed of action (ranging from several hours for a hormone signal to produce expression of a protein to milliseconds for a neurotransmitter to open an ion channel).

Three main types of cell-surface receptor are found in glial cells: ion channel-linked receptors, receptors coupled to G-proteins, and tyrosine kinase receptors (see Figure 1.3)

1) Ion channel or ligand gated receptors. Signal transmission at synapses involves specific receptors that transduce neurotransmitter binding into electrical signals, ie. alterations in the membrane potential. Receptors containing integral ion channels mediate rapid transduction. Astrocytes contain various Na\(^+\), K\(^+\) and Ca\(^{2+}\) channel-linked receptors (Bevan, 1990, Sontheimer, 1994), including glutamate- and GABA-gated ion channels (Kim et al, 1994, Fraser et al, 1994).

2) G-protein coupled receptors interact with a heterotrimeric GTP-binding protein, consisting of \(\alpha\), \(\beta\) and \(\gamma\) subunits (Birnbaumer, 1990, Simon et al, 1991). Receptor activation promotes GTP binding to the \(\alpha\) subunit, causing the \(\beta\gamma\) complex to dissociate
from the α subunit, both parts then go on to regulate effector proteins including ion channels, adenylyl cyclases and phospholipases. Activation of these effector proteins results in the production of second messengers such as Ca\(^{2+}\) or cyclic nucleotides cAMP or cGMP. Examples of G-protein coupled receptors in astrocytes include various isoforms of the metabotropic glutamate receptor (Balazs et al, 1998, Silva et al, 1999), PLA\(_2\)-coupled P2 nucleotide receptors (Bruner and Murphy, 1993, Bolego et al, 1997), D2 dopamine receptors (Luo, 1998) and endothelin receptors (Lazarini, et al, 1996, Sasaki et al, 1998).

The rapid generation of a second messenger at a high concentration, results in
amplification of the signal from the first messenger, a neurotransmitter or hormone. The second messengers modulate the activity of effector systems via their direct activation of targets such as protein kinases or protein phosphatases. For example, cAMP acts via its dedicated protein kinase, PKA, while Ca^{2+} (which may be more correctly termed a 'third messenger', being released by the action of the second messenger IP_3) has many roles in astrocytes (Finkbeiner, 1993) including the activation of the Ca^{2+}/calmodulin-dependent protein kinases or the Ca^{2+}/calmodulin-dependent protein phosphatase, calcineurin.

3) Receptor tyrosine kinases, when activated by various cytokines and hormones, dimerise and autophosphorylate to initiate several signal transduction pathways by recruiting key proteins to the plasma membrane which bind to the receptors via SH2 domains. These SH2-containing proteins include Src, Grb2 and Shc. Grb2 then recruits Sos, a nucleotide exchange protein, which activates Ras by converting it to its GTP-bound form. Once activated, Ras then activates Raf to initiate the MAPK (or extracellular signal regulated kinase, ERK) cascade. Two cytoplasmic tyrosine kinase families that are recruited to the membrane include Src and janus kinase (JAK). Downstream targets include the nuclear transcription factors Jun, Fos, Elk-1 and Myc. (See Figure 1.3)

Most growth factor receptors expressed in astrocytes (Ridet et al, 1997) are tyrosine kinases, activation of which initiates intracellular signal transduction pathways via various enzymes including phospholipase Cγ (PLCγ) and the enzymes comprising the mitogen-activated protein kinase (MAPK) cascade (Tournier et al, 1994). Tyrosine-phosphorylated PLC γ hydrolyses inositol phospholipids to generate two second messengers, IP_3 and DG, which trigger the release of Ca^{2+} from intracellular stores, and DG activates protein kinase C (PKC) (Berridge, 1993, Figure 1.3).

The downstream effects of receptor activation are mediated by various protein kinases and phosphatases, which often form a cascade where each successive protein kinase is
phosphorylated and activated by the one before it. One important example of such a
cascade is the MAPK cascade, outlined in Figure 1.3, demonstrated in astrocytes by
several groups (Cazaubon et al, 1993, Tourier et al, 1994, Willis et al, 1996). These
general signal transduction pathways are common to most cell types.

1.5 Signalling via phospholipid hydrolysis

An example of the generation of intracellular second messengers is the generation of IP₃ and
DG from the cleavage of membrane phospholipids (Berridge, 1993). IP₃ is formed from the
cleavage of a specific class of membrane phospholipids, the phosphoinositides, which consist
of phosphatidyl inositol (PI), phosphatidyl inositol-4-phosphate (PIP) and phosphatidyl
inositol-4,5-bisphosphate (PIP₂). Noradrenaline-induced activation of the α₁-adrenoceptor,
coupled via a G protein to PLC leads to the activation of PLC (Pearce et al, 1986). The
phosphorylated inositol ring is then cleaved from the glycerol backbone of PIP₂ by PLC to
yield two products, DG, which remains in the membrane, and IP₃, which is soluble in the
cytosol. Both DG and IP₃ are second messengers, and IP₃ acts on intracellular receptors to
release another messenger, Ca²⁺, from intracellular stores (Berridge, 1993, Verkhartsky
and Kettenmann, 1996). Thus IP₃ production can impact on many cellular processes
through the activation of Ca²⁺-dependent PKC isozymes, Ca²⁺-calmodulin activated
targets, or through activation of Ca²⁺-dependent phospholipases such as cytosolic
phospholipase A₂ (cPLA₂) to liberate arachidonic acid (AA) from membrane
phospholipids. IP₃ is dephosphorylated by specific inositol phosphatases back to free
inositol, unless Li⁺ is present, in which case dephosphorylation of the final phosphate
group is prevented and IPs are allowed to build up (Berridge et al, 1982). This property
was used in the present study as a means of measuring production of IPs by cultured
astrocytes in response to various stimuli. The main source of DG appears to be
phosphatidyl choline (PC) rather than PI, presumably because PC is much more abundant
than PI (Exton, 1990, Billah and Anthes, 1990). DG may be liberated from PC by the
action of various phospholipases, including phospholipase D (PLD) to form phosphatidic acid (PA), which is then hydrolysed to DG by phosphatidic acid phosphohydrolase, or directly from PC by the enzyme PC-specific PLC (PC-PLC) (Asaoka et al, 1992).

In addition to its role as a second messenger in which it activates a number of PKC isoforms, DG can be cleaved by DG lipase to liberate free fatty acids such as AA, which is a major precursor for the synthesis of eicosanoids. In astrocytes, however, AA is mainly obtained by the action of PLA$_2$ on phospholipids rather than via PLD (Bruner and Murphy, 1990, Pearce et al, 1987). The cytosolic form of the enzyme, cPLA$_2$, is thought to control receptor-mediated eicosanoid production and to participate in intracellular signal transduction processes (Lin et al, 1992). This form of the enzyme is particularly enriched in astrocytes of the grey matter (Stephenson et al, 1994).

The main source of eicosanoids in the CNS appears to be astrocytes (Murphy et al, 1988, Seregi et al, 1987) so this pathway in astrocytes is of importance to the function of the brain as a whole. Eicosanoids are a large family of unsaturated fatty acids, consisting of prostanoids and leukotrienes, and their main precursor is AA (Smith, 1989). The functions of the eicosanoids in the CNS are wide-ranging, as would be expected from such a large class of mediators. These functions include modulation of behaviour, blood flow, water balance, thermoregulation, immune function and neurotransmission (Chiu and Richardson, 1985). Interestingly, astrocytes not only synthesise and release eicosanoids, they also possess functional receptors for many of them, although the reasons for these are not yet known (Murphy and Pearce, 1987).

The exact mechanism(s) leading from receptor activation to production of AA in astrocytes are still unclear. Ca$^{2+}$-influx following receptor activation is necessary for activation of cPLA$_2$, which also requires phosphorylation and translocation to the membrane if it is to liberate AA from membrane phospholipids (Channon and Leslie,
1990, Qui et al, 1993). Phosphorylation and activation of cPLA₂ is Ca²⁺-dependent and appears to involve PKC-phosphorylated MAPK and probably other PKC- and MAPK-independent mechanisms (Lin et al, 1993, Chen and Chen, 1998). Since phosphorylation by PKs is balanced by dephosphorylation by PPs, the activation of cPLA₂ is likely to be reversed by PPs. In the present study, the involvement of PPs in agonist-induced AA release and IP production was investigated using the PP inhibitor, okadaic acid.

1.6 Reversible protein phosphorylation

The most important means of acute regulation of protein function in eukaryotic cells is now acknowledged to be reversible protein phosphorylation (Hunter, 1995). Protein phosphorylation is catalysed by protein kinases and reversed by protein phosphatases. Changes in phosphorylation state often induce conformational changes, which can change the function of the target protein.

1.7 Protein phosphatases

Protein phosphatases can be broadly classified according to their ability to dephosphorylate either serine/threonyne, tyrosine, or all three residues. More than 97% of protein-bound phosphate in mammalian cells is on serine or threonyne residues (Shenoliker, 1994), and those PPs that dephosphorylate serine/threonyne will be considered here. Cohen initially classified ‘cytosolic’ PPs into four types, although it is now clear that PP1 and PP2B can be targeted to cell bodies/organelles (Cohen, 1989, Ingebritsen and Cohen, 1983). The most abundant serine/threonyne PPs in mammalian cells were classified according to properties such as the ability to dephosphorylate the α-
(type 1 PP) or β- (type 2 PP) subunit of phosphorylase kinase and their requirements for
divalent metal cations (PP2A is independent of cations, while PP2B and PP2C require
Ca\(^{2+}\) and Mg\(^{2+}\), respectively) (Cohen, 1989, Ingebritsen and Cohen, 1983). With hindsight
this system has proved to be insufficient in that certain serine/threonine PPs exist which
do not fit into this classification, for example mitochondrial PPs, and of those that do,
molecular cloning has revealed that PP2A is more closely related to PP1 than to the other
'type 2' PPs, while PP2C is structurally unrelated to PP1/PP2A/PP2B (Cohen and Cohen, 1989).

The activity due to the various PPs in a physiological system can be determined using
properties such as substrate specificity, requirement for Ca\(^{2+}\) or Mg\(^{2+}\) or by the use of
inhibitors. Two endogenous inhibitors exist for PP1, known simply as inhibitor-1 (I-1)
and inhibitor-2 (I-2). These are readily purified from skeletal muscle and are useful for
identifying dephosphorylation due to PP1 (Huang and Glinsmann, 1976, Nimmo and
Cohen, 1978). Both I-1 and I-2 are small, heat- and acid-stable proteins with molecular
weights of 19 and 23 kDa, respectively (Aitken et al, 1982, Macdougall et al, 1988),
although these are much greater if estimated by SDS-PAGE due to unusually low binding
to detergent (Nimmo and Cohen, 1978). I-1 becomes inhibitory towards PP1 only when
it has been phosphorylated on threonine-35 by PKA. I-2 is a subunit of the cytosolic form
of PP1, and when bound to the catalytic subunit inactivates it.

The properties of I-1 are very similar to those of DARPP-32 (dopamine- and cAMP-
regulated phosphoprotein, apparent M, 32 kDa as determined by SDS-PAGE), an acid-
and heat-stable phosphoprotein that is highly enriched in D1-dopaminergic neurons
in mammalian brain (Walaas and Greengard, 1984, Hemmings et al, 1984c, Ouimet et
al, 1984). DARPP-32 is converted to a potent inhibitor of PP1 on phosphorylation of
threonine-34 by PKA (Hemmings et al, 1984a,b, Williams et al, 1986). Both I-1 and
DARPP-32 are dephosphorylated by calcineurin (Hemmings et al, 1984a, King et al,
However, in spite of their similarity, I-1 and DARPP-32 represent two distinct physiological inhibitor proteins of PP1, having different but often overlapping tissue distribution, indicating that their functions are not fully interchangeable (Hemmings et al, 1992).

The inhibitor that has provided the most information about PP function over the past decade is a toxin derived from marine dinoflagellates (plankton), which concentrate in the black sponge *Halichondria okadaii* by filter feeding. Okadaic acid (OA) is a polyether fatty acid that causes diarrhoetic shellfish poisoning (Cohen et al, 1990). The mechanism of its diarrhoetic effect is by long-lasting contraction of smooth muscle, causing stomach cramps. Inhibition of protein phosphatases by OA prevent dephosphorylation of myosin light chain, preventing smooth muscle relaxation (Takei et al, 1987, Bialojan and Takei, 1988). OA is a specific inhibitor of PP1 and PP2A (Bialojan and Takei, 1988, Haystead et al, 1989, Suganuma et al, 1988), while calcineurin (PP2B) is far less sensitive to OA inhibition and PP2C is unaffected (Bialojan and Takei, 1988). OA is cell-permeable, and is a popular first-choice reagent for finding out whether a particular cellular process is regulated by reversible phosphorylation. For example, the use of OA in intact adipocytes provided the first indication that glucose transport was stimulated by a phosphorylation event, since glucose uptake was substantially increased in the presence of OA (Haystead et al, 1989). The accumulation of OA in intact cells is impossible to quantify so OA cannot be used to discriminate between these two PPs *in vivo* (Cohen et al, 1989). However, in a cell-free system the differential potency of inhibition of PP1, PP2A and PP2B (Ki = 20 nM, 0.2 nM and 10 μM respectively) makes it an ideal choice to distinguish between PP1 and PP2A (Cohen et al, 1989). Other PP inhibitors have since been discovered, including tautomycin, dinophysistoxin, calyculin, microcystin, nodularin and cantharidin (MacKintosh et al, 1990, Li and Casida, 1992, Luu et al, 1993, Honkanen, 1993), although OA remains the most widely used. Effects of OA are listed in Table 1.1.
### Table 1.1 Some effects of okadaic acid on biochemical parameters in intact cells

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Effect of OA</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatocytes</td>
<td>Stimulates glycogenolysis and gluconeogenesis</td>
<td>Haystead et al, 1989</td>
</tr>
<tr>
<td></td>
<td>Inhibits insulin stimulation of glycogen synthesis</td>
<td>Agius and Peak, 1991, Peak and Agius, 1992</td>
</tr>
<tr>
<td></td>
<td>Activates acyl-CoA:cholesterol acyltransferase (ACAT)</td>
<td>Hernández et al, 1997</td>
</tr>
<tr>
<td></td>
<td>Inhibits phosphatidyl choline biosynthesis</td>
<td>Hatch et al, 1992</td>
</tr>
<tr>
<td></td>
<td>Inhibits microtubule-based vesicle movement</td>
<td>Hammalvarez et al, 1996</td>
</tr>
<tr>
<td>Adipocytes</td>
<td>Stimulates lipolysis and inhibits fatty acid synthesis</td>
<td>Haystead et al, 1989</td>
</tr>
<tr>
<td></td>
<td>Stimulates glucose transport, activates MAPK</td>
<td>Rondinone et al, 1996</td>
</tr>
<tr>
<td>Mouse skin</td>
<td>Promotes phosphorylation of acetyl-CoA carboxylase and thereby inactivates it</td>
<td>Fujikui and Suganuma, 1994</td>
</tr>
<tr>
<td>Neurons</td>
<td>Increases phosphorylation of microtubule-associated proteins MAP2 and tau</td>
<td>Arias et al, 1993</td>
</tr>
<tr>
<td>Human and Rat mast cells and Human basophils</td>
<td>Stimulates LPS-mediated NF-κB activation</td>
<td>Pahan et al, 1998</td>
</tr>
<tr>
<td>Macrophages</td>
<td>Activates MAPK-activated protein kinase-2 leading to increased phosphorylation of vimentin</td>
<td>Cheng and Lai, 1998</td>
</tr>
<tr>
<td>Astrocytes</td>
<td>Stimulates LPS-mediated NF-κB activation, expression of inducible nitric oxide synthase (iNOS), production of nitric oxide (NO)</td>
<td>Pahan et al, 1998</td>
</tr>
<tr>
<td></td>
<td>Increases nerve growth factor mRNA content, gene transcription and secretion</td>
<td>Pshenichkin and Wise, 1995</td>
</tr>
<tr>
<td></td>
<td>Increased staining of glial fibrillary acidic protein, changes in morphology from flat to a stellate</td>
<td>Arias et al, 1993</td>
</tr>
<tr>
<td></td>
<td>Prevents change in morphology by β-amyloid protein</td>
<td>Salinero et al, 1997</td>
</tr>
<tr>
<td></td>
<td>Reverses phosphorylation of PEA-15 (phosphoprotein enriched in astrocytes, 15 kDa) by Ca(^{2+})/Calmodulin-dependent Protein Kinase II (CaM kinase II) in vitro.</td>
<td>Kubes et al, 1998</td>
</tr>
</tbody>
</table>
1.7.1 Protein phosphatase 1

The catalytic subunit of PPI, PP1c exists as various isoforms (α, β, γ1, γ2 and δ, Cohen, 1988, Dombradi et al, 1990, Sasaki et al, 1990), the α, β, γ1 and γ2 isoforms have all been detected in brain, where they show distinct subcellular localisation: PP1β localizes to a discrete area of the soma, while PP1γ1 is highly enriched in dendritic spines and presynaptic terminals of cultured neurons (Strack et al, 1999). This subunit forms a 1:1 complex with a regulatory subunit (Hubbard and Cohen, 1993) of which several types exist, targeting the enzyme to specific subcellular locations. The glycogen-bound regulatory subunit of PPI is termed the G-subunit, forming the holoenzyme PP1G (Strålforset al, 1985) while muscle contains PP1M (Alessi et al, 1992), the nucleus PP1N (Beullens et al, 1992, 1993) and the cytosol PP1S, which has I-2 as its regulatory subunit (DePaoli-Roach et al, 1994).

PP1c is regulated by binding to its various regulatory/targeting subunits and to inhibitor proteins such as I-1, I-2 and the brain form of I-1, DARPP-32 (Hemmings et al, 1984a). The best-characterized targeting protein is the glycogen-binding or G subunit, which confers association of PPI with skeletal muscle glycogen. PP1G dephosphorylates the enzymes of glycogen metabolism. It is active when the two subunits are bound together; this binding is regulated by phosphorylation of G at two sites. Phosphorylation of site 1 (serine 48) is required for the association of G and PP1c (Hubbard and Cohen, 1989), while site 2 (serine 67) phosphorylation promotes dissociation of the free catalytic subunit into the cytosol, greatly reducing its ability to dephosphorylate glycogen phosphorlyase and glycogen synthase (Hubbard and Cohen, 1989, Dent et al, 1990). The activating effect of site 1 phosphorylation is overridden by phosphorylation of site 2. Application of adrenaline to skeletal muscle cells causes a rise in intracellular cAMP via the action of adenylate cyclase, leading to activation of PKA and phosphorylation of the G subunit of PP1G at site 2 resulting in its dissociation and inactivation (Dent et al, 1990).

PKA also phosphorylates and activates I-1, the cytosolic PPI inhibitor, which binds to
and inhibits PP1c. Thus hormone-induced dissociation of PP1G is coordinated with I1-1 activation to inhibit protein dephosphorylation in mammalian skeletal muscle. I-1-phosphate is dephosphorylated by PP2B, causing it to dissociate from PP1c, while site 2 phosphorylated by PKA on the G subunit is dephosphorylated by PP2A, PP2B and PP2C in vitro (Hubbard and Cohen, 1989).

PP1 is widely distributed in neurons, being present in membrane fractions (Sim et al, 1994), dendritic spines (Ouimet et al, 1995), the postsynaptic density (Strack et al, 1997a), synaptic junctions (Shields et al, 1985), and associated with neurofilaments (Strack et al, 1997b). PP1c also associates with brain microtubules. A PP1 targeting subunit recently identified is the microtubule-associated protein tau; PP1 and possibly other PPs are involved in regulating microtubule stability (Liao et al, 1998). A recently identified neuronal PP1 binding protein, spinophilin, localises PP1 to dendritic spines in the vicinity of PP1 targets such as AMPA-type glutamate channels (Allen et al, 1997, Ouimet et al, 1995). It has recently been discovered that PP1c binds to the PKA anchoring protein AKAP220, thus co-localising with its opposing enzyme PKA in the vicinity of shared substrates (Schillace and Scott, 1999).

PP1 is involved in diverse cellular processes including glycogen metabolism, calcium transport, muscle contraction, protein synthesis and intracellular transport (Cohen, 1989, Shenoliker and Nairn, 1990) and also appears to have an important role in the regulation of mitosis (Doonan and Morris, 1989, Axton et al, 1990). In the brain, as in other tissues, PP1 is mainly associated with the particulate fraction (Cohen and Cohen, 1989, Sim et al, 1994), although in contrast to other tissues this membrane-bound PP1 appears to have a low specific activity (Sim et al, 1994). Functions of PP1 in brain appear to include regulation of the neuronal cytoskeleton by dephosphorylation of microtubule-associated and neurofilament proteins, and regulation of CaM kinase II, which is instrumental in gene expression, neurotransmitter synthesis and release, postsynaptic responses and cytoskeletal rearrangements (Strack et al, 1997a, Shields et al, 1985, Kennedy, 1998, Braun and Schulman, 1995). The synaptic localization of PP1γ1 indicates that this
isoform is involved in the regulation of synaptic phosphoproteins such as neurotransmitter receptors and ion channels implicated in synaptic plasticity (Strack et al, 1999). The three inhibitor proteins of PP1, I-1, I-2 and DARPP-32 are present in brain (Macdougall et al, 1989, Hemmings et al, 1992, Ouimet et al, 1984).

PP1 has a well-characterised role in regulation of dopaminergic signalling cascades in which its effects are opposed by DARPP-32. DARPP-32 is found at high concentrations in certain brain regions and is absent from virtually all other tissues (Hemmings and Greengard, 1986). The distribution of DARPP-32 and I-1 is often complementary, suggesting some difference in function (Hemmings et al, 1992, Alder and Barbas, 1995). The main site of DARPP-32 expression is in the basal ganglia, which contains neurons having dopamine D1 receptors coupled to adenylate cyclase (Hemmings and Greengard, 1986). It is also found in some glial cells, including astrocytes, which are known to contain dopamine-sensitive adenylate cyclase (Walaas and Greengard, 1984, Ouimet et al, 1984) and in which the neuronal form of I-1 could not be detected (Gustafson et al, 1991).

Dopamine, acting on D1 receptors causes activation of PKA which phosphorylates DARPP-32 on threonine-34 (Hemmings et al, 1984b). In its phosphorylated form, DARPP-32 inhibits PP1, which controls the phosphorylation state and consequently the physiological activity of various neuronal phosphoproteins such as neurotransmitter receptors, ion channels, ion pumps and transcription factors (Shenoliker and Nairn, 1991). In astrocytes, the role of PP1 also includes the dephosphorylation of most sites on GFAP, an intermediate filament protein which is restricted mainly to astrocytes and undergoes multisite phosphorylation (Vinadé and Rodnight, 1996). The role of DARPP-32 in dopamine neurotransmission was studied using mice that lack the protein (Fienberg et al, 1998). Fienberg and co-workers found that mutation of the DARPP-32 gene markedly reduced, and in some cases abolished, various responses to dopaminergic agonists and antagonists. The authors conclude that a cascade involving dopamine receptor-mediated activation of DARPP-32, inhibition of PP1, and increased phosphorylation of neuronal
substances is an obligatory component in dopaminergic neurotransmission. Since disturbances in dopaminergic neurotransmission have been implicated in several major neurological and psychiatric disorders including Parkinsonism, drug addiction and schizophrenia, drugs that mimic or block the inhibitory effects of DARPP-32 on PP1 might prove useful pharmacological agents (Fienberg et al, 1998).

1.7.2 Protein Phosphatase 2A

PP2A is thought to exist primarily as a trimeric enzyme of variable subunit composition, consisting of a catalytic (C) subunit and two regulatory subunits (A and B), although dimers have been purified, which may suggest that the regulatory subunits can dissociate (Cohen, 1989). The 36 kDa catalytic subunit, PP2Ac, of which two 97-98 % identical mammalian isoforms, α and β, exist (Da Cruz e Silva and Cohen, 1987, Stone et al, 1987), is complexed with the 65 kDa A subunit and one of at least 15 distinct regulatory B subunits ranging in molecular weight from 54 kDa to 130 kDa (Healy et al, 1991, Mayer-Jaekel et al, 1991, Pallas et al, 1992, Hendrix et al, 1993). As with the catalytic subunit, two distinct isoforms of the A subunit (α and β) are present in mammalian tissues (Hemmings et al, 1990, Walter et al, 1989, 1990). The amino acid sequences of the human α- and β-isoforms are 87 % homologous. It is likely that the B subunits influence substrate specificity and subcellular localisation. For example, the 72 kDa B subunit contains a potential nuclear localisation signal in its primary sequence (Hendrix et al, 1993), which may account for the presence of PP2A in the nucleus (Turowski et al.). Several isoforms of each B subunit of PP2A exist (Khew-Goodall and Hemmings, 1988, Axton et al, 1990), and while most subunits are ubiquitously expressed, two isoforms of the 55 kDa B subunit, β and γ are mainly found in neuronal tissue (Mayer-Jaekel and Hemmings, 1994, Zolnierowicz et al, 1994).

Various mechanisms of regulation of PP2A have been identified. The main means of long-term PP2A regulation is by the differential expression of regulatory B subunit proteins that control enzyme specificity and activity by formation of oligomeric
complexes with the common AC core (Mumby and Walter, 1993). While the expression of A and C in different tissues is coordinated, it does not match that of the B subunits (Mayer-Jaekel et al., 1992, Ruediger, 1991). Association of the core complex with different B subunits confers distinct enzymatic properties to the complexes, such as alterations in substrate specificity (Chen et al., 1989, Imaoka et al., 1983), substrate targeting (Kosik, 1993, Sontag et al., 1996), kinetic parameters (Kamibayashi et al., 1994), subcellular localisation (McCright et al., 1996), cellular function (Mawal-Dewan et al., 1994, Zhao et al., 1997) and specific activity (Kamibayashi et al., 1992).

As with PP1, reversible phosphorylation has an important role in the short-term regulation of PP2A. The catalytic subunit or the AC heterodimer is inactivated by phosphorylation by various receptor tyrosine kinases including pp60src, p56^ck, the epidermal growth factor receptor, and the insulin receptor in vitro (Chen et al., 1992), so the activity of PP2A can be modified by extracellular signals. The inhibition of PP2A by tyrosine phosphorylation is interesting since PP2A is thought to suppress cell growth (Felix et al., 1990, Lee et al., 1991, Picard et al., 1991). Phosphorylation and inactivation of the PP2A C subunit is enhanced by OA, suggesting that the protein is rapidly autodephosphorylated (Chen et al., 1992). The activation of MAPK is a key event in signalling by growth factors (Sturgill and Wu, 1991), prolonged activation of this enzyme is required to trigger mitogenesis (Pages et al., 1993, Cowley et al., 1994). MAPK and its activator, MAPK kinase (MEK) are both inhibited by dephosphorylation by PP2A (Alessi et al., 1995). In this way, PP2A opposes entry into the cell cycle, which may explain its inhibitor, OA being a tumour-promoter (Suganuma et al., 1988).

The role of PP2A in opposing cell division does not end at inhibition of MAPK, the enzyme has potential functions in controlling several aspects of the cell cycle. PP2A has an inhibitory effect on progression through the cell cycle from G2 to M-phase (mitosis). Entry into mitosis is controlled by the cyclin-dependent protein kinases, more specifically by the complex formed between cyclin B and p34^cd2 (Langan et al., 1989) which is regulated by reversible phosphorylation. Phosphorylation of p34^cd2 on thr-161 is required
for activity (Gould et al, 1991), this is a similar site to that required for activation of MAPK, (Cobb et al, 1991, Payne et al, 1991), whereas phosphorylation of thr-15 and tyr-14 is inhibitory (Lundgren et al, 1991, Parker et al, 1991, 1992). Phosphorylation of thr-161 is catalysed by a p34\textsuperscript{cdk2}-activating kinase, also known as CAK, and the pathway leading to thr-161 phosphorylation is inhibited by PP2A. Thr-161 is not directly dephosphorylated by PP2A (Lee et al, 1994). p34\textsuperscript{cdk2} activation requires dephosphorylation of thr-15 and tyr-14 by a protein phosphatase, cdc25. cdc25 is only active in the phosphorylated state and is kept inactive by PP2A (Clarke et al, 1993).

Studies on the physiological function of PP2A were initially concentrated on its role in the regulation of metabolism; it inhibits gluconeogenesis and lipolysis and stimulates fatty acid synthesis (Cohen, 1989). The enzymes that control the rate of fatty acid and cholesterol synthesis and lipolysis, ie. ACC, hydroxy methyl-glutaryl-coenzyme A reductase (HMG-CoAR) and hormone-sensitive lipase (HSL) are all dephosphorylated by PP2A (Ingebritsen et al, 1983a, Gaussin et al, 1997, Olsson and Belfrage, 1987). These three enzymes are also phosphorylated and inactivated by the same protein kinase, AMPK (Hardie, 1992, Garton et al, 1989). Other reports have suggested a role for PP2A in synaptic transmission and RNA splicing (Sim, 1992, Walter and Mumby, 1993) and dephosphorylation of DARPP-32, the inhibitor of PP1 on thr-34, the site at which phosphorylation is required for activity (Desdouits et al, 1995). Barnes et al (1995) found PP2A localised at the synapses of CNS neurons, where it may alter the functions of phosphoproteins involved in synaptic plasticity, and where it was shown to dephosphorylate autophosphorylated CaM kinase II. PP2A selectively dephosphorylates the soluble form of CaM kinase II rather than that associated with the postsynaptic density where it is a substrate for PP1 (Strack et al, 1997a).

**1.7.3 Protein phosphatase 2B**

PP2B, otherwise known as calcineurin, is the only serine/threonine protein phosphatase dependent on Ca\textsuperscript{2+}/calmodulin, and has a vital role in the transmission of Ca\textsuperscript{2+} signals in
response to receptor activation (Klee et al, 1979). It is enriched in neural tissue but is also broadly distributed in the periphery (Su et al, 1995, Blumenthal and Krebs, 1986, Ingebritsen et al, 1983c). Calcineurin is a heterodimer consisting of calcineurin A, a 58-64 kDa catalytic and calmodulin-binding subunit, and calcineurin B, a 19 kDa tightly bound regulatory subunit, which is itself a Ca^{2+}-binding protein (Klee et al, 1988). Three isoforms of calcineurin A have been reported, encoded by 3 separate genes, where α is the neural isoform, β is broadly distributed, and γ is testis-specific (Yokoyama et al, 1990). Two isoforms of calcineurin B have also been identified, the B1 form and the B2 form (Klee et al, 1998).

Calcineurin is regulated by its two Ca^{2+}-binding proteins, calmodulin and calcineurin B (Klee et al, 1988). Calcineurin B contains four Ca^{2+}-binding sites; one of these is a high affinity site (Kd < 0.1 μM) and three are low affinity sites (Kd = 0.5-1 μM). A low intracellular Ca^{2+} concentration is sufficient for occupancy of the high affinity site and binding of calcineurin B to the catalytic subunit, but not for activity. Occupancy of the low affinity Ca^{2+}-binding sites results in a small activation, which is then increased 20-fold by the binding of calmodulin (Klee et al, 1998).

Study of the functions of calcineurin was hampered by its resistance to inhibition by the PP inhibitors, inhibitor-1, inhibitor-2, OA (Ki = 10 μM), calyculin and microcystin (Shenoliker, 1994), until it was found to be the target of the immunosuppressants FK506 (tacrolimus) and cyclosporin A. This enabled the elucidation of the role of calcineurin in T cell activation (Liu et al, 1991). FK506 and cyclosporin form complexes with FKBP12 and cyclophilin respectively, which bind to and inhibit calcineurin (Liu et al, 1991). Inhibition of calcineurin blocks Ca^{2+}-induced activation of the NF-AT (nuclear factor of activated T cells) transcription factors (Flanagan et al, 1991), causing immunosuppression in T cells (Fruman et al, 1992). Roles of calcineurin so far discovered include the regulation of the transcription of various growth factors and cytokines, including interleukin-2 (IL-2), TNFα, interferon-γ (IFN-γ) (O’Keefe et al, 1992, Frantz et al, 1994, Staruch et al, 1998), IL-4, IL-5, and IL-13 (Pacocha et al, 1999), dephosphorylation of the
PP1 inhibitor DARPP-32 (Halpain et al, 1990), inhibition of neurotransmitter release; including glutamate and GABA (Nichols et al, 1994, Stelzer, 1992), noradrenaline and the neuropeptide cholecystokinin-8 (Hens et al, 1998), and regulation of NMDA receptor function (Lieberman and Mody, 1994) and Ca$^{2+}$-channel function of ryanodine and IP$_3$ receptors (Snyder et al, 1998). An interesting new role for calcineurin in the CA1 region of the hippocampus, where calcineurin is present at high levels, is in the transition from short-term to long-term memory (Winder, et al, 1998, Mansuy et al, 1998).

Until recently, calcineurin was assumed to be absent from glial cells (Billingsley et al. 1994, Dawson et al. 1994, Usuda et al. 1996). However, Vinadé et al. (1997) found a low level of both calcineurin A and B which was detectable using immunoblotting, but too low to be detected using immunocytochemistry. The calcineurin Aβ isoform was more recently shown by immunofluorescence to be present in gerbil hippocampal activated astrocytes, appearing between days 4-7 after transient ischaemia (Hashimoto et al, 1998), which suggests that the amount of calcineurin present in astrocytes may increase in response to stress stimuli. The authors believed this to be the first evidence of calcineurin Aβ expression in astrocytes. Another group, Matsuda et al (1998) have recently shown the presence of calcineurin A, probably the Aβ2 isoform, and the B subunit in cortical astrocytes using both immunoblotting and immunofluorescence at about 5-10 % of the level seen in whole rat brain. However, it is not clear what the physiological role of calcineurin in astrocytes might be.

1.7.4 Protein Phosphatase 2C

The Mg$^{2+}$-dependent phosphatase, PP2C, is a monomer of molecular weight 43-48 kDa. It is structurally unique among the four main serine/threonine PPs and, possibly for this reason, is insensitive to OA-inhibition (Cohen, 1989). Two isoforms of PP2C, α and β have been identified in mammalian tissues and both are ubiquitously expressed (McGowan et al, 1987, McGowan and Cohen, 1987). Little is known about the localisation, regulation or substrate specificity of PP2C since no inhibitor has been found
and no activator with which to study the effects of PP2C in physiological systems. A novel mechanism of regulation of PP2C has been described recently in bovine retina. The retina have a high lipid content, and PP2C activation was observed in response to the lipid second messenger arachidonic acid (AA) (Klumpp et al, 1998), which is also known to inhibit myosin light chain phosphatase activity (Gong et al, 1992, Gailly et al, 1996) and to activate PP5 (Chen and Cohen, 1997). Enzyme activity of PP2C is barely measurable at physiological Mg\(^{2+}\) concentrations. Application of AA and of various other unsaturated fatty acids, especially oleic, linoleic and γ-linoleic acid reduces the Mg\(^{2+}\) requirement of PP2C tenfold to within the physiological range and allows Ca\(^{2+}\) to become inhibitory at low concentrations (Klumpp et al, 1998).

Proteins such as Hydroxy Methyl-Glutaryl-Co A Reductase (HMG-CoAR) (Ingebritsen et al, 1983a,c), AMP-activated protein kinase (AMPK) (Clarke et al, 1991) and CaM kinase II (Fukanaga et al, 1993) have been shown to be good substrates for PP2C in vitro. OA has no effect on AMPK activity in vivo, so PP2C, which is resistant to OA inhibition, is implicated in the regulation of lipid biosynthesis in mammalian cells by catalysing the dephosphorylation and inactivation of AMPK (Moore et al, 1991, Marley et al, 1996). Inactivation of AMPK results in a decrease in phosphorylation of its substrates, HMG-CoAR and ACC, and therefore an increase in the rates of cholesterol and fatty acid synthesis, respectively.

The use of mutant recombinant PP2C has suggested a role for the enzyme in cellular processes such as tRNA splicing, sporulation, mitosis, and the heat-shock response and osmoregulation in Schizosaccharomyces pombe (Robinson et al, 1994, Shiozaki et al, 1994, Shiozaki and Russell, 1995). Recently, it has been reported that PP2C is responsible for dephosphorylation of DARPP-32 at serine-137 in vivo (Desdouits et al, 1998). This site is phosphorylated by casein kinase I (CKI) (Desdouits et al, 1995). Phosphorylation of DARPP-32 at serine-137 inhibits dephosphorylation of threonine-34 by calcineurin (Desdouits et al, 1995). Phosphorylation of threonine-34 by PKA is necessary for inhibition of PP1 (Hemmings et al, 1984). Essentially, dephosphorylation
of DARPP-32 by PP2C allows its inactivation by calcineurin and so promotes the activity of PP1. In astrocytes, no specific role of PP2C has been reported.

1.8 Protein Kinases

PKs are a large family of diverse enzymes which play vital roles in the regulation of cell processes and growth (Taylor et al, 1990, Krebs, 1985, Hanks et al, 1988). The majority of PKs catalyse the transfer of phosphate from ATP to a serine or threonine residue on their target protein, while others preferentially phosphorylate tyrosine residues. Dual-specificity PKs also exist, which are able to phosphorylate both types of residue. Many PKs have major regulatory functions, while being tightly regulated themselves. PK activity is regulated in various ways; including the binding of ligands such as hormones (eg. insulin), second messengers (eg. cAMP) or metabolites (eg. AMP), by reversible phosphorylation, or by proteolytic degradation. Considerable diversity exists in the size, subunit structure, subcellular localization, regulatory mechanism and substrate specificity of different PKs. One feature common to the PKs, defined by sequence similarities, is their catalytic core. This is the area around the active site of the enzyme containing all the elements necessary to carry out catalysis (Hanks et al, 1988).

The effects of PKs can be powerful and far-reaching due to the signal amplification achieved by PK cascades. An extracellular signal acting on a receptor may induce the production or release of many molecules of second messenger per molecule of bound ligand. PKs are then activated by this second messenger, and phosphorylate and activate several target PKs, and so on. For example, in the MAPK cascade (see Figure 1.1, Ferrell, 1996) each stage can achieve a theoretical amplification factor of 100, although 10 may be more realistic. Therefore, the four-enzyme MAPK kinase cascade represents a possible amplification factor of 10,000 (Hunter, 1995). Additional regulation of PK cascades is provided by negative feedback, ie, when the final product of the pathway inhibits an upstream PK, or when a protein kinase phosphorylates and inhibits an upstream
component of the pathway.

A further mechanism of regulation of PK function is produced by targeting of the enzyme to specific subcellular compartments by anchoring proteins. This compartmentalization allows selectivity of the PKs by favouring their accessibility to certain substrate proteins (Lester and Scott, 1997). Proteins to which PKs are anchored in this way include structural proteins that are components of membranes, the cytoskeleton or cellular organelles (Faux and Scott, 1996).

1.9 The cyclic AMP-dependent protein kinase (PKA)

1.9.1 PKA structure and mechanisms of regulation

\[ R_2C_2 + 4\text{cAMP} \rightarrow R_2(\text{cAMP})_4 + 2C \]

The most extensively studied PK was purified as the kinase activating phosphorylase kinase and found to be cyclic AMP (cAMP)-dependent (Krebs et al, 1959, Walsh et al, 1968). It is known as the cAMP-dependent protein kinase, or protein kinase A (PKA). In the absence of cAMP the inactive PKA holoenzyme consists of two catalytic (C) subunits bound to a dimer of two regulatory (R) subunits. The C subunits are bound via their active sites to pseudosubstrate consensus sequences in the R subunits. Elevation of intracellular cAMP levels leads to binding of two molecules of cAMP to each of the R subunits. This produces a conformational change in the area of each R subunit to which the C subunit is bound, known for this reason as the ‘hinge region,’ and the C subunit dissociates, exposing its active site. The binding of cAMP to the R subunit is subject to positive cooperativity: binding of cAMP to one site encourages a second molecule of cAMP to bind to the second site (Taylor et al, 1990). The equation for the activation is shown above.
The active site of the C subunit contains a peptide binding site, a nucleotide binding site and a catalytic site, and these three regions are found in all protein kinase active sites (Taylor et al, 1990). The nucleotide binding site contains some invariant amino acids required for MgATP binding eg. Lys 72, and secondary structural features required for orientation of MgATP as it is a large, charged molecule. The peptide binding site varies between different PKs as would be expected since they have different substrate specificities. The peptide binding sites of the PKs each recognise a particular sequence of amino acids surrounding the serine/threonine to be phosphorylated. These sequences are the preferred recognition sequence, although slight variations allow the PK to phosphorylate other targets with reduced affinity. In PKA negatively charged Glu residues interact with basic residues in the substrate N-terminal to the phosphorylation site so that the consensus sequence for PKA is:

- arg/lys - arg/lys - X - (X) - S - Φ -

(Where arg = arginine, lys = lysine, X = any small residue, Φ = a large hydrophobic residue and S is the serine to be phosphorylated, there can be one or two small residues N-terminal to S) (Daile et al, 1975, Zetterqvist et al, 1976, Maller et al, 1978). The R subunit hinge region contains a modified consensus sequence for recognition by the C subunit so that it binds to the R subunit and is inactivated.

The active site of PKA consists of a groove in the tertiary structure of the C subunit, containing the peptide binding, nucleotide binding and the catalytic site. The peptide binding site positions the substrate so that the serine residue to be phosphorylated is adjacent to the terminal (γ) phosphate group of ATP, the latter positioned by the nucleotide binding site. Once both ATP and a substrate have bound, there is a conformational change in the C subunit which closes the groove in which the active site is situated, enabling phosphorylation to occur. The resulting ADP molecule dissociates, along with the phosphorylated peptide (Knighton et al, 1991).
PKA is a multisubstrate, ubiquitous enzyme which is activated by cAMP produced in response to hormone stimulation. The most obvious mechanism of regulation of PKA is by changes in the level of cAMP. This is increased following stimulation of adenylate cyclase by G protein-coupled receptors and decreased by degradation by cAMP phosphodiesterases (PDE). Various isoforms of these two enzymes exist, of the adenylate cyclase that synthesises cAMP nine forms are known, while at least thirty forms of PDE are known (Houslay and Milligan, 1997). The main isozyme of PDE in astrocytes appears to be PDE IV which is subject to rapid short-term regulation by elevation of intracellular cAMP levels. In this way the signal is switched off by a mechanism that appears to involve phosphorylation of PDE IV by PKA (Madelian and La Vigne, 1996).

While protein targets with the correct consensus sequence must be present in a cell to elicit a physiological response, there is a specificity associated with PKA activation that suggests more complex control. An important means of adjusting the level, localisation and response to increases in cAMP is via the multiple isoforms of PKA. Three isoforms of the C subunit of PKA exist, α, β and γ, the α and β forms being highly homologous (93 % amino acid sequence identity, Uhler et al, 1986a,b, Showers and Maurer, 1986) while the γ isoform, is slightly different, and human testis specific (Beebe et al, 1990). No differences in substrate specificity have been detected between these isoforms (Walsh and van Patten, 1994), therefore this is perhaps not the source of specificity of the cAMP response. Cβ is most highly expressed in brain and reproductive tissues (Uhler et al, 1986, Cadd and McKnight, 1989).

Two main types of R subunit exist, and they are referred to as R₁ and R₁I, based on the order in which they elute from anion exchange resins (Corbin et al, 1975). The two types are functionally different in their method of binding the C subunit. In R₁, there is a pseudosubstrate site in the hinge region which contains an alanine instead of a serine residue, so the C subunit binds (-arg-arg-arg-gly-ala-ile-), while R₁I contains an autophosphorylation site (-asp-arg-arg-val-ser-val-), where the C subunit binds and phosphorylates R₁I (Hofmann et al, 1975, Walter et al, 1977). An α and β form of each
R subunit exist, with the \( \beta \) forms showing greater tissue specificity (Cadd et al, 1990, Jahnsen et al, 1986). In rat brain mature primary cultures, neurons contain both \( \alpha \) and \( \beta \) forms, while astrocytes contain only \( \alpha \) forms (\( R_\alpha \), \( R_\alpha \alpha \)) (Massa et al, 1991).

The relative amounts of the two R subunit isoforms may alter the speed at which PKA activation is seen in a particular cell type, since it has been reported that the autophosphorylated PKA\( _{\text{II}} \) holoenzyme dissociates at a lower cAMP concentration than PKA\( _{\text{I}} \) (Rosen and Erlichman, 1975, Hofmann et al, 1975, Rangel-Aldao and Rosen, 1977). However, the situation may be more complex than this due to the fact that much of the PKA\( _{\text{II}} \) is to be found anchored to specific subcellular compartments via the R \( \alpha \) subunit (Scott and McCartney, 1994). A family of dedicated A-Kinase Anchoring Proteins (AKAPs) have been identified whose role is to tether PKA\( _{\text{II}} \) to specific subcellular structures (Lohmann et al, 1984). This regulates PKA phosphorylation by limiting the availability of substrates to those in the immediate vicinity. AKAPs are localised in many different compartments of a wide range of cell types and range in size from 15 to 420 kDa (Murphy and Scott, 1998). Although AKAPS were originally defined by their ability to bind R\( _{\text{II}} \), a new class of dual specificity AKAPS has been described which can also bind the R\( _{\text{I}} \) subunit (Huang et al, 1997). PKA anchoring to specific subcellular locations is required for modulation of ion channels. AKAP 15 is present in purified rat brain sodium channels, while partially purified preparations of the channel co-purify with PKA R\( _{\text{II}} \) and C subunits (Tibbs et al, 1998). In the brain a higher proportion of PKA is found in the particulate fraction than in other tissues (Hofmann et al, 1977), being equally distributed between the cytosolic and particulate fractions (Naim et al, 1985) (in other tissues it is mainly cytosolic). Its highest specific activity is found in the cytosol and synaptic membrane-enriched fractions (Walter et al, 1978). AKAP 79 and 150 have been found localised to the postsynaptic densities, a network of proteins on the internal surface of the postsynaptic membrane, suggesting that AKAP-targeted-PKA plays an important role in synaptic function (Carr et al, 1992).

Like PP1, PKA has its own endogenous inhibitor protein, known as the Walsh Inhibitor
(Walsh et al, 1971, Ashby and Walsh, 1972), which binds to the C subunit to form a 1:1 complex (Van Patten et al, 1986). The Walsh Inhibitor is a 16 kDa protein which functions as a competitive inhibitor of the C subunit, and contains a pseudosubstrate site (Whitehouse and Walsh, 1983). A short (20 amino acid) peptide taken from the sequence of the Walsh Inhibitor near its amino terminus contains the active portion of the inhibitor protein, and this peptide is known as the protein kinase inhibitor, or PKI (Scott et al, 1985).

The effects of PK phosphorylation must be balanced by dephosphorylation by protein phosphatases. In the case of PKA, the PP most usually responsible for reversing its effects is PP1. Two inhibitors of PP1, inhibitor-1 and DARPP-32, are only active when phosphorylated by PKA, so PKA is able to prolong its own signal by inhibition of PP1 since the two enzymes share many substrates. Conversely, PKA is able to preserve the phosphorylation state of proteins phosphorylated by other PKs, by its inhibition of PP1. This inhibition of PP1 by PKA is achieved in two ways in skeletal muscle, in addition to its activation of I-1 and DARPP-32 as mentioned above, PKA phosphorylates the G subunit of PP1G, thus promoting dissociation of the catalytic subunit of PP1 into the cytosol, away from its site of action (Hubbard and Cohen, 1989).

1.9.2 Functions of PKA

It is well established that PKA mediates most of the actions of hormones that exert their effects by elevating the intracellular concentration of cAMP. PKA is implicated in numerous cell processes including:

Regulation of metabolism The most well-known function of PKA is in its regulation of glycogenolysis. Phosphorylation of phosphorylase kinase by PKA (Walsh et al, 1968) converts it to its active state in which it phosphorylates and activates its target protein, glycogen phosphorylase, making the latter enzyme more sensitive to its allosteric
activator, AMP. Glycogen phosphorylase catalyses the first step in the degradation of glycogen, and is dephosphorylated and inactivated by PP1, which is inhibited by PKA. Astrocytes store most of the brain's glycogen. Glycogenolysis in astrocytes is elicited by vasoactive intestinal peptide, noradrenaline (NA) and adenosine, all of which elevate intracellular cAMP (Sorg and Magistretti, 1991). Similarly, the method by which adrenalin stimulates lipolysis in adipose tissue is via activation of PKA, which phosphorylates and activates HSL (Strålfors and Belfrage, 1984). Astrocytes are the only brain cell population that oxidise fatty acids to ketone bodies (Edmond et al, 1987, Edmond, 1992, Staub et al, 1995). Cultured astrocytes produce ketone bodies at rates similar to those of hepatocytes (Blázquez et al, 1998, Guzmán and Geelen, 1992, Guzmán et al, 1995). The synthesis of these fatty acids is discussed on page 68.

Gene transcription The transcription factor CREB is a major nuclear substrate for PKA (Lalli and Sassone-Corsi, 1994), but is also phosphorylated on the same serine residue (ser-133) by MAPK in response to Ca^{2+}-influx or growth factor activation (Pende et al, 1997). Phosphorylated CREB binds to a response element known as the cAMP-responsive element (CRE), in cAMP-inducible genes. This consists of an 8-base pair palindrome, and mediates the transcriptional induction of many genes including somatostatin, phosphoenolpyruvate carboxykinase (PEPCK), vasoactive intestinal peptide (VIP), enkephalin and synapsin I (Montminy, 1997). cAMP-induced gene expression is important in many cellular responses, including the establishment of long-term memory (Hunter, 1995), (or long-term potentiation, LTP). It is likely that different forms of LTP employ different isoforms of the PKA R subunit (Woo et al, 2000). In astrocytes, PKA suppresses the induction of inducible nitric oxide synthase (Feinstein et al, 1993); whereas expression of the Na^{+}-dependent glutamate transporters, GLUT-1, and to a lesser extent the expression of the glutamate/aspartate transporter, GLAST, in astrocytes is increased by activation of PKA (Schlag et al, 1998).

Signal transduction CNS substrates for PKA involved in signal transduction include ion channels and receptors. K^+ channels in neurons are regulated either positively or
negatively by PKA phosphorylation, depending on their type (Greengard, 1987). Voltage
gated Ca\(^{2+}\) channel conductance is increased by PKA phosphorylation (Greengard, 1987).
Phosphorylation of the nicotinic acetylcholine receptor by PKA increases its rate of
desensitization and thereby modulates synaptic transmission postsynaptically (Huganir
et al, 1986). The \(\beta\)-adrenergic receptor (\(\beta\)-AR) mediates the stimulatory effects of
catecholamines on many tissues through activation of adenylate cyclase. Prolonged
exposure to \(\beta\)-adrenergic agonists results in desensitization of its adenylate cyclase
activity, caused by phosphorylation of the \(\beta\)-AR by the \(\beta\)-AR kinase (Stadel et al, 1983).
These effects can be reproduced by exogenous cAMP, suggesting that desensitization is
stimulated by PKA. The phosphorylation of synapsin I, which is localized to presynaptic
nerve terminals, increases neurotransmitter release and thereby modulates synaptic
transmission presynaptically. Synapsin I is a substrate of PKA (Hemmings et al, 1989).
DARPP-32, the PP1 inhibitor is also a substrate for PKA, phosphorylation by PKA
activates DARPP-32, enabling it to inhibit PP1 (Hemmings et al, 1984b).

Cell motility Phosphorylation of myosin light chain (MLC) causes smooth muscle
contraction. The enzyme responsible for this phosphorylation is MLC kinase (MLCK),
which is inactivated by phosphorylation by PKA. In this way, adrenergic stimulation of
smooth muscle produces muscle relaxation via activation of PKA and its inactivation of
MLCK (Pato et al, 1995, Verin et al, 1998). MLCK has also been identified in many
regions of rat brain, as well as in cultured astrocytes and cerebellar granule cells, where
it may have motility-related functions (Edelman et al, 1992). Approximately 30 % of
PKA activity found in brain cofractionates with neuronal microtubule-associated protein
MAP-2 is a high molecular weight protein (~ 270 kDa) which co-purifies with brain
microtubules, and is a major substrate of PKA (Sloboda et al, 1975, Vallee, 1980). In
astrocytes PKA phosphorylates the intermediate filament proteins GFAP and vimentin,
major components of the glial cell cytoskeleton (McCarthy et al, 1985, Mobley and
Combs, 1992). The intermediate filament proteins assemble to form filaments and
undergo dynamic reorganisation during mitosis or differentiation (Jones et al, 1985, Lim

**Cell proliferation.** The stimulation of cell proliferation by growth factors is mediated via MAPK activation. In some cell types, including astrocytes, PKA inhibits cell proliferation through its inhibition of MAPK activity (Graves et al, 1993, Cook and McCormick, 1993, Sevetson et al, 1993, Wu et al, 1993), and also by blocking the translocation of MAPK from the cytosol to the nucleus (Kurino et al, 1996). PKA appears to phosphorylate and inactivate the upstream kinases, Raf-1 and B-Raf (see Figure 1.1, Peraldi et al, 1995, Ramstad et al, 2000, Liebmann, 2001) However, cAMP is a positive intracellular signal for cell proliferation in many differentiated cells (Dumont et al, 1989).

1.10 The AMP-activated protein kinase

AMPK has been quoted as the ‘fuel gauge’ of the cell responsible for maintaining energy status in times of stress (Hardie and Carling, 1997). AMPK is activated by agents that increase the AMP:ATP ratio eg. anoxia, ATP depletors, $\text{H}_2\text{O}_2$, heat shock, fructose (Corton et al, 1994), and also by an increasing number of conditions that do not affect the nucleotide ratio eg. osmotic shock (Fryer et al, 2002), leptin (Minokoshi et al, 2002), insulin (Witters and Kemp, 1992), and the antidiabetic drug metformin (Zhou et al, 2001). AMPK responds by ‘switching off’ non-essential, energy-utilising processes (such
as cholesterol and fatty acid synthesis) whilst ‘switching on’ ATP-generating systems (such as fatty acid oxidation and glucose utilisation) (Hardie et al, 1998). The enzyme was first identified in rat liver as the protein kinase responsible for the inactivation of both ACC and HMG-CoAR, the enzymes regulating fatty acid and cholesterol synthesis, respectively (Carling et al, 1987, 1989). In any situation, the goal of AMPK is to protect and replenish intracellular ATP levels.

1.10.1 Structure of AMPK

AMPK has been extensively purified; affinity chromatography using ATP-γ-sepharose revealed that AMPK is a heterotrimer consisting of a catalytic subunit, α, and two non-catalytic subunits, β and γ of molecular weights 63, 38 and 35 kDa respectively, all three of which are required for catalytic activity (Davies et al, 1994). The β and γ subunits not only bind to the α subunit, but also to each other, increasing the stability of the complex (Dyck et al, 1996). Isoforms of each subunit have been identified, termed α1, α2 (Stapleton et al, 1996), β1, β2 (Thornton et al, 1998) and γ1, γ2, γ3 (Gao et al, 1996, Cheung et al, 2000) suggesting many possible combinations of the αβγ holoenzyme could exist.

The potential diversity of AMPK holoenzymes may confer differences in properties, for example substrate recognition has been reported to differ slightly in α1 compared with α2 (Woods et al, 1996a, Michell et al, 1996), while a difference in tissue distribution of the subunit isoforms (Stapleton et al, 1997, Thornton et al, 1998, Turnley et al, 1999) suggests tissue-specific roles for the isoforms. The γ subunit is involved in binding of AMP to the enzyme complex, and complexes containing the γ2 isoform have a much greater dependence on AMP (Cheung et al, 2000). Brain contains similar amounts of each γ isoform, while the γ1 isoform predominates in the periphery (Cheung et al, 2000). The β subunit is thought to act as a scaffold protein to which the α and γ subunits bind in the correct orientation for the binding of AMP and activation (Woods et al, 1996b).
The human skeletal muscle form of AMPK has been cloned; human AMPK α subunit cDNA is 90% homologous to the rat sequence, with a predicted molecular mass of 62.3 kDa, the gene has been localised to chromosome 1 (Beri et al, 1994). AMPK is closely related to a family of yeast protein kinases which are required for the response to nutritional stress. The 63 kDa α subunit of rat AMPK is analogous to the SNF1 (sucrose non-fermenter) kinase of *Saccharomyces cerevisiae*, which is essential for the release of genes from glucose repression (Carling et al, 1994). Similarly, the β subunit has 3 related yeast homologues; SIP1 (or SNF1 interacting protein 1), SIP2 and GAL83, leading to the suggestion that the β subunit may regulate transcription in mammalian cells as these proteins do in yeast (Stapleton et al, 1994). The yeast SNF4, which is required for activation of SNF1, is homologous to the AMPK γ subunit (Stapleton et al, 1994, Woods et al, 1996b).

### 1.10.2 Regulation of AMPK

AMPK is activated in a number of ways by AMP, firstly by its binding to an allosteric site on the enzyme, for which it competes with ATP if the latter nucleotide is present at higher concentrations. The activity of AMPK increases 4-fold in the presence of the standard assay concentrations of AMP (200 μM) and ATP (200 μM), with a half-maximal effect ($K_a$) at 2-4 μM AMP (Carling et al, 1987, Corton et al, 1995). The more physiological concentration of 4 mM ATP produces a tenfold increase in the $K_a$ for AMP to 30 μM (Corton et al, 1995). This is the basis for the recognition that AMPK is activated by an increase in the AMP:ATP ratio (Corton et al, 1994). AMPK is further activated at least 20-fold by phosphorylation by a specific upstream protein kinase, AMPK kinase (AMPKK, Moore et al, 1991, Weekes et al, 1994 Carling et al, 1987). This phosphorylation occurs at threonine-172 on the α subunit (Hawley et al, 1996, Stein et al, 2000), although other sites are also thought to be involved. These effects are independent, making a total of 100-fold activation possible (Corton et al, 1994). The phosphorylation and activation of AMPK is achieved both by making AMPK a better
substrate for AMPKK and also by a direct allosteric activation of AMPKK by AMP (Hawley et al, 1995). A further means by which AMP promotes AMPK activity is by its inhibition of dephosphorylation of the enzyme by PP2C, apparently by reducing the ability of phosphorylated AMPK to act as a substrate for PP2C rather than a direct action of AMP on the phosphatase (Davies et al, 1995). A diagrammatic representation of the AMPK cascade is shown in Figure 1.4.

![Figure 1.4 AMPK subunit association and mechanisms of regulation.](image)

The consensus sequence recognised by AMPK has been investigated by three different groups using site-directed mutagenesis of a peptide substrate. Early studies used the sequence surrounding serine-79 of ACC, HMRSSMSGLHLVKRR (“SSMS”) where the serine underlined is that phosphorylated by AMPK. In ‘SAMS’ peptide, the serine phosphorylated by PKA was replaced with alanine: HMRSAMSGLHLVKRR so the latter peptide is ideal for use in crude preparations as a specific AMPK substrate (Davies et al, 1989). Sullivan et al (1994) compared AMPK substrate specificity of a peptide derived from the sequence surrounding serine 872 on HMG-CoAR (HMIHNRSKINLQNRR) to SAMS peptide. The former was found to be a better substrate for both rat and human liver AMPK. The HMG-CoAR peptide, like SAMS, has methionine at -5. Instead of the basic, hydrophilic arginine at -4, it has a hydrophobic isoleucine residue. However, it has histidine at -3, which is basic, so the basic residue may be at either -4 or -3. Toomey et
al (1995) analysed a range of synthetic peptides based on the sequence surrounding serine-79 of ACC. AMPK from rat liver (containing a mixture of α1 and α2 catalytic subunit isoforms (Woods et al, 1996) was tested against peptides with modifications to the first four amino acids of HMRSSMSGLHLVKRR. A hydrophobic residue at the -5 position was found to be important. Replacement of the methionine at -5 with alanine produced a seven-fold increase in Km, while there was no increase when it was replaced with another hydrophobic residue. A basic residue at -4 is also important. This data was in agreement with the findings of Weekes et al (1993) that hydrophobic side chains at -5 and +4 and basic residues at -4 or -3 were important. Dale et al (1995) concluded that AMPK prefers the sequence: hyd-X-bas-XXSXXX-hyd, or hyd-bas-XXXSXXX-hyd, where hyd is a hydrophobic residue (M, V, L, I or F) and bas is a basic residue (R>K>H). Michell et al (1996) confirmed the findings of Dale et al (1995), while reporting that the α1 isoform of the catalytic subunit does not require a hydrophobic residue at the +4 position. Woods et al (1996a) reported that the α1 and α2 isoforms of the AMPK catalytic subunit exhibit a subtle difference in substrate specificity in vitro. Compared to α1, α2 appears to prefer leucine and isoleucine over methionine at the -5 position and isoleucine over leucine at +4.

1.10.3 Functions of AMPK

Whenever ATP is depleted, AMP concentration rises dramatically due to the action of adenylate kinase, which attempts to increase the ATP concentration according to the reaction 2ADP ↔ ATP + AMP. Corton et al (1994) found that activation of AMPK in response to heat shock or arsenite exposure was directly proportional to an increase in the AMP:ATP ratio, caused by both a decrease in ATP and an increase in AMP concentration. AMPK attempts to maintain cellular energy status by inhibiting ATP-utilising processes and activating alternative pathways for ATP generation (Hardie and Carling, 1997). AMPK inactivates ACC by phosphorylation of serine-79 (Munday et al, 1988), and HMG-CoAR by phosphorylation of serine-871 (Clarke and Hardie, 1990), resulting in inhibition of fatty acid and cholesterol synthesis, respectively.
Tissues such as the heart and skeletal muscle are mainly oxidative, while mammary gland and adipose tissue are mainly lipogenic. The liver is capable of expressing either high rates of fatty acid oxidation or high rates of lipogenesis, depending on the nutritional status of the animal (Velasco et al, 1997a), so the regulation of fatty acid oxidation is more complex in liver (reviewed by Guzmán and Geelen, 1993) than in heart (reviewed by Lopaschuk et al, 1994) and skeletal muscle (reviewed by Winder, 1998). The most important regulatory site in the process of fatty acid oxidation is the entry of fatty acids into the mitochondria, a step catalysed by two carnitine:palmitoyl-CoA acyltransferases, CPT I and CPT II. The transferase on the cytosolic side of the mitochondrial membrane, CPT I, is potently inhibited by malonyl-CoA, the product of the reaction catalysed by ACC (McGarry and Foster, 1979, Brown et al, 1994, McGarry and Brown, 1997). In this way, inhibition of ACC by AMPK activates fatty acid oxidation by removing its inhibitor, malonyl-CoA, and results in increased energy production (Awan and Saggerson, 1993, Saddik et al, 1993, Hutber et al, 1997).

AMPK is not only activated during pathological stresses; in skeletal muscle the process of fatty acid oxidation, stimulated by AMPK activation is a normal occurrence during muscle contraction. Muscle contains different isoforms of both CPT I and ACC to those found in lipogenic tissues (see section 1.12.1), and its CPT I is much more susceptible to malonyl-CoA inhibition than the liver CPT I (Weis et al, 1994a, b, Thampy, 1989, Bianchi et al, 1990).

In muscle the concentration of malonyl-CoA diminishes during exercise (Winder et al, 1990). Electrical stimulation of muscle via its nerve supply has revealed that this decrease in malonyl-CoA is associated with a loss of ACC activity, which is evident within seconds and persists for more than an hour after contraction has ceased (Duan and Winder, 1992, Vavvas et al, 1997, Winder and Hardie, 1996). This inhibition of ACC activity appears to be due to phosphorylation (Vavvas et al, 1997) and is associated with reciprocal changes in the activity of the α2 (but not the α1) isoform of AMPK (Vavvas et al, 1997). Free AMP levels are increased during contraction and exercise (Aragon et
al, 1980, Dudley and Terjung, 1985), thus it is likely that this change in the AMP:ATP ratio is what activates AMPK in these conditions (Ponticos et al, 1998, Vavvas et al, 1997). This conclusion is strengthened by the finding that contraction-induced stimulation of AMPK can be mimicked by the AMP analogue, ZMP, on incubation with 5-aminomidazole-4-carboxamide ribonucleoside (AICAR) (Merrill et al, 1997, see next paragraph). Cardiac muscle fatty acid oxidation appears to be regulated in a similar manner: hearts subjected to ischaemia followed by reperfusion exhibited increased AMPK activity, decreased ACC activity and increased fatty acid oxidation (Kudo et al, 1996).

Identification of physiological substrates of AMPK has been hampered by the lack of specific methods for activating the kinase in intact cells. One method employed was to activate AMPK in hepatocytes by treatment with fructose or arsenite (Hardie and Carling, 1997). While these treatments effectively deplete ATP, this has many nonspecific side effects. A more specific method for activating AMPK in intact cells has been made possible by the use of AICAR. AICAR is taken up and metabolised to its monophosphorylated form, 5-aminimidazole-4-carboxamide ribonucleoside monophosphate (ZMP) (Corton et al, 1995) by many, although not all, cell types (Javaux et al, 1995). ZMP has been shown to mimic the effect of 5'-AMP on the activation of AMPK in rat liver without altering the levels of ATP, ADP or AMP (Corton et al, 1995, Vincent et al, 1991). Exposure to AICAR has been shown to inactivate ACC and HMGCoAR in isolated hepatocytes (Henin et al, 1995, Vincent et al, 1991) and HSL in isolated adipocytes (Sullivan et al, 1994). Incubation of isolated rat skeletal muscle with AICAR led to activation of glycogen phosphorlase and glycogenolysis (Young et al, 1996), and stimulation of glucose uptake as seen during exercise (Merrill et al, 1997, Bergeron et al, 1999). AICAR treatment of muscle has been recently used to demonstrate that AMPK activation leads to the increases in GLUT-4 and hexokinase proteins induced by exercise, possibly by phosphorylation of transcription factors (Holmes et al, 1999, Ojuka et al, 2000).
Anaerobic conditions are known to activate AMPK. These conditions also stimulate glycolysis; a phenomenon known as the Pasteur effect. The role of AMPK in the Pasteur effect was investigated in perfused rat hearts and cultured cells, in which ischaemia was induced by oligomycin (Marsin et al, 2000). AMPK and 6-phosphofructo-2-kinase (PFK-2) were both activated. PFK-2 controls the synthesis of fructose 2,6-bisphosphate, a potent stimulator of the key glycolytic enzyme 6-phosphofructo-1-kinase (PFK-1) (Okar, and Lange, 1999). PFK-2 was activated following phosphorylation by AMPK, so AMPK is instrumental in the stimulation of glycolysis in the ischaemic heart (Marsin et al, 2000).

Treatment of isolated hepatocytes with AICAR has revealed that the role of AMPK in the regulation of fatty acid oxidation via CPT I activation in liver is in fact twofold (Velasco et al, 1997a). In the first case, AMPK-induced ACC inactivation would lead to the depletion of intracellular malonyl-CoA, thus reducing the concentration of this potent inhibitor of CPTI. This mechanism, also found in the ischaemic heart (Kudo et al, 1996), perfused rat hindlimb (Merrill et al, 1997) and working muscle (Winder and Hardie, 1996) makes a major contribution to the AICAR-induced stimulation of hepatic long-chain fatty acid oxidation. The remaining 20-25 % (in vitro, not necessarily the same in vivo) of the AICAR-induced stimulation of CPT I is by a malonyl-CoA-independent mechanism (Velasco et al, 1997a), which may involve direct phosphorylation of cytoskeletal components by AMPK (Velasco et al, 1998b). Disruption of intermediate filament proteins produces a significant stimulation of CPT I (Velasco et al, 1998a); AMPK has been shown to phosphorylate intermediate filament proteins in hepatocytes (Velasco et al, 1998b), an ability it shares with CaM kinase II (Velasco et al, 1998a).

AICAR has recently been used by Witters and co-workers who report a novel substrate for AMPK (Muoio et al, 1999). The committed step in the pathway of glycerolipid biosynthesis is catalysed by the activity of sn-glycerol-3-phosphate acyltransferase (GPAT). In their study, AICAR appeared to inhibit mitochondrial GPAT in rat hepatocytes. Purified recombinant AMPK was then found to inhibit hepatic mitochondrial GPAT in a time- and ATP-dependent manner. These data provide evidence that AMPK
phosphorylates and inhibits mitochondrial GPAT and promotes channelling of acyl-CoA towards β-oxidation and away from glycerolipid biosynthesis (Muoio et al, 1999).

The same group report identification of a further new substrate for AMPK in rat heart, endothelial nitric oxide synthase (eNOS) was found to be phosphorylated by AMPK both in vitro and during ischaemia in rat hearts (Chen et al, 1999). Paradoxically, this phosphorylation is capable of either activation or inhibition of eNOS depending on the presence or absence of Ca²⁺/CaM; the physiological significance of this phosphorylation is unclear at present (Chen et al, 1999). However, since eNOS controls synthesis of the vasodilator NO, this is potentially an important role for AMPK in cardiac ischaemia.

Regulation of gene expression
A number of genes involved in glucose and lipid metabolism are up-regulated by high glucose concentrations in both liver and adipose tissues (reviewed by Girard et al, 1997, Towle et al, 1997). In two studies prompted by the similarity of the yeast enzyme SNF1 to the AMPK α subunit, Salt et al (1998a, b) investigated whether AMPK had a similar role to that of SNF1 in the regulation of gene expression in response to glucose concentration in mammalian cells. The α₂ subunit isoform of AMPK was found to be enriched in the nucleus, suggesting a role in the regulation of gene expression like that of its yeast homologue, SNF1 (Salt et al, 1998a). AMPK is activated 5-fold by the removal of glucose in the glucose-sensitive pancreatic β cell lines HIT-T15 and INS-1, and may be involved in the regulation of insulin release (Salt et al, 1998b). Further work on the regulation of glucose-dependent genes in primary hepatocytes using the AMPK-activator, AICAR, has shown that AMPK inhibits glucose activation of three glucose-responsive genes, namely L-type pyruvate kinase, Spot 14 and fatty acid synthase, suggesting that AMPK probably controls the regulation of glucose-responsive genes in mammals as its homologue, SNF1 does in yeast (Leclerc et al, 1998, Foretz et al, 1998, Woods et al, 2000, Delzenne et al, 2001).

Raf-1 is a serine/threonine PK which is an effector-protein of Ras, and stimulates the
MAPK cascade, thus relaying signals from receptor activation to the nucleus. Little is known about the activation of Raf-1 by phosphorylation, but phosphorylation of Ser-621 appears to be important in Raf-1 structure and regulation, and this residue is primarily phosphorylated by AMPK in cytosolic extracts of NIH-3T3 cells, and possibly in intact CHO cells (Sprenkle et al, 1997). This may be one mechanism by which AMPK influences gene expression in the nucleus. The Raf-1 kinase has been implicated in apoptosis (Wang et al, 1996), a process recently shown to be inhibited by AICAR in rat thymus cells (Stefanelli et al, 1998). It is clear much remains to be discovered of the role of AMPK in regulation of gene expression and signalling pathways.

1.10.4 AMPK in brain

AMPK has a wide tissue distribution in the rat, where its activity has been measured in liver, lung, heart, kidney, brain, lactating mammary gland, adrenal gland, adipose tissue and skeletal muscle (Davies et al, 1989, Stapleton et al, 1996). A recent study by Turnley et al (1999) investigated the cellular distribution of the AMPK α1, α2, β1, β2, γ1 and γ2 isoforms in mouse CNS. It appears from their immunostaining that α2 is the predominant catalytic subunit in the CNS, while β1 and β2 expression varied. The γ1 noncatalytic subunit was found in most neural tissue, but not in astrocytes, while the antibody used to detect the γ2 isoform detected very little, if any expression in all areas of brain examined, with the exception of Bergmann glia, radial glia found in the cerebellum. It remains to be seen which γ subunit is present in astrocytes, since the γ1 and γ2 could not be detected, although γ3 was not used in this study, and is therefore a likely candidate. One of the most significant findings of Turnley et al (1999) was the nuclear localization of α2, β1 and γ1 in some neurons, but not in astrocytes, suggesting a possible nuclear role for the α2β1γ1 heterotrimer. Expression of the α2 subunit was increased in reactive astrocytes (Turnley et al, 1999). The discovery that AMPK is present in astrocytes is important in view of its role in energy conservation and the regulation of metabolism. Astrocytes are highly metabolically active and regulate the availability of fuel such as lactate to the neurons.
Recent work on the role of AMPK in the brain has found new roles for the enzyme in protection of cells from damage. Culmsee et al (2001) found that treatment of hippocampal neurons with AICAR protected them against death induced by glucose deprivation, chemical hypoxia, and exposure to glutamate and amyloid β-peptide. This effect of AICAR was abolished when levels of AMPK were suppressed. AICAR was also used by Blázquez et al (2001) to study the involvement of AMPK in apoptosis in astrocytes. AICAR blunted fatty acid-mediated induction of serine palmitoyltransferase and ceramide synthesis de novo, without affecting fatty acid synthesis and oxidation. The build-up of ceramide normally activates the Raf-1/ERK cascade leading to fatty acid-induced apoptosis.

1.11 Carbohydrate and lipid metabolism in brain

As part of the BBB, astrocytes separate the other cells of the brain from the bloodstream. This means that all nutrients required by the brain for energy production, such as glucose and oxygen, have to enter through these cells. Glucose is virtually the only fuel used by the brain, except during prolonged starvation. The brain has limited fuel stores and so requires a continuous supply of glucose, which enters freely from the bloodstream. The brain accounts for 60% of the utilization of glucose by the whole body in the resting state. During activation of a given cortical area, the increase in glucose uptake can be ascribed predominantly, if not exclusively, to astrocytes (Magistretti and Pellerin, 1996), whose endfeet line the capillaries. If glucose was subsequently released by astrocytes there would be increased uptake of glucose by neurons, which was not seen. This observation implies that energy substrates other than glucose must be released by astrocytes for use by other cells.

Lactate and pyruvate are adequate substrates for brain tissue in vitro (Teller et al, 1977, Schurr et al, 1988), although they have limited permeability across the BBB (Pardridge and Oldendorf, 1977), so must be formed inside this barrier. Lactate is quantitatively the main metabolic intermediate released by astrocytes (Pellerin and Magistretti, 1994). It is
taken up from the extracellular space by neurons (Fellows et al, 1993, Dringen et al, 1993b) in addition to glucose. Other fuel molecules released by astrocytes into the extracellular space to be taken up by neurons, but to a much smaller extent than lactate, include pyruvate, α-ketoglutarate, citrate and malate (Shank and Campbell, 1984, Selak et al, 1985, Sonnewald et al, 1991, Shank et al, 1993). In conditions of hypoxia, astrocytes release ketone bodies, as explained later (Auestad, et al, 1991, Blázquez et al, 1998).

Glycogen is the largest energy reserve of the brain; it is mainly localized in astrocytes, although certain ependymal, endothelial and choroid plexus cells and some large neurons contain small quantities of glycogen (Cataldo and Broadwell, 1986a, b). As sites of glycogen storage, astrocytes protect co-cultured neurons from anoxia and glucose deprivation (Vibulsreth et al, 1987, Swanson and Choi, 1993). Glycogen degradation in cultured astrocytes results in the release of lactate, not glucose, as a fuel that can be used by neighbouring cells (Wender et al, 2000, Walz and Muckerji, 1988, Dringen and Hamprecht, 1993, Magistretti et al, 1993), indeed, some astrocytes lack the enzyme glucose-6-phosphatase required for the production of glucose from glycogen (Bell et al, 1993). Breakdown of glycogen in astroglial-rich primary cultures can be induced by several hormones, including some that elevate cAMP, such as adenosine (Magistretti et al, 1986), noradrenaline (Quach et al, 1978, Cambray-Deakin et al, 1988), histamine (Quach et al, 1980, Arbones et al, 1990), serotonin (Quach et al, 1982), and VIP (Magistretti et al, 1981). The cAMP released enables PKA activation of the glycogen cascade (section 1.7.1).

Energy metabolism in the brain is characterised by high and rapidly fluctuating rates of ATP synthesis and utilization. Creatine kinase (CK) plays an important role in maintaining intracellular ATP levels (Wallimann et al, 1992). Four main types of CK exist, the cytosolic MM-CK, found mainly in muscle and B-CK which is most highly expressed in the brain, and two mitochondrial CKs (MiCK) (Benfield et al, 1988, Schlegel et al, 1988, Haas and Strauss, 1990). In common with other cell systems
characterised by fluctuating energy requirements, the brain contains both cytosolic creatine kinase (B-CK) and mitochondrial CK (MiCK) isozymes (Jacobus and Lehninger, 1973, Wyss et al, 1992). The CK isozymes catalyse the reversible phosphotransfer reaction between creatine phosphate (PCr) and ADP to regenerate ATP, according to the following equation:

\[
\text{Creatine} - (\text{P}) + \text{ADP} + \text{H}^+ \rightleftharpoons \text{Creatine} + \text{ATP} \\
\uparrow \quad \text{CK}
\]

and therefore have an important role in maintaining intracellular ATP levels in cells with high energy demands (Hemmer and Wallimann, 1993, Manos and Bryan, 1993).

The CK/PCr system is involved in maintaining the balance of ATP:ADP within cells and hence has an impact on intracellular AMP levels also. Since the AMP:ATP ratio is important in the regulation of AMPK, Ponticos et al (1998) investigated whether the two systems were linked. AMPK phosphorylated and inactivated MM-CK, while an excess of PCr inhibited AMPK activity. Muscle contraction results in an increase in concentration of AMP coupled with a decrease in PCr, resulting in activation of AMPK by AMP and loss of inhibition of AMPK by PCr. It is likely that a similar mechanism exists for the regulation of B-CK by PCr in the CNS, although this has not been published at the time of writing.

Astrocytes in primary culture contain B-CK, as do primary cultured neurons and oligodendrocytes, although reports vary as to the relative amounts in each cell type.
(Manos et al, 1991, Molloy et al, 1992, Manos and Bryan, 1993). In the study by Molloy et al (1992), B-CK mRNA was detectable in homogeneous, primary cultures of neurons; the level was about 17-fold higher in oligodendrocytes and 15-fold higher in astrocytes than in neurons. The authors suggest that the high levels of B-CK mRNA detected in astrocytes may be related to their ATP requirements for ion transport, such as the uptake of ions from the synapse. B-CK protein has been found in rat brain synaptic plasma membranes, where it appears to be coupled to Na⁺,K⁺-ATPase (Saks et al, 1983). Activity levels of B-CK appear to differ from its mRNA levels. Manos et al, (1991) studied the cellular distribution of B-CK using primary cultures of purified neurons, astrocytes and oligodendrocytes. They found 3-4 fold higher CK activity in oligodendrocytes than in astrocytes or neurons, which had similar activity.

Immunofluorescence studies have been carried out to show the subcellular localisation of B-CK in astrocytes (Manos and Bryan, 1993). Low density, dividing astrocyte cultures contained high B-CK activity and immunoreactivity, mainly localised to the nucleus. Both activity and immunofluorescence were significantly reduced in confluent, nondividing cultures. B-CK co-localises with DNA in the nucleus; cytosolic B-CK does not co-localise with the cytoskeletal proteins tubulin or GFAP. The nucleus is not known to generate energy itself; it must rely on phosphate transfer as a source of energy. PCr, phosphorylated in the mitochondria can enter the nucleus via nuclear pores. It would then be available for nuclear ATPases to produce ATP for processes such as nucleic acid synthesis, macromolecular transport, contractile events or other movements within the nucleus (Manos and Edmond, 1992, Cande, 1983).

Certain metabolic intermediates, under particular conditions, can substitute for glucose as alternative substrates for brain energy metabolism (Sokoloff, 1989). Starvation, diabetes or breast-feeding in neonates lead to increased plasma levels of the ketone bodies acetoacetate and D-3-hydroxybutyrate that can be used by the brain as metabolic substrates in addition to glucose (Patel and Owen, 1977, Yeh et al, 1977, Robinson and Williamson, 1980). In fact, during pathophysiological situations when there is glucose
deprivation, ketone bodies may become the major source of brain energy (Edmond, 1992, Zammit, 1994). Astrocytes in culture produce ketone bodies from fatty acids (Auestad, et al, 1991, Blázquez et al, 1998), under the control of AMPK and ACC, during hypoxia (Blázquez et al, 1999). The situation in vivo, including the relative importance of ketone bodies or lactate as the energy source produced by astrocytes in response to pathophysiological conditions, remains to be seen.

HMG-CoAR, the rate-limiting enzyme in cholesterol synthesis is present in astrocytes (Langan et al, 1987, Bobryshev et al, 1995). In fact, the astrocyte appears to be a key cell in CNS cholesterol metabolism (Pitas et al, 1987, Swanson et al, 1988, Poirier et al, 1991). CNS cholesterol is regulated separately from the peripheral cholesterol levels by an intra cerebral system of lipid synthesis due to the low permeability of the BBB to plasma lipoproteins (Swanson et al, 1987, Malavolti, 1991, Dietschy, 1993). Recently, use of the HMG-CoAR inhibitor, lovastatin, has reinforced the role of astrocytes in the pathogenesis of multiple sclerosis (MS) by reducing the expression of iNOS, TNF-α and IFN-γ, thus improving clinical signs, in the experimental model of MS, experimental allergic encephalomyelitis (EAE) (Stanislaus et al, 1999).

The brain synthesize fatty acids de novo (Brady, 1960, Volpe and Kishimoto, 1972) and by chain elongation of preexisting fatty acids (Aeberhard et al, 1969). In the developing brain, ketone bodies and lactate are preferred over glucose as substrates for lipid synthesis (Yeh et al, 1977, Bolaños and Medina, 1992). The de novo synthesis of fatty acids is regulated by ACC, first measured in brain by Gross and Warshaw (1974). The main site of lipogenesis in the brain is in the oligodendrocytes which synthesise fatty acids for myelin (Spencer et al, 1993, Pleasure et al, 1984, Morell and Toews, 1984). Astrocytes synthesise fatty acids to a lesser extent (Koper et al, 1984), which they may release for neuronal uptake (Moore, S, et al, 1991). ACC was measured in astrocytes recently by Blázquez et al (1998), where it may have a role in the regulation of fatty acid oxidation and ketone body production.
1.12 Acetyl-Coenzyme A Carboxylase (ACC)

ACC catalyses the first committed step in fatty acid biosynthesis, the carboxylation of acetyl-CoA to form malonyl-CoA (Wakil et al, 1990). It is the regulatory enzyme in this pathway and is also involved in the regulation of fatty acid oxidation via the potent inhibitory action of malonyl-CoA on CPT I, the enzyme responsible for mitochondrial uptake of fatty acids for β oxidation (McGarry and Foster, 1979, Moir and Zammit, 1993).

1.12.1 Structure and Function of ACC

ACC contains a biotin prosthetic group, covalently attached via its carboxyl group to a lysine residue on the enzyme. It is one of four known mammalian biotin-containing enzymes where biotin serves as a carrier of activated CO₂. The other three biotin-containing enzymes are in the mitochondria (pyruvate carboxylase, propionyl-CoA carboxylase, 3-methyl crotonyl CoA carboxylase). ACC is the only cytosolic enzyme in this group. The reaction catalysed by ACC can be partitioned into discrete half-reactions (Moss and Lane, 1971):

\[
\text{ACC.biotin} + \text{HCO}_3^- + \text{ATP} \rightarrow \text{ACC.biotin.CO}^2- + \text{ADP} + \text{Pi} \quad (I)
\]
\[
\text{ACC.biotin.CO}_2^- + \text{acetyl-CoA} \rightarrow \text{malonyl-CoA} + \text{ACC-biotin} \quad (II)
\]

The initial step involves the MgATP-dependent carboxylation of the biotinyl prosthetic group of the enzyme to form a 1′N-carboxybiotinyl enzyme intermediate. In the second step, carboxyl transfer from the carboxybiotinyl enzyme to acetyl-CoA gives rise to malonyl-CoA. Both half-reactions are activated by citrate, and enhanced reactivity of the carboxybiotinyl moiety has been correlated with citrate-induced conformational changes at the prosthetic site. (Moss and Lane, 1971). In Escherichia coli, ACC can be resolved into three domains (Guchait et al, 1974): a biotin carboxylase domain, in which reaction (I) is catalysed, a carboxyl transferase domain, in which reaction (II) is catalysed and a biotin domain, containing a carboxyl carrier protein which is a non-enzymic protein.
containing the covalently bound biotin. Mammalian ACC is a single polypeptide and the model of its domain structure (Munday and Hemingway, 1999) is based on homologies of the primary amino acid sequence with carbamoyl phosphate synthetase (which catalyses ATP-dependent carboxylation) and propionyl-CoA carboxylase (Haase et al., 1982, Hardie and Carling, 1997, Lopez-Casillas et al., 1988), the sequencing of phosphorylation sites (Munday et al., 1988a) and the known locations of the ATP, biotin and acyl-CoA binding sites (Lopez-Casillas et al., 1988, Abu-Elheiga et al., 1995, 1997, Kim, 1997).

Much evidence from purification (Ahmad et al., 1978, Thampy, 1989, Winz et al., 1994, Trumble et al., 1995), immunological analysis (Bianchi et al., 1990) and cDNA cloning (Lopez-Casillas et al., 1998, Abu-Elheiga et al., 1995, 1997, Ha et al., 1996, Widmer et al., 1996) agrees that two major isoforms of ACC exist, having approximate molecular weights of 265 kDa and 280 kDa. These have been variously termed ACC1/ACCα/ACC-265 and ACC2/ACCβ/ACC-280, but will be referred to here by their molecular weights, ie. ACC-265 and ACC-280. The two isoforms of ACC represent distinct gene products; the ACC-265 gene has been localized to human chromosome 17 (Abu-Elheiga et al., 1995), and the ACC-280 gene to human chromosome 12 (Widmer et al., 1996, Abu-Elheiga et al., 1997). In spite of this fact, the isoforms are strikingly similar in their domain structure and amino acid sequence of their catalytic regions (Munday and Hemingway, 1999), which are 85% homologous (Abu-Elheiga et al., 1997). The main difference between the two isoforms is an additional N-terminal sequence found in ACC-280 but not ACC-265 (Abu-Elheiga et al., 1997). This N-terminal moiety has been suggested to function in anchoring ACC-280 to the mitochondrial membrane in order to produce malonyl-CoA adjacent to CPT I (Kim, 1997). Wakil and colleagues have recently visualised the association of ACC-280 with the mitochondria using immunofluorescent microscope analysis (Abu-Elheiga et al., 2000). The same group report significantly lowered levels of malonyl-CoA and elevated rates of fatty acid oxidation in hearts and muscle of ACC-280 knockout mice (Abu-Elheiga et al., 2001).
As would be predicted from the fact that two isoforms of the same enzyme exist, the ACC isoforms appear to have slightly different roles, and also exhibit different tissue distribution. ACC-280 has lower Km values for ATP and HCO$_3^-$ and a higher Km for acetyl-CoA and higher Ka for citrate than ACC-265 (Trumble et al, 1995, Bianchi et al, 1990). The difference in tissue distribution of the two isoforms may suggest discrete functions for each, since predominantly lipogenic tissues such as white adipose tissue and lactating mammary gland mainly express ACC-265, while oxidative tissues such as heart and skeletal muscle contain mostly ACC-280. Both fatty acid oxidation and synthesis are important in liver, and this tissue expresses both isoforms (Trumble et al, 1995, Bianchi et al, 1990, Abu-Elheiga et al, 1997). There has been speculation, therefore, that ACC-280 may be involved in the control of fatty acid oxidation, while ACC-265 may function mainly in the control of fatty acid synthesis. However, it has recently been reported that in astrocytes, the only site of fatty acid oxidation in the brain (Edmond et al, 1987, Edmond, 1992, Staub et al, 1995), 90-95 % of total ACC is ACC-265 and only 5-10 % is ACC-280 (Blázquez et al, 1998). This would appear to suggest that the ACC isoform profile of astrocytes is closer to that of the lipogenic white adipose tissue (Bianchi et al, 1990) than it is to that of hepatocytes (Bianchi et al, 1990, Guzmán et al, 1995). The process of fatty acid oxidation is well documented in astrocytes (Auestad et al, 1991, Blázquez et al, 1998, Spurway et al, 1997), as is the synthesis of fatty acids (Koper et al, 1984). It is not known whether elongation or oxidation of fatty acids predominates in astrocytes, so any conclusions linking roles of ACC to isoform expression can only be speculation at present. However, it appears from use of an ACC-280 knockout mouse that ACC-280 is responsible for the regulation of oxidation, the knockout mice had higher rates of oxidation and lower body fat than the wild-type (Abu-Elheiga et al, 2001). This suggests that fatty acid synthesis may predominate in astrocytes.

The minimum molecular weight of native ACC suggests that the smallest form that exists \textit{in vivo} is a dimer. Immunoprecipitation with antibodies for each isoform suggests that this could be a homodimer, or a heterodimer of both isoforms (Bianchi et al, 1990). The dimer (also termed a protomer) in turn polymerises into chains of up to 30 protomers in
the presence of high concentrations of citrate (Ahmad et al, 1978), and this polymerisation may be involved in regulation of the enzyme (Ashcraft et al, 1980, Borthwick et al, 1987, Thampy and Wakil, 1988).

1.12.2 Regulation of ACC

ACC is regulated in several ways, in the long term the enzyme concentration itself is controlled by nutritional and hormonal effects on ACC gene expression. Short-term regulation of ACC enzyme activity occurs by allosteric regulation by citrate and long chain acyl-CoA esters, and reversible phosphorylation, which is itself a highly regulated process. The bulk of knowledge concerning ACC regulation was obtained before the second isoform was discovered, and relates to ACC-265. A summary of the regulation of ACC is presented in Figure 1.5.

1.12.2.1 Long term regulation of ACC concentration

1.12.2.2 Short term regulation of ACC activity

Allosteric regulation

Two main allosteric activators of ACC have been identified, citrate and long-chain fatty acyl-CoA esters. Citrate stimulates both partial reactions of ACC (Stoll et al, 1968). It can increase ACC activity in vitro 50-fold within 1 min, acting as a feedforward activator (Hardie and Guy, 1980). The Ka for citrate of pure ACC is approximately 2 mM (Hardie and Guy, 1980); the physiological citrate concentration range is 0.1-1 mM, so citrate is potentially an allosteric activator of ACC in vivo. Citrate causes polymerization of ACC (Beatty and Lane, 1983), which renders it resistant to phosphorylation (Munday and Hemingway, 1999). Conversely, phosphorylated ACC displays reduced citrate sensitivity (Munday et al, 1988a), so there is cooperation between the regulation of ACC by citrate and by phosphorylation.

Feedback inhibition of ACC by saturated and unsaturated long-chain fatty acyl-CoA esters varies according to chain length, from 1-150 nM (Nikawa et al, 1979). The physiological importance of this ability to regulate ACC is difficult to assess, however, since fatty acyl-CoAs exist in the cell bound to proteins (Faergeman and Knudsen, 1997). Exogenous fatty acids are known to inhibit lipogenesis, but it is not clear whether this is through the allosteric inhibition of ACC (Halestrap and Denton, 1974, Mayes and Topping, 1974).

Reversible phosphorylation

The first protein kinase found to phosphorylate ACC was purified PKA (Hardie and Guy, 1980). ACC is now known to contain several phosphorylation sites which can be phosphorylated by various protein kinases in vitro, but probably only by PKA and AMPK in vivo (Munday and Hemingway, 1999, Munday et al, 1988a). Rat ACC-265 is phosphorylated by AMPK on serines 79, 1200 and 1215, and by PKA on serines 77 and 1200 (Munday et al, 1988a, Davies et al, 1990). Phosphorylation of ACC by either protein kinase doubles its Ka for citrate, but phosphorylation by AMPK reduces the V_max.
of the enzyme by 80-90% and phosphorylation by PKA by only 15% (Munday et al., 1988a). From this it could be inferred that serine 1200, the common site of phosphorylation might control sensitivity to citrate, while the phosphorylation of serines 77 and 79 by AMPK or PKA respectively affects the $V_{max}$ to different extents. However, this hypothesis is not supported by experimental evidence. Truncation of the enzyme at the N-terminal, thus removing serines 77 and 79 results in full reactivation, so phosphorylation of serine 1200 may not be implicated in inhibition of ACC (Davies et al., 1990). Conversely, point mutations of ACC carried out by Ha et al. (1994) revealed that the inhibitory effects of AMPK were mediated by its phosphorylation of serine 79, while only the mutation of serine 1200 abolished PKA inhibition of ACC. The phosphorylation of serine 77 and serine 79 is mutually exclusive (Munday et al., 1988b), and the importance of serine 1200 in ACC regulation is as yet unclear. Dephosphorylation of ACC and hence reactivation is mediated by the protein phosphatase PP2A (Ingebritsen et al., 1983a). Gaussin et al. (1997) have reported that a novel PP2A, activated by glutamate, is responsible for activation of ACC in rat hepatocytes.

Physiological regulation of phosphorylation of ACC follows the diurnal rhythm. During the dark period when the rats are feeding, ACC is dephosphorylated and activated (Davies et al., 1992), while in the light period when they are fasting, the enzyme is phosphorylated and inactivated. The same inactivation by phosphorylation is seen in response to a high fat diet (Davies et al., 1992). The hormones glucagon and adrenaline stimulate phosphorylation and inactivation of ACC, both in isolated cells (Witters et al., 1979b, Holland et al., 1984, Brownsey et al., 1979) and in vivo (Mabrouk et al., 1990, Lee and Kim, 1979) by a currently unknown mechanism. This inactivation, in all cases where it was determined, was characterised by a large increase in the $K_a$ for citrate and a large decrease in $V_{max}$. These changes are characteristic of phosphorylation by AMPK and not by PKA. Indeed, there is much evidence to support the fact that the physiological inactivator of ACC is not PKA but AMPK, and it is serine 79 that becomes phosphorylated in vivo in response to physiological manipulations (Davies et al., 1992, Sim and Hardie, 1988, Haystead et al., 1990).
ACC-280 is a phosphoprotein (Thampy, 1989, Winz et al, 1994) and there is plenty of evidence that this isoform is also regulated by reversible phosphorylation. Rat liver ACC-280 is a better substrate for PKA than for AMPK (Winz et al, 1994), and although it lacks the serine-1200 site of ACC-265, it contains consensus sequences for PKA in its N-terminal extension (Abe et al, 1998). There are conflicting reports as to whether in vitro phosphorylation by PKA can alter ACC-280 activity. Winder et al (1997) claim PKA phosphorylation has no effect on ACC-280 activity, while Dyck et al (1999) report significant inactivation of ACC-280 by PKA phosphorylation.

![Figure 1.5 Regulation of ACC by the AMPK cascade](image)

ACC-280 is inhibited 200-300 % by AMPK phosphorylation in skeletal muscle in vitro, which is similar to the inhibition of ACC-265 by AMPK (Winder et al, 1997, Winder and Hardie, 1996). The phosphorylation and inactivation of ACC-280 by AMPK in reperfused rat hearts following ischaemia results in 3 to 4-fold activation of fatty acid oxidation (Kudo et al, 1996). The phosphorylation of ACC-280 by AMPK is reversible by PP2A just as for the ACC-265 isoform (Vavvas et al, 1997).
1.12.3 Functions of ACC in brain and astrocytes

The main functions of ACC in fatty acid synthesis and the inhibition of fatty acid oxidation have been outlined previously in this section. In the brain, ACC has long been known to be involved in lipogenesis (Gross and Warshaw, 1974, Patel and Tonkonow, 1974) but the brain enzyme has not been studied as extensively as in other tissues. Since the main CNS cell type involved in lipid synthesis is the oligodendrocyte, it has been assumed that brain ACC is exclusively localised to these cells (Spencer et al, 1993). However, this is unlikely to be true, particularly in the developing brain since all cells require lipids for the construction of membranes. ACC activity in brain is at a maximum during early development, corresponding with the period of myelination (ie. a week before birth in chick brain and a week after birth in rat brain), thereafter it falls and remains low throughout adulthood (Patel and Tonkonow, 1974, Thampy and Koshy, 1991).

As recently as 1991 it was not widely known that the brain could oxidise fatty acids, so the discovery that brain ACC was almost exclusively the 265 kDa isoform was not unexpected in what appeared to be a purely lipogenic tissue (Thampy and Koshy, 1991). Since the discovery that only one CNS cell type, the astrocyte, is able to oxidise fatty acids (Edmond et al, 1987, Auestad et al, 1991, Edmond, 1992), renewed efforts to discover ACC-280 in these cells have proved disappointing; a recent study reports only 5-10 % of total astrocyte ACC was ACC-280 (Blázquez et al, 1998).

CPT I has been shown to regulate flux through the ketogenic pathway in astrocytes in a similar way to hepatocytes (Blázquez et al, 1998, Spurway et al, 1997), in fact the CPT I isoform appears to be the same in the two tissues. CPT I is regulated in astrocytes by inhibition by malonyl-CoA produced by ACC in the same way as in hepatocytes (Blázquez et al, 1998). Presumably ACC is a substrate for AMPK in astrocytes, as is
HMG-CoAR, demonstrated in primary astrocytes by Volpe and Hennessy (1977) and Volpe et al (1985), and known to be regulated to some extent in astrocytes by lipoproteins (Langan et al, 1987).

1.13 Aims of this thesis

The main aims of this project were:

To identify the PPs present in astrocytes and examine changes in their activity over the course of astrocyte development in culture. To use OA to examine the functions of PP2A and PP1 in astrocytes, particularly in cell signalling pathways involving PI metabolism and AA production.

To compare the properties of purified rat brain AMPK with those of the well-characterised rat liver AMPK. To examine the regional distribution of AMPK activity in the brain and changes over a time course of brain development, and to compare these with changes in ACC (the best substrate for AMPK) and with PP2A (the PP that antagonises AMPK action). To identify AMPK activity in cultured astrocytes and examine changes in activity over a time course of development.

To determine the regional distribution of PKA activity in the brain and changes over a time course of brain development and to determine the relative proportions of the two major isoforms of PKA under these conditions. To measure PKA activity in astrocyte culture and examine changes in activity over a time course of development.
CHAPTER 2

MATERIALS AND METHODS
2.1 Materials

Earles Balanced Salt Solution (EBSS) and Minimal Essential Medium (MEM) without glutamine were obtained from GIBCOBRL Life Technologies, Paisley, Scotland. Redivue [γ-\(^{32}\)P]-ATP, \(^3\)H-arachidonic acid, \(^3\)H-inositol and NaH\(^{14}\)CO\(_3\) were obtained from The Radiochemical Centre, Amersham, Bucks, UK.

Antibiotic antimycotic solution, phosphorylase kinase, phosphorylase b, Dowex AG1-X8 anion exchange resin, Dowex 50W-X8 400 mesh strong cation exchange resin, casein, dimethyl sulfoxide (DMSO), calmodulin, calcineurin, salmon testes DNA, A23187, isobutyl methyl xanthine (IBMX), cAMP, Hepes buffer, acetyl Coenzyme A (AcCoA), inositol, 4.9 M MgCl\(_2\), Kemptide, EGTA, bovine serum albumin (BSA), benzamidine, trypsin, poly-D-lysine, D-glucose, L-glutamine, foetal calf serum, trypsin, deoxyribonuclease (DNase), soya-bean trypsin inhibitor (SBTI), phenylmethylsulphonyl fluoride (PMSF) and protein kinase A inhibitor (PKI) were from Sigma Chemical Company, Poole, Dorset, UK.

Mono Q quaternary ammonium strong anion exchanger was from Pharmacia Biotech, St. Albans, Herts, UK.

Cellulose nitrate membrane filters, DE52 pre swollen ion exchange cellulose, P11 cellulose phosphate, fast-flow DEAE sepharose beads and P81 phosphocellulose paper were from Whatman International Ltd, Maidstone, Kent, UK.

A peptide derived from the R\(_n\) subunit of PKA, used to assay PP2B, and Optiphase 'Safe' scintillation fluid were from Fisons Chemicals, Loughborough, Leics, UK.

Okadaic acid, AMP and ATP were from ICN Biomedicals Ltd, Thame, Oxon, UK.

Bisbenzimide was from Hoechst UK Ltd, Hounslow, Middx, UK. Polyethylene glycol-6,000 (PEG) was from Avocado Research Chemicals Ltd, Heysham, Bucks, UK.

Dithiothreitol (DTT) was obtained from Melford Laboratories Ltd, Ipswich, Suffolk, UK. or Calbiochem-Novabiochem Ltd, Nottingham, UK.

Human Glial Fibrillary Acidic Protein (GFAP) and vimentin were obtained from
Calbiochem-Novabiochem Ltd, Nottingham, UK.
N-p-tosyl-L-lysine chloromethyl ketone (TLCK) and N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) were from Acros Organics, Geel, Belgium.
SAMS peptide (HMRSAMSGLHLVKRR) and other AMPK substrate peptides based on the sequence around serine-79 of ACC were synthesised at the School of Pharmacy by Dr. N. Flinn, and was >98 % pure.
Other reagents were from BDH Chemicals, Poole, Dorset, UK.

2.2 Animals

Wistar rats and rat pups, both male and female were used throughout this study. Animals were maintained on a standard chow diet, in temperature controlled conditions with a twelve hour light/dark cycle. Pups were killed by decapitation without prior stunning; older animals by stunning and cervical dislocation.

2.3 Preparation of Primary Cultured Rat Cortical Astrocytes

Astrocyte-enriched cultures of neonatal rat cerebral cortex were prepared according to the method of Dutton et al. (1981). Equipment was sterilised and placed in a positive pressure laminar flow hood. All solutions were sterilised by filtration through a 0.2 μm cellulose nitrate membrane filter immediately before use. Enzyme solutions were adjusted to neutral pH by bubbling with 5% CO₂ / 95% O₂. Neonatal rat pups, aged 0-3 days were decapitated and the head sprayed with a solution of 10 % chlorhexidine in 70 % ethanol. The head was quickly transferred into the hood where the cerebral cortices were removed.

The cortices were placed on a sintered glass plate and the meninges removed using fine forceps. The prepared cortices were placed in disaggregation medium (DM) containing
14 mM D-glucose, 3 mg/ml BSA, 1.5 mM MgSO₄, in EBSS on small plates while the remaining cortices were prepared. The DM was poured off the plates and the cortices were chopped coarsely with a scalpel. The chopped tissue was transferred into 50 ml tubes containing 10 ml trypsin solution (0.25 mg/ml trypsin in DM), swirled and poured into a trypsinisation flask which was shaken at 37°C for 15 min. Dilute DNase (6.4 μg/ml DNase, 192 μg/ml trypsin inhibitor and 240 μM MgSO₄ in DM) (10 ml) was filtered into each flask. This was shaken gently, poured back into the 50ml tubes and centrifuged briefly at 1,000 rpm to sediment cell bodies.

The supernatant was removed from the cell pellet, to which was added a few drops of filtered concentrated DNase (40 μg/ml DNase, 1.2 mg/ml trypsin inhibitor, 240 μM MgSO₄ in DM). A 1.5 mm bore sterile steel cannula was used to gently triturate the suspension by creating a shear force against the side of the tube. Once settled, the top layer containing cells was removed with a sterile plastic pipette into a 15 ml sterile tube. A few more drops of concentrated DNase were filtered onto remaining suspension and the process repeated to harvest any remaining cells. The final suspension was underlaid with 1 ml 4% BSA containing 1.2 mM MgSO₄ in DM using a cannula. Cells were sedimented through the BSA leaving cell debris above it by centrifugation for 5 min at 1,000 rpm. The resulting supernatant containing cell debris was poured off leaving intact cells. Sterile filtered growth medium (GM) (2 mM L-glutamine, 33 mM D-glucose, 10 units/ml penicillin, 10 μg/ml streptomycin, 25 ng/ml amphotericin B (Antibiotic antimycotic solution, Sigma), 10 % foetal calf serum, in MEM without glutamine was used to resuspend the cells, which were made up to a volume equivalent to 2 ml per well of the 6-well plates or 20 ml per 75 cm² flask, with an approximate seeding density of 100,000 cells per well or 800,000 cells per flask. This cell suspension was mixed thoroughly before plating out onto poly-d-lysine (50 μg/ml) pre-coated plasticware. The cells were maintained in a humidified environment of 5 % CO₂ in air at 37°C. Culture medium was renewed by replacing half of the existing GM with fresh GM every 3-5 days.
2.4 Cell Harvesting

When harvesting cells or tissues, any stress, such as dissection/cell harvesting at ambient temperature might result in hypoxia, and artificial AMPK activation by altered AMP:ATP levels. This could lead to inaccurate measurement of AMPK activity and activities of target enzymes. Ideally, the tissues should be rapidly cooled in order to preserve the phosphorylation state at isolation (Hardie and Carling, 1997). This may be achieved with whole tissues by cold-clamping or freeze-clamping (Easom and Zammit, 1984, Davies \textit{et al}, 1991) and in cultured cells by the addition of a large volume of ice-cold lysis buffer (Gillespie and Hardie, 1992, Hardie and Carling, 1997). Similarly, the phosphorylation states of target proteins will not be preserved if they are exposed to phosphatase activity. Thus buffers contained the phosphatase inhibitors NaF and NaPPi (although these were subsequently removed when phosphatase activity was to be assayed). Astrocyte cultures were washed with ice-cold 0.9 % NaCl, 50 mM NaF, 2 mM NaPPi then scraped in a small volume of the same buffer. The resulting suspension was centrifuged at 3,000 rpm for 30 s. The supernatant was discarded and the pellet was either used immediately or snap frozen in liquid nitrogen then stored at -80°C. For statistical purposes a ‘determination’ (see Figures) constitutes the use of material from one rat or one cell culture, ie. comparison was always made between separate animals or separate cultures.

2.5 Measurement of $^3$H-arachidonic acid release from cultured astrocytes

Cultures grown on 6-well plates were prelabelled for 18 h with 0.2 μCi / ml $^3$H-arachidonic acid ($^3$H-AA) in serum free MEM, then washed twice in Krebs Henseleit Buffer (KHB; 116 mM NaCl, 26 mM NaHCO$_3$, 1.5 mM MgSO$_4$, 5 mM KCl, 1 mM NaH$_2$PO$_4$, 1.3 mM CaCl$_2$ and 20 mM glucose, pH 7.4 at 37°C), once in KHB supplemented with 1 % BSA then again with normal KHB. Incubations were in a final volume of 2 ml KHB for various lengths of time at 37°C in a 5 % CO$_2$/ air atmosphere with additions of pharmacological agents as indicated. At the end of the incubation period...
0.5 ml buffer was collected for liquid scintillation counting in 5 ml Optiphase to measure the amount of $^3$H released by the cells, the remaining buffer was removed from the wells and the cultures digested in 1 ml NaOH (0.1 M) for 30 min. Aliquots (0.2 ml) of the digested cells were also taken for counting to give a measure of the total radiolabel remaining in these cells. The radiolabel released into the incubation medium was then calculated as a percentage of the total incorporated into the cells (ie $^3$H released plus $^3$H remaining in cells).

2.6 Production of inositol phosphates by cultured astrocytes

This method is used to study the production of inositol phosphates by cultured astrocytes in response to various stimuli. It measures total $^3$H-inositol phosphates ($^3$H-IP). The cells were incubated in the presence of Li$^+$ to inhibit inositol-1-phosphatase, thus preventing the hydrolysis of the $^3$H-inositol phosphates formed (Berridge et al. 1982). After a 45 min incubation period the bulk would be $^3$H-IP$_1$, with some $^3$H-IP$_2$ but very little $^3$H-IP$_3$.

Cultures grown on 6-well plates were incubated with 4 μCi/ml [$^3$H] myo-inositol in GM for 24 h to prelabel membrane inositol phospholipids. Cells were washed twice with 2ml aliquots of KHB (see section 2.5) then allowed to equilibrate for 30 min in KHB at 37°C in a 5% CO$_2$/air atmosphere. 2 ml fresh buffer containing 5 mM LiCl was then added and the cultures returned to the incubator for 15 min. Cultures were then incubated for a further 45 min in the presence of pharmacological agents as indicated, at 37°C in a 5% CO$_2$/air atmosphere. Incubations were terminated by the removal of the incubation medium and the addition of 0.5 ml ice-cold methanol to each well. Cells were harvested (as described in section 2.4) and the suspension in methanol transferred to 1.5 ml tubes to which 0.3 ml chloroform and 0.3 ml distilled water were added. The contents were vortexed for 30 s and the phases separated by centrifugation for 5 min at 11,000 rpm. 0.5 ml of the upper aqueous phase containing free $^3$H-IP was diluted to 3 ml in distilled water.
before being applied to a column containing 1 ml 50% slurry of AG 1-X8 strong anion exchange resin. The columns were washed with 5 x 2 ml volumes of 10 mM inositol to remove free $^3$H-inositol, leaving $^3$H-IPs as the only labelled substance, since they were attached to the column via negatively charged phosphate groups. Then the $^3$H-IP were eluted directly into scintillation vials with 1 ml 1 M ammonium formate in 0.1 M formic acid. Aliquots (50 μl) of the lower organic phase were also taken as a measure of radiolabel incorporation into phospholipids. There is some variation in lipid labelling between different batches of culture, which is probably due to differences in cell density. To overcome this problem so that data from different cultures can be combined, the total radiolabel recovered in lipids per dish was determined. For a range of 8,000 to 12,000 dpm per dish, the radioactivity recovered in IP was then corrected to a standard incorporation of $10^4$ dpm into the lipids (Pearce et al, 1986).

2.7 Protein Phosphatase assays

2.7.1 Preparation of radioactive phosphorylated $[^{32}P]$-Phosphorylase a substrate

Radiolabelled phosphorylase a was prepared according to the method of Resink et al. (1983). Phosphorylase kinase was resuspended at a concentration of 2,000 units/ml in 70 mM Tris HCl, pH 7.4 at 37°C, 0.1 mM EDTA, 10% glycerol, 1 mM DTT. This was used immediately or snap frozen in liquid nitrogen and stored at -80°C. Phosphorylase b was dissolved at a concentration of 10 mg/ml in the same buffer.

A pilot phosphorylation of phosphorylase showed that incubation with phosphorylase kinase and [γ-32P]-ATP for 1 h gave maximal labelling. The phosphorylation reaction contained 50 mM Tris HCl, pH 7.4 at 37°C, 0.1 mM EDTA, 10% glycerol, 0.2 mM [γ-32P]-ATP (specific radioactivity $1.5 \times 10^6$ cpm/nmole phosphate), 4 mM MgCl$_2$, 100 μM
CaCl₂, 5 mg/ml phosphorylase b, and 200 units/ml phosphorylase kinase in a volume of 1 ml. After 1 h at 37°C the mixture was dialysed in 2 l buffer A (50 mM Tris HCl, pH 7.4 at 4°C, 10% glycerol, 0.1 mM EGTA, 1 mM DTT), to remove excess [γ-^32P]-ATP for 48-72 h at 4°C with about five buffer changes. Protein phosphatase contamination was reduced by dialysing the labelled phosphorylase into buffer A containing phosphatase inhibitors 50 mM NaF and 5 mM NaPPi overnight at 4°C. The phosphatase inhibitors NaF and NaPPi irreversibly inhibited phosphatases that contaminate the substrate. The inhibitors were then removed by dialysing back into the buffer A without NaF and NaPPi. It was found to be more effective to treat the ^32P-phosphorylase a in this way than to pre-treat the phosphorylase b before phosphorylation. Amberlite monobed resin was added to the dialysis buffer to sequester free [γ-^32P]-ATP and thereby increase the effective buffer volume. Dialysis was continued until the radioactivity in a TCA precipitate of 10 μl of the protein solution was at least fifty times greater than in the supernatant. The ^32P phosphorylase a was stored at 4°C before use.

In the assay, the amount of phosphate released from the substrate was determined by the specific activity of the ATP solution used to phosphorylate the substrate, calculated at the time each assay was performed. The method for calculating the specific radioactivity of the [γ-^32P]-ATP is described in section 2.8.3. This is also used to calculate the stoichiometry of phosphorylation of the ^32P phosphorylase a substrate. Assuming the concentration of phosphorylase kinase still present to be negligible, the final protein concentration of the ^32P-phosphorylase solution was determined using the Bradford assay (section 2.14). An aliquot (5 μl) of phosphorylase a was counted in scintillation fluid in a Beckman LS 5000CE liquid scintillation counter. Since the concentration of phosphorylase in the solution was known, the stoichiometry of phosphorylation could be calculated as mole ^32P phosphate per mole phosphorylase a on each occasion the substrate was prepared.
Example:

Specific activity of ATP solution: 1,800,000 cpm / nmole phosphate

$^{32}$P content of 5 μl $^{32}$P phosphorylase a 93,800 cpm

Concentration of $^{32}$P phosphorylase a 2.09 mg / ml

= 10.45 μg / 5 μl

= 93,800 cpm / 10.45 μg

MW of phosphorylase a = 97.4 kDa

= 97.4 μg

= 10.45 μg

\[
\frac{10.45 \mu g}{97.4 \mu g}
\]

= 0.1 nmol phosphorylase a

\[
\frac{93800 \text{ cpm}}{1,800,000 \text{ cpm}} = 0.05 \text{ nmol }^{32}\text{P}
\]

= 0.05 nmol $^{32}$P / 0.1 nmol phosphorylase a

= 0.5 nmol phosphate / nmol $^{32}$P phosphorylase a

This is a typical value for the specific activity of $^{32}$P phosphorylase a.

2.7.2 $^{32}$P-Casein phosphorylation

Casein was phosphorylated by the catalytic (C) subunit of cAMP-dependent protein kinase (PKA) purified from bovine heart by the method of Reimann and Beham (1983). The phosphorylation reaction contained 50 mM Tris HCl, pH 7.4 at 37°C, 0.1 mM EDTA, 10% glycerol, 5.6 mg/ml casein, 0.02 mg/ml C subunit, 0.2 mM $[\gamma^{32}\text{P}]$-ATP (specific activity $1.5 \times 10^6$ cpm/nmole phosphate), 4 mM MgCl$_2$. 

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The phosphorylation mixture was incubated for 1 h at 37°C then dialysed into 50 mM Tris HCl, pH 7.4 at 25°C, 0.1 mM EDTA, 1 mM DTT, 10 % glycerol for 48-72 h at 4°C with frequent buffer changes. The first batch of buffer solution contained protein phosphatase inhibitors NaF (50 mM) and NaPPi (5 mM) to inactivate contaminating phosphatase (see section 2.7.1). Amberlite monobed anion exchange resin was added to the buffer to sequester excess [γ-32P]-ATP. Dialysis was continued until the radioactivity in a TCA precipitate of 10 µl of the protein solution was at least fifty times greater than from the supernatant. 32P-Casein was stored at 4°C before use.

Phosphate release from 32P-casein was determined using the specific activity of the ATP solution used to phosphorylate the casein in the same way as for phosphorylase (section 2.7.1) Calculation of the stoichiometry of phosphorylation of casein cannot be done because it is not a single molecular weight species (MacKintosh, 1993).

2.7.3 [32P]-R II Peptide phosphorylation

Phosphorylation Method

PP2B dephosphorylates the site on the PKA R II subunit which is phosphorylated by the C subunit (Blumenthal et al, 1986). A 19 amino acid peptide (DLDVPIPGRFDRRVSVAAE) (R II peptide) containing this part of the R II subunit was 32P-phosphorylated and used to assay PP2B in astrocytes. The peptide was phosphorylated according to the method of Hubbard and Klee (1991). The peptide was dissolved in deionised water at a concentration of 2 mM and stored at -20°C before use.

The phosphorylation mixture contained 400 µM R II peptide, 100 mM Tris HCl, pH 7.4 at 37°C, 0.8 mM 32P-ATP (specific activity of 0.8 x 10⁶ cpm/nmole phosphate), 16 mM
MgCl₂ and 0.02 mg/ml C-subunit of PKA. Half of the ATP and enzyme were added at the start and half after 45 min incubation at 37°C. The reaction was allowed to proceed for 105 min in total before separation of the phosphorylated peptide from excess ³²P-ATP using Dowex anion exchange resin.

**Preparation of Dowex AG1-X8 anion exchange resin.**

About 100 ml Dowex anion exchange resin was stirred into 250 ml 1M NaOH and collected over filter paper. The beads were extensively washed with water until the eluate was pH 8-8.5, then resuspended in 250 ml 1M HCl and filtered. The beads were washed with at least 3 l deionised water until the pH was greater than 4 and stored in a little water at 4°C.

**Peptide separation**

To separate unincorporated ³²P-ATP from the phosphorylated peptide, 5 ml resin slurry was poured into a polypropylene chromatography column and washed with at least 3 column volumes of 30 % glacial acetic acid before use. This resin binds the negatively charged ³²P-ATP but not the peptide, which will have a net positive charge in acidic conditions. The reaction mixture was slowly pipetted onto the Dowex column followed by dropwise addition of 30 % acetic acid. Fractions (250 µl) were collected immediately and their cpm determined by Cerenkov counting. The fractions containing the peak of radioactivity were pooled. These were then added to 20 ml deionised water and lyophilised. The amount of peptide recovered, assuming no losses, was about 40 nmoles and so the residue was dissolved in 0.8 ml deionised water to make a nominal 50 µM solution.

Specific activity was calculated for the ATP and used to measure phosphate release as
before (sections 2.7.1, 2). The specific activity of the $^{32}$P-Rn-peptide can be estimated from nmoles phosphate/µl assuming complete recovery of 40 nmoles peptide, since the relative molecular mass of the peptide is known (2154.6 Da).

2.7.4 Preparation of tissue and cell extracts

Brain samples for PP assay were prepared by homogenisation as described in section 2.8.1, but using the following buffer: 0.25 M mannitol, 100 mM Tris HCl, pH 7.4 at 4°C, 1 mM EDTA, 1 mM DTT, 1 mM benzamidine, 1 µg/ml SBTI, 0.2 mM PMSF. Frozen astrocyte pellets (section 2.4) were resuspended in homogenisation buffer before lysis. Homogenisation was used initially to liberate intracellular proteins but this method resulted in loss of material, which was already limited. Lysis by freeze / thawing was subsequently used, i.e. alternately snap freezing in liquid nitrogen and warming to 37°C three times. This proved as effective as homogenisation, but caused minimal loss of cellular material. The lysate was centrifuged for 1 min at 13,000 rpm and the supernatant retained. In this method the cell weight and volume of buffer added was adjusted so that final protein concentration was approximately 1 mg/ml.

2.7.5 Phosphorylase phosphatase assay (PP1, PP2A)

Glycogen phosphorylase is a substrate for both PP1 and PP2A. Release of $^{32}$P-phosphate from $^{32}$P-labelled phosphorylase a prepared as in section 2.7.1 was used to determine the activity of these phosphatases, according to the assay method of Cohen et al. (1989). Dephosphorylation reactions contained 37.5 mM Tris HCl pH 7.4 at 37°C, 0.75 mM EDTA and 1 mg/ml $^{32}$P-phosphorylase a. Astrocytes scraped from three to six plates depending on the number of days in culture were used in the PP assays. The protein
The concentration of astrocyte cell lysate in the assay was approximately 0.3 mg/ml, assay volume was 0.1 ml. Okadaic acid (OA), stored in 20% DMSO was used at 1nM to inhibit PP2A and 2 μM to inhibit both PP1 and PP2A. Control assays included the DMSO vehicle. Inhibitor-1 was used at a final concentration sufficient to achieve maximal inhibition.

The assays were incubated at 37°C and 40 μl aliquots taken at 3 and 6 min. The aliquots were pipetted into 40 μl 40% TCA, mixed then placed on ice for 5 min. After centrifuging at 13,000 rpm for 3 min to separate denatured protein from free phosphate, a 70 μl aliquot of supernatant was added to 1 ml scintillation fluid and counted for 1 min to measure $^{32}$P-phosphate release. A blank incubation was included which contained no astrocyte lysate to measure free $^{32}$P in the substrate. This was subtracted from each test assay. The cpm obtained for each aliquot was converted into nmoles $^{32}$P-labelled phosphate released. An aliquot of the ATP solution used to phosphorylate the substrate was counted. Since the phosphate content of the aliquot was accurately known the cpm/nmole phosphate could be calculated. Phosphate release was linear over 6 min.

Example of calculation:

Specific activity of ATP (see section 2.8.3):
1 nmole $^{32}$P-ATP counted: 1,650,000 cpm/nmole phosphate.

Total dephosphorylation (ie in the absence of inhibitors) of $^{32}$P phosphorylase a by astrocyte phosphatases after 3 min incubation: 127,505 cpm.

127,505 cpm - blank 6,820 cpm = 120,685 cpm.

\[
\frac{120,685}{1,650,000} = 0.073 \text{ nmoles phosphate released}
\]
Protein concentration of astrocyte lysate: 1.1 mg/ml, diluted 35/100 in assay
= 0.38 mg/ml in assay
= 38 µg/100 µl assay volume, of which only 35 µl was counted
= 13.3 µg protein counted

\[
\frac{0.073}{13.3 \mu g} \text{ nmoles phosphate released} \times 1000
\]

= 5.49 nmoles \(^{32}\)P-phosphate released / mg astrocyte protein.

2.7.6 Casein phosphatase assay

This assay measures \(^{32}\)P release from the \(^{32}\)P-casein substrate prepared as in section 2.7.2. Phosphorylated casein is a substrate for PP2A and PP2C. The methodology was identical to that described for phosphorylase phosphatase assays (section 2.7.5). Incubations contained 37.5 mM Tris HCl, pH 7.4 at 37°C, 0.75 mM EGTA, 20 mM MgCl\(_2\) and 1 mg/ml \(^{32}\)P-casein, and about 0.3 mg/ml astrocyte protein. 20 mM MgCl\(_2\) was present because PP2C is Mg\(^{2+}\)-dependent. 2 µM OA in DMSO was included in some assays to inhibit PP2A. DMSO vehicle was added to all other assays. Calculations were similar to those for phosphorylase.

2.7.7 R\(_{II}\) Peptide phosphatase assay

This assay measures the release of \(^{32}\)P from \(^{32}\)P-labelled R\(_{II}\) peptide and was adapted from Fruman et al (1992). The identity of the peptide as a substrate for PP2B (calcineurin) was checked by using pure PP2B at a concentration of 12 nM. PP2C, is resistant to OA inhibition but PP2C is Mg\(^{2+}\) dependent, so its possible activity towards the peptide was limited by excluding Mg\(^{2+}\) ions from all solutions. PP2B can be inhibited by OA, but at a higher concentration than that necessary to inhibit PP1 or PP2A. Initially OA was included in the assays at a concentration of 0.5 µM as suggested by Fruman et al (1992).
However this concentration of OA virtually abolished all PP activity against the peptide, and was found to interfere with dephosphorylation of the peptide by pure calcineurin. Therefore 1 nM OA was used in the assay to inhibit PP2A and inhibitor-1 to inhibit PP1. These conditions now allowed measurement of a Ca^{2+}-dependent PP activity against the R_{II} phosphopeptide.

Assays were performed in the presence and absence of 1 mM CaCl₂ and 0.5 μM calmodulin. Assays in the absence of Ca^{2+} and calmodulin contained 5 mM EGTA to chelate any free Ca^{2+} ions present. All reactions contained 5 μM ^{32}P-R_{II} peptide, astrocyte lysate (0.1 mg protein/ml), 50 mM Tris HCl, pH 7.4 at 37°C, 0.1 M NaCl, 1 μM DTT, 0.1 mg/ml BSA. The reactions were incubated at 37°C. Aliquots of 40 μl were removed at 2 and 4 min, into 160 μl 12.5% TCA/100 mM potassium phosphate (KPi) and placed on ice. After centrifuging for 3 min at 13,000 rpm, 180 μl supernatant was pipetted onto a 0.5 ml Dowex 50W-X8 column. The ^{32}P-R_{II} peptide is positively charged and binds to the Dowex, while the ^{32}P-phosphate is negatively charged and washes straight through. The column was washed with 0.8 ml deionised water and the eluate collected into a 5 ml scintillation vial. 4 ml scintillation fluid was added and mixed thoroughly and the cpm determined in a scintillation counter.

Preparation of Dowex 50W-X8 400 mesh strong cation exchange resin

Dowex 50W-X8 400 mesh strong cation exchange resin was prepared as follows: 500g resin was suspended in 1 l deionised water, collected over filter paper using a Buchner funnel, then resuspended in 1 l of 1M NaOH and filtered. The beads were washed on filter paper with 2 litres 1M HCl, then 4 l deionised water. The resin was recovered and stored in 250 ml deionised water at 4°C. To make columns for the separation of free phosphate, 2 ml syringes were plugged with glass wool onto which was pipetted 0.5 ml cation exchange resin.
2.8 Protein kinase assays

2.8.1 Assay for cyclic AMP-dependent protein kinase (PKA)

The method for the assay of PKA was based on that described by Maller et al. (1978). PKA activity was measured as the incorporation of $^{32}$P from $[\gamma-^{32}\text{P}]$-ATP into the synthetic peptide substrate Kemptide (LRRASLG). PKA activity in cell or tissue extracts was taken to be the activity that was sensitive to the specific pseudosubstrate inhibitor of PKA (PKI, Walsh et al., 1971). PKA activity was measured in the absence of cAMP (initial activity) and in the presence of cAMP (total activity) and expressed as an initial: total activity ratio.

Brains or astrocytes (from three to six culture plates depending on the age of the culture) were homogenised 1 in 3 in 0.25 M mannitol, 100 mM Tris HCl, pH 7.4 at 4°C, 50 mM NaF, 2 mM NaPPi, 1 mM EDTA, 1 mM DTT, 1 mM benzamidine, 1 μg/ml SBTI, 0.2 mM PMSF, 1 mM IBMX (a cAMP phosphodiesterase inhibitor). The suspension was centrifuged at 13,000 rpm for 1 min at 4°C and the supernatant diluted 1 in 100 in Heps buffer (50 mM Hepes, pH 7.0, 100 mM NaCl, 50 mM NaF, 1 mM EDTA, 1 mg/ml BSA, 1 mM DTT, 1 mM benzamidine, 1 μg/ml SBTI). Phosphorylation of Kemptide (Kemp et al., 1977) was assayed in a final volume of 100 μl Hepes buffer containing 200 μM Kemptide, 1 mM IBMX, 4 mM MgCl$_2$, 0.2 mM $[\gamma-^{32}\text{P}]$-ATP (2-400,000 cpm /nmole phosphate) in the presence or absence of 10 μM cAMP and/or 50 units/μl PKI. Aliquots were removed at 3 min intervals over a 12 min incubation period at 37°C. The aliquots (20 μl) were spotted onto 1 cm$^2$ P81 phosphocellulose paper squares which were dropped into a bath of 75 mM phosphoric acid to stop the assay. In the acid the peptide becomes positively charged and binds to the P81 paper while unreacted $^{32}$P-ATP is washed away. The papers were stirred in the acid for 8 min then the acid was changed and the stirring continued for a further 8 min before washing with water. The papers were then transferred.
to scintillation vials for counting, to which 4 ml scintillation fluid was added.

PKA activity was calculated as the activity sensitive to PKI inhibition in the presence or absence of cAMP. The activity ratio of PKA represents the degree of dissociation of the C subunit at the time of isolation compared to the total amount of C subunit present and was calculated by dividing the activity in the absence of cAMP by the activity in the presence of cAMP.

2.8.2 Assay for AMP-activated protein kinase (AMPK)

Preparation of extract

Astrocytes from a whole single culture (about 10 rat pups, 20 six-well culture dishes) or rat brains weighing between 0.5 and 1.2 g were homogenised by hand using 30 strokes of a Teflon glass homogeniser (10 ml volume) 1 in 3 in 0.05 M Tris HCl, pH 7.4 at 4°C, 1 mM EDTA, 0.1 mM EGTA, 2 mM NaP Pi, 50 mM NaF, 10 % glycerol, 1 mM DTT, 1 mM benzamidine, 1 µg/ml SBTI, 0.2 mM PMSF, and mixed with 50 % polyethylene glycol (PEG) to make a final PEG concentration of 6 %. PEG precipitates proteins without denaturing them and is a useful method of separating proteins (by varying the PEG concentration, different proteins are precipitated), in this case it was used as a concentrating step. The PEG solution was placed on ice for 5 min in order for the precipitate to form, then centrifuged for 3 min at 13,000 rpm and the supernatant discarded. The pellet was either snap frozen in liquid nitrogen and stored at -80°C or used immediately. The pellet was resuspended in buffer A (50 mM Tris HCl, pH 7.4 at 4°C, 1 mM EDTA, 0.1 mM EGTA, 2 mM NaP Pi, 50 mM NaF, 10 % glycerol, 1 mM DTT), applied to a 1 ml DEAE column and washed with 15-20 column volumes buffer A. The partially purified enzyme was eluted with buffer B (50 mM Tris HCl, pH 7.4 at 4°C, 200 mM NaCl, 1 mM EDTA, 0.1 mM EGTA, 2 mM NaP Pi, 50 mM NaF, 1 mM DTT). Six
fractions of approximately 250-300 μl were collected and the protein concentration of each fraction was determined (section 2.14). The fraction containing the peak protein concentration was diluted to about 1 mg/ml using assay buffer (0.5 M Tris HCl, pH 7.4 at 37°C, 0.1 mM EDTA, 50 mM NaF, 1 mM DTT) and this 1 mg / ml solution was used at a 1 in 5 final dilution in the assay.

**Assay Method**

The method was similar to that described by Davies et al. (1989). The synthetic peptide SAMS was used as a substrate:

\[
\text{His-Met-Arg-Ser-Ala-Met-Ser}^{79}\text{-Gly-Leu-His-Leu-Val-Lys-Arg-Arg}
\]

The peptide sequence is based on a section of ACC, a physiological substrate of AMPK. AMPK phosphorylates ACC at serine-79 (represented in bold), while PKA phosphorylates ACC at the nearby serine-77. SAMS peptide was designed to be specific for AMPK by the substitution of an alanine residue at the ‘77’ position so that it is not phosphorylated by PKA (Davies et al, 1989). The peptide has two arginine residues at its C-terminus which aids peptide binding to the phosphocellulose paper in the assay.

Phosphorylation of SAMS peptide was assayed in a final volume of 100 μl containing 0.2 mg/ml AMPK partially purified through PEG and (usually) DEAE steps, 0.1 M Tris HCl, pH 7.4 at 37°C, 0.2 mM EDTA, 10 mM NaF, 0.02 mM DTT, 0.2 mM SAMS, 4 mM MgCl₂, 0.2 mM [γ-32P]-ATP (2-400,000 cpm/nmole phosphate) A blank incubation without SAMS was also assayed to determine non-specific binding of 32P-labelled species to the phosphocellulose P81 paper. Aliquots were removed at intervals over 3-4 min incubation at 37°C. The aliquots (20 μl) were spotted onto 1 cm² phosphocellulose P81 paper squares which were dropped into a bath of 75 mM ortho-phosphoric acid to stop
the assay. The papers were stirred in the acid for 8 min then the acid was changed and the stirring continued for a further 8 min before washing with water. In acid conditions the peptide is positively charged so it attaches to the phosphocellulose. Negatively charged species such as unused \( [\gamma^{32}\text{P}]-\text{ATP} \) do not bind. The papers were then transferred to scintillation vials for counting, to which 4 ml scintillation fluid was added. AMPK activity was calculated as the difference in cpm above blank in the presence or absence of AMP. This was converted into nmoles phosphate incorporated into SAMS/min/mg protein.

Example:

Protein concentration of DEAE peak fraction = 1 mg/ml, diluted 1 in 5 in the assay to 0.2 mg/ml.

20 \( \mu \)l aliquot from assay contained 4 \( \mu \)g protein

20 \( \mu \)l aliquot from test assay = 1472 cpm

20 \( \mu \)l aliquot from blank assay = 830 cpm

\[ \text{642 cpm incorporated into SAMS} \]

ATP specific activity (see section 2.8.3) = 300,000 cpm/nmole \( ^{32}\text{P} \)-phosphate

\[ \therefore \frac{642}{300,000} \times \frac{1}{4 \mu g} \times 1000 = 0.54 \text{nmoles}^{32}\text{P} \text{-phosphate incorporated into SAMS / mg protein} \]

2.8.3 ATP cocktail for kinase assays

A solution of ATP was prepared containing 1 mM non-radioactive ATP, 20 mM MgCl\(_2\), and ‘Redivue’ \( [\gamma^{32}\text{P}] \)-ATP of a sufficient volume that on the day the solution was used it had a specific activity of 2-400,000 cpm / nmole phosphate.
Calculation of specific activity of $^{32}$P-ATP

In order to quantify the number of moles of phosphate transferred to a substrate peptide or protein, the amount of radioactivity per mole phosphate in the ATP cocktail at the time of counting the assay samples had to be determined. The concentration of $\gamma$-phosphate present was equal to the concentration of ATP in the solution. Since the Redivue dye was found to absorb at 260 nm it was impossible to directly measure the ATP concentration in the final solution. Instead it was determined by measuring the adenosine content of the non-radioactive ATP stock solution by the absorbance of a known dilution at 260 nm. (The radioactive ATP from Amersham was considered as having a negligible adenosine concentration).

Absorbance, $A_{260} = \xi C l$

where $\xi$ is the millimolar absorption coefficient (for adenosine = 15), $C$ is the concentration in mM, and $l$ is the path length in cm (=1)

Example:

For a 1 in 2,000 dilution of stock ATP

$A_{260} = 15C \quad C = \frac{A_{260} \times \text{dilution factor (2,000)}}{15}$

$A_{260} = 0.120, \quad C = \frac{0.120 \times 2,000}{15}$

$=16 \text{ mM}$

Therefore the non-radioactive stock ATP was diluted 1 in 16 in the ATP cocktail to produce a final concentration of 1 mM.
2.9 Assay for Acetyl-CoA Carboxylase

The reaction being measured by this assay is the incorporation of radioactive CO₂ from bicarbonate into malonyl-CoA, catalysed by acetyl-CoA carboxylase (ACC):

\[
\text{ACETYL-CoA + BICARBONATE} \rightarrow \text{MALONYL-CoA + Mg\textsuperscript{2+} CITRATE}
\]

The assay was performed over a range of citrate concentrations (Witters et al, 1979) in brain, mammary gland and cultured astrocytes using protein partially purified by 6 % PEG or 40 % ammonium sulphate precipitation.

The cells or tissue were homogenised 1 in 3 in 0.25 M mannitol, 0.1 M Tris HCl, pH 7.4 at 4°C, 1 mM EDTA, 50 mM NaF, 2 mM NaPPi, 1 mM DTT, 0.1 mM PMSF, 2 μg/ml SBTI, 1 mM benzamidine and the suspension centrifuged at 13,000 rpm for 1 min to remove unbroken cells and debris. The supernatant volume was measured and 0.13 vol PEG or 0.243 g/ml ammonium sulphate were added. The solution was then placed on ice for 5 min in order for the precipitate to form. The suspension was centrifuged for 5 min at 13,000 rpm and the supernatant discarded. The pellet was either snap frozen in liquid nitrogen or used immediately.

The pellet was resuspended in a small volume of homogenisation buffer (0.25 M mannitol, 0.1 M Tris HCl, pH 7.4 at 4°C, 50 mM NaF, 2 mM NaPPi, 1 mM EDTA, 1 mM
DTT) and assayed immediately in 100 μl final volume containing 0.3-0.5 mg protein/ml for the liver or mammary gland or 1-2 mg protein/ml for brain or astrocyte samples, 0.1 M Tris HCl, pH 7.4 at 37°C, 0.2 mM DTT, 0.3 mM Acetyl CoA, 0.4 mM ATP, 0.2 mM Mg²⁺, 1 % BSA, 20 mM NaH¹⁴CO₃ (specific activity 0.75 mCi/mmole = 1700 cpm/nmole) and 0-20 mM concentrations of equimolar MgCl₂ and citrate. Incubations at 37°C were terminated after 90 s to ensure the rate of ¹⁴C incorporation was still linear, by the addition of 50 μl ice-cold 6 % perchloric acid (PCA). A control incubation was carried out without the substrate, acetyl-CoA to account for any non-specific ¹⁴CO₂ binding. After cooling on ice for 5 min the suspension containing the PCA precipitate was centrifuged at 13,000 rpm for 3 min and 125 μl supernatant was removed and evaporated to remove volatile substances including any remaining NaH¹⁴CO₃. The pellet, which contained the involatile ¹⁴C-malonyl-CoA product, was resuspended in 100 μl water then 1 ml scintillation fluid was added and the samples were counted for ¹⁴C for 10 min using a Beckman LS 5000CE liquid scintillation counter.

Vₘₐₓ and Kₜₐ citrate (the concentration of citrate producing half maximal activity) were calculated using a computer programme to fit the data to the following equation:

\[ v = \frac{V_{max}[C].h}{K_a + [C].h} \]

Where \( v \) = initial reaction velocity (rate)

\( h \) = Hill coefficient

\( [C] \) = citrate concentration
Calculation of specific activity of $^{14}$C-bicarbonate.

200 mM NaH$^{14}$CO$_3$ was prepared, with an approximate specific radioactivity of 0.75 mCi/mmole

10 µl 200 mM NaH$^{14}$CO$_3$ was diluted in water to 20 mM

Of this solution, 10 µl was taken and added to 90 µl 0.1 M NaOH and 1 ml Optiphase and the cpm counted over 10 min, giving 341,382 cpm.

10 µl contains 200 nmoles $^{14}$C-labelled bicarbonate

so the specific activity = $\frac{341,382}{200}$ = 1707 cpm/nmole $^{14}$C-bicarbonate.

Example of calculation of ACC activity:

ACC activity was calculated using the following calculation:

$$\text{cpm} \times \frac{1}{^{14}\text{C Sp. act}} \times \frac{150}{2} \times \frac{1}{3 \text{ mg protein}} = \text{nmol } ^{14}\text{C incorporated into malonyl-CoA}$$

where 150/125 is the correction for the 125 µl taken from 150 µl final assay volume, 2/3 is to convert from 90 s incubation time to per min, ‘mg protein’ refers to the amount of protein in an assay. The calculation was modified as follows to calculate activity per gram wet weight of tissue:

$$\text{cpm} \times \frac{1}{^{14}\text{C sp. act}} \times \frac{150}{2} \times \frac{1}{3 \text{ g brain}} = \text{nmoles } ^{14}\text{C incorporated into malonyl-CoA}$$

/min/g wet weight
where 'g brain' is the weight of tissue from which the protein in one assay was derived, for example:

\[
\text{weight of tissue} \times \frac{\text{proportion of homogenate used}}{\text{for PEG pellet (eg 200/3000 µl)}} \times \frac{\text{vol resuspended PEG pellet in assay (10 µl)}}{\text{total vol resuspended PEG pellet (eg 110 µl)}}
\]

\[
= 0.95 \times \frac{200}{3000} \times \frac{10}{110} = 5.75 \times 10^{-3} \text{ g wet weight / assay}
\]

2.10 Separation of the R_I and R_{II} subunit isoforms of PKA in different tissues

The R_2C_2 holoenzyme of PKA binds to DEAE at pH 7 via the negatively charged R subunits. Over a salt gradient of 0-0.4 M NaCl the R_I-containing PKA elutes first and the R_{II}-containing enzyme second (Reimann et al, 1971, Corbin 1977)

A 2 ml column of fast-flow DEAE sepharose beads was prepared and equilibrated in 50 mM Tris HCl, pH 7.0 at 25°C, 1 mM EDTA, 1 mM DTT, 1 mM benzamidine, 1µg/ml SBTI. The same buffer was used to homogenise 1 in 5 the tissue samples of bovine heart, rat heart, rat brain and astrocytes. The homogenates were centrifuged at 13,000 rpm for 1 min and filtered through a 0.2 µm cellulose nitrate membrane filter immediately before loading onto the column. The column was connected to a fast performance liquid chromatography system (FPLC, Pharmacia). After washing to remove unbound protein, the enzyme was eluted over a 0-0.4 M NaCl gradient at 1 ml/min and fractions were assayed for PKA activity (as in section 2.8.1) in the presence of 10 µM cAMP and in the presence or absence of 50 units/µl PKI.
2.11 Preparation of Inhibitor-1

Inhibitor-1 is an endogenous, thermostable, cytosolic protein which inhibits PPI at nM concentrations (Cohen and Cohen, 1989). The purification method described by Cohen et al (1988) was followed, with some modifications. The inhibitor-1 produced is an approximately equal mixture of phosphorylated and dephosphorylated forms. This method uses PKA to fully phosphorylate and activate the inhibitor-1. Although inhibitor-1 is heat stable, where practical, all steps were performed at 4°C unless otherwise stated.

250-300 g frozen rat skeletal muscle was defrosted, minced and then homogenised with 4 vol (1200 ml) 2 % trichloroacetic acid (TCA) for 45 s. The resulting suspension was centrifuged at 5,000 g for 45 min. The supernatant was filtered through glass wool and adjusted to 15 % TCA with stirring. After stirring for 3 h, the TCA precipitate was recovered by centrifuging the suspension at 10,000 g for 20 min. The precipitate was resuspended in a minimum volume of 0.5M Tris HCl, pH 8.5 at 5°C (solution A), adjusted to pH 7.4 then diluted to 250 ml / kg muscle with water and dialysed against 0.005 M Tris HCl, pH 8.5 at 25°C, 0.1 M EGTA (solution B). Space for expansion was left in the dialysis tube as a large amount of precipitate formed. The precipitate was removed by centrifuging for 10 min at 20,000 g. The supernatant was heated in a conical flask for 15 min at 100°C then cooled to 10°C on ice and centrifuged for 10 min at 20,000 g. The supernatant was decanted through glass wool and solid ammonium sulphate was added to 60 % saturation. This was stirred at 4°C for 4 h, then centrifuged for 15 min at 20,000 g. The precipitate was redissolved in 50 mM Tris HCl, pH 8.5 at 5°C, 1 mM EGTA (50 ml). The solution was dialysed against the same buffer containing 200 mM NaCl and 45 % ethanol. The suspension was centrifuged at 2,000 g for 10 min, and the supernatant dialysed against solution B containing 80 % ethanol for 24 h. After 24 h, the suspension was stored at -20°C for 24 h, then more solution B with 80 % ethanol was added, giving a final volume of 50 ml. This was centrifuged for 10 min at 20,000 g, and
the supernatant discarded. The precipitate was dissolved in solution A then dialysed into solution A to remove any traces of ethanol. The resulting solution of partially purified inhibitor-1 was a mixture of phosphorylated and dephosphorylated protein (Cohen and Cohen, 1989). Since inhibitor-1 is active when phosphorylated by PKA, the solution was then incubated with the C subunit of PKA (0.02 mg/ml), non-radioactive (0.2 mM) ATP and MgCl₂ (4 mM) at 30°C for 2 h. The reaction was stopped by the addition of 1/10 reaction volume of ice-cold 100 mM EDTA, pH 7.0 at 25 °C. Inhibitor-1 was stored at 4°C before use.

2.12 Purification of AMP activated protein kinase

2.12.1 Preparation of DEAE anion exchange column

DE52 preswollen ion exchange cellulose was slowly stirred into 15 volumes (w/v) 0.5M HCl, and left for 30 min before washing on a Buchner funnel with water until the filtrate was pH 4.0. The ion-exchanger was then stirred into 15 volumes 0.5 M NaOH and left for a further 30 min before filtering off the supernatant and resuspending in a fresh 15 volumes 0.5 M NaOH. After 30 min the ion-exchanger was washed until the filtrate was near neutral pH. The prepared DEAE was equilibrated in Buffer A (50 mM Tris HCl, pH 7.4 at 4°C, 1 mM EDTA, 0.1 mM EGTA, 2 mM NaPpI, 50 mM NaF, 10 % glycerol) and stored at 4°C.

2.12.2 Preparation of phosphocellulose

P11 cellulose phosphate, a fibrous cation exchanger, was slowly stirred into 20 volumes 0.5 M NaOH for 5 min and immediately washed until the filtrate was pH 11.0 or less. It was then stirred into 20 volumes 0.5 M HCl for 5 min before washing with water until the pH of the filtrate was pH 3.0 or greater. The prepared phosphocellulose was
equilibrated in Buffer C (50 mM Tris HCl, pH 7.4 at 4°C, 1 mM EDTA, 0.1 mM EGTA, 50 mM NaF, 1 mM DTT, 2 μg/ml SBTI, 1 mM benzamidine, 10 % glycerol) and stored at 4°C.

2.12.3 AMPK Purification Method

The purification of AMPK was based on the method described for rat livers by Carling et al, (1989). 12 adult Wistar rats were killed by exposure to carbon dioxide. The livers and brains were homogenised in 150 ml ice cold homogenisation buffer (25 mM Tris HCl, pH 7.4 at 4°C, 0.25 M sucrose, 50 mM NaF, 2 mM NaP Pi, 1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 2 μg/ml SBTI, 1 mM benzamidine, 0.1 mM TLCK) and the homogenate centrifuged at 40,000 g for 20 min at 4°C. The supernatant was filtered through glass wool. 50 % PEG was stirred in to make a 2.5 % PEG final concentration. This was centrifuged as before and the supernatant adjusted to 6 % final PEG concentration. After a further centrifugation, the pellet was retained and resuspended in a minimum volume of buffer A (50 mM Tris HCl, pH 7.4 at 4°C, 1 mM EDTA, 0.1 mM EGTA, 2 mM NaP Pi, 50 mM NaF, 1 mM DTT, 2 μg/ml SBTI, 1 mM benzamidine, 10 % glycerol), then applied to a DEAE column (10 ml) at 4°C. The column was washed with buffer A until the absorbance at 280 nm of the flow-through was less than 0.05 units (ie there was very little unbound protein still to elute).

The enzyme was batch eluted with 3 column volumes of buffer B (50 mM Tris HCl, pH 7.4 at 4°C, 200 mM NaCl, 0.2 mM ATP, 4 mM MgCl₂, 1 mM EDTA, 0.1 mM EGTA, 2 mM NaP Pi, 50 mM NaF, 1 mM DTT, 2 μg/ml SBTI, 1 mM benzamidine, 10 % glycerol) and dialysed at 4°C into buffer C to de-salt and remove NaP Pi. The buffer C also contained 0.2 mM ATP and 4 mM MgCl₂ to promote phosphorylation and activation of the AMPK by the endogenous AMPK kinase. The dialysate was stirred with 20 ml phosphocellulose for 20 min at 4°C. The phosphocellulose was washed on a Buchner
funnel with 20 column volumes of buffer C containing 0.2 mM ATP and 4 mM MgCl₂ then poured into a glass column and washed with the same buffer until the absorbance of the flow-through was less than 0.05 units. AMPK was batch eluted with 3 column volumes buffer E (50 mM Tris HCl, pH 7.4 at 4°C, 200 mM NaCl, 0.2 mM ATP, 4 mM MgCl₂, 1 mM EDTA, 0.1 mM EGTA, 50 mM NaF, 1 mM DTT, 2 µg/ml SBTI, 1 mM benzamidine, 10 % glycerol). The eluate was dialysed into buffer A, then loaded onto a Mono Q anion exchange column on FPLC and washed with buffer A until the absorbance returned to baseline. The enzyme was then eluted over a 0-400 mM NaCl gradient. The peak of AMPK activity eluted around 200 mM NaCl and was pooled and concentrated using centricons before dialysing into storage buffer (50 mM Tris HCl, pH 7.4 at 4°C, 1 mM EDTA, 0.1 mM EGTA, 2 mM NaPPi, 50 mM NaF, 50 % glycerol, 0.1 mM PMSF, 1 mM DTT, 2 µg/ml SBTI, 1 mM benzamidine, 0.1 mM TLCK, 0.1 mM TPCK) and stored at -20 °C.

2.13 Assay for determination of DNA concentration

The method used was that described by Labarca and Paigen (1980). A fluorimeter was used to quantify the enhancement of fluorescence seen when the fluorochrome bisbenzimide binds to DNA. RNA does not interfere with this assay.

A Perkin-Elmer 3000 spectrofluorimeter was set at an excitation wavelength of 355 nm and emission wavelength of 445 nm. DNA stock solution was stored until needed in aliquots of 200 µg/ml in 2 mM EDTA at -70°C. A calibration curve was first constructed (Figure 2.1) using a range of 0-6 µg DNA in assay buffer (2 M NaCl, 50 mM NaH₂PO₄·2H₂O, pH 7.4 at 25°C) and 1 µg/ml bisbenzimide in a final volume of 3 ml. Samples of brain were homogenised 1 in 3 in homogenisation buffer (0.25 M mannitol, 100 mM Tris HCl, pH 7.4 at 4°C, 50 mM NaF, 2 mM NaPPi, 1 mM EDTA, 1 mM DTT,
Figure 2.1 Calibration curve for the determination of DNA concentration in samples of rat brain.

Samples were prepared containing the amounts of DNA indicated on the graph. Increase in fluorescence of the fluorochrome bisbenzimide in the presence of increasing amounts of DNA was measured.

1 mM benzamidine, 1 μg/ml SBTI, 0.2 mM PMSF) and 10 μl samples were stored at -70°C until needed. Once defrosted, the samples were diluted in assay buffer, to a final 1 in 800 dilution in the assay.

2.14 Assay for determination of protein concentration

Protein concentration was measured using the principles of the method of Bradford (1976). Binding of proteins to Coomassie Brilliant Blue-G250 via basic amino acids shifts the absorbance of the dye from 495 to 595 nm. The increase in absorbance at 595nm was used to determine the amount of protein present relative to BSA standard solutions. The Coomassie reagent was prepared as follows: 30 mg Coomassie Brilliant
Blue G-250 was stirred into 100 ml absolute ethanol. Once this had dissolved 55 ml phosphoric acid was added, then deionised water was added to a final volume of 1 l. The reagent was stored at 4°C and protected from light.

A standard curve was prepared using known concentrations of BSA. A stock solution of BSA was freshly prepared with an absorbance at 280 nm of 0.13, equivalent to 0.2mg/ml protein. Seven solutions of 100 µl volume containing from 0 to 6 µg BSA in water were prepared. To each solution was added 1 ml Coomassie reagent (warmed to room temperature before use). The solution without protein was used to zero the UV spectrophotometer (Perkin-Elmer) at 595 nm, then the absorbances of the other solutions were measured and the standard curve plotted. Small aliquots of unknown protein samples were diluted in a final volume of 100 µl water to which 1 ml reagent was added. The A$_{595}$ was measured and the amount of protein determined from the calibration curve. The dilution of unknown protein was adjusted so that the A$_{595}$ of the sample was within the linear portion of the calibration curve, usually between 0.05 and 0.15 units. This dilution was corrected for in calculations.

2.15 Polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE is a rapid and sensitive method of separation, capable of a high degree of resolution of proteins and macromolecules. Polyacrylamide gels are ideal supporting media for electrophoresis since they are chemically inert and can be readily formed in a range of pore sizes by varying the concentrations of acrylamide and methylene bis-acrylamide. The anionic detergent sodium dodecyl sulphate (SDS) disrupts nearly all noncovalent interactions in native proteins and renders the negative charge on the protein insignificant since the negative charge acquired on binding SDS is usually much greater. Different proteins can therefore be separated purely on the basis of size, with small molecules moving through the gel more readily than larger molecules. The proteins can
be visualised by Coomassie blue or silver staining depending on the amount of proteins present. Radioactively labelled proteins can also be visualised using autoradiography of the gel onto photographic film, as described in section 2.15.4.

### 2.15.1 Preparation of protein samples for SDS-PAGE

Protein samples were solubilised in SDS sample buffer containing 62.5 mM Tris HCl, pH 6.8 at 37°C, 2% w/v SDS, 10% glycerol, 5% v/v 2-mercaptoethanol, 0.05 mg/ml bromophenol blue. The samples were boiled for 3 min and stored at -20°C until needed.

### 2.15.2 Preparation of polyacrylamide gel

The method for PAGE used was according to Laemmli (1970), using an SDS Tris/glycine discontinuous buffer system. A vertical slab gel (8 cm x 10 cm) was prepared with running gel (5 ml): 7.5% w/v acrylamide, 0.066% N’N’-methylene bisacrylamide, 0.1% SDS, 37.5 mM Tris HCl, pH 8.8 at 37°C. The addition of 0.05% v/v NN-N’N’-tetramethylene-diammine (TEMED) was use to cross-link the acrylamide. This was catalysed by 25 µl freshly prepared 10% ammonium persulphate which was added just prior to use.

A standard casting kit was used to prepare the gel between two glass plates which had been cleaned with ethanol and dried. The running gel was loaded to approximately 6 cm in height and overlaid with a thin layer of isobutanol to flatten the surface and prevent a meniscus forming. The running gel polymerised after about 45 min at room temperature after which time the isobutanol was removed by rinsing with distilled water. A less dense stacking gel (5% w/v acrylamide, 0.066% N’N’-methylene bisacrylamide, 0.1% SDS, 12.5 mM Tris HCl, pH 6.8 at 37°C.) was loaded on top of the running gel to approximately 2 cm in height and a 10 comb well was inserted to form the sample wells.
The comb was carefully removed after the stacking gel had set (about 30 min). The sample wells were washed with reservoir buffer containing 25 mM Tris HCl, pH 8.3, 0.19 M glycine, 0.1% SDS. The gel was transferred to the electrophoresis equipment where the samples were loaded.

2.15.3 Separation of proteins on SDS-PAGE

Samples prepared as described in section 2.15.1 were loaded into the wells. SDS sample buffer was loaded into any empty wells to ensure an even distribution of current across the gel. Standard molecular weight markers were run concurrently with the samples, these contain a mixture of proteins of known molecular weights over a range in which the molecular weight of the protein(s) of interest in the samples are expected to lie. Protein separation was achieved after about 45 min at a constant current of 14 mA. The gel was carefully removed from the glass plates and placed into a fixer solution of 50% methanol, 10% acetic acid for 30 min. Coomassie blue R (0.6%) in fixer was used to stain the gel for 30 min. Destaining was achieved using 10% methanol, 10% acetic acid, with the addition of small pieces of sponge or absorbent paper to soak up the dye. The destainer was changed several times until the bands of protein were visible. The gel was dried between cellophane sheets using a gel drier.

2.15.4 Autoradiography

${}^{32}$P-labelled proteins separated on SDS-PAGE were visualised using autoradiography. The gel was exposed to X-ray film (Hyperfilm MR) in a Kodak X-Omatic cassette at -80°C. The film was developed in the dark for 2 min using LX-24 developer then fixed using FX-40 fixer.
CHAPTER 3

SERINE/THREONINE PROTEIN PHOSPHATASES IN CULTURED ASTROCYTES
3.1 Introduction

All four of the main cytosolic serine/threonine PPs found in mammalian cells have been demonstrated in brain (Ingebritsen et al, 1983c). PP1 accounts for 18-40% of phosphorylase phosphatase activity in rabbit brain homogenates, which is half as much activity per mg protein as a similar extract from rabbit skeletal muscle (Cohen et al, 1989). However, mRNA levels for PP1 are similar in each tissue (Stemmer and Klee, 1991). Sim et al (1994) studied the localization of PP1 and PP2A in cytosolic and particulate fractions of rat forebrain. They found that the phosphorylase phosphatase activity of each enzyme was far higher in the cytosolic fraction, while the PP1 and PP2A protein concentrations in the samples were higher in the particulate fraction than the cytosol. There may therefore be potential for further activation of brain PP1 and PP2A by dissociation from membranes (Sim et al, 1994).

PP2A is more abundant in whole brain than PP1, comprising 60-75% of brain phosphorylase phosphatase activity (Cohen et al, 1991, Ingebritsen et al, 1983c). At the mRNA level, PP2A is ten times more abundant in brain than in skeletal muscle, liver, kidneys and ovaries (Khew-Goodall and Hemmings, 1988). Calcineurin was discovered in brain in the 1970's (Klee and Krinks, 1978, Klee et al, 1979) and identified as PP2B in 1982 (Stewart et al, 1982). The brain isozyme is generally referred to as calcineurin, whereas outside the brain it is often referred to as PP2B (Klee et al, 1988). According to both protein and mRNA levels, calcineurin is present at ten to twenty times higher concentrations in brain (where it accounts for 1% of total protein) than in other tissues (Stemmer and Klee, 1991). PP1, PP2A and PP2B have been shown in astrocytes by immunocytochemistry, with increases in PP2A and PP2B occurring in astroglial cells of Alzheimer's disease temporal cortex (Pei et al, 1997). There have been no studies to measure the activity of any individual PP in astrocytes, only in whole brain.
A single study by Ingebritsen et al (1983c) demonstrated the existence of PP2C in whole rabbit brain by the PP2C-mediated dephosphorylation of phosphorylase kinase. The activity measured was much lower than that of the other PPs: PPI and PP2B had six-fold higher activity and PP2A had 20-fold higher activity than PP2C, although PPI and PP2A were measured using a different substrate, phosphorylase a.

PP1 has two endogenous inhibitors. These are heat-stable proteins known as inhibitor-1 and inhibitor-2, first described by Huang and Glinsmann (1976). One of these, inhibitor-1, was purified from rat skeletal muscle (as described in Materials and Methods, section 2.11, Cohen et al, 1988) for this study and was used to identify PP1 activity. Another useful way of quantifying individual phosphatase activities in cell extracts is by the use of different substrates. Three have been used here: phosphorylase-a is a major substrate for PP1 and PP2A, casein-phosphate for PP2A and PP2C, and a peptide derived from the Rn subunit of PKA, which, when phosphorylated by the C subunit of PKA, is a substrate for PP2B (in addition to other PPs).

PP1, PP2A and PP2B have different sensitivities to inhibition by the toxin okadaic acid (OA) (Cohen et al, 1990), while PP2C is not inhibited by the toxin. IC\text{50} values have been reported for the three enzymes as follows: 15-20 nM for PP1, 0.1 nM for PP2A (Cohen et al, 1989) and 5 μM OA for PP2B (Bialojan and Takai, 1988). Therefore the inclusion of 1 nM OA in an assay completely inhibits PP2A, while PP1 is unaffected. PP2A activity is then taken as the proportion of total phosphatase activity which is inhibited by 1 nM OA. PP1 is the activity resistant to 1 nM OA but inhibited by 1 μM OA. The two PPs resistant to inhibition by 1 μM OA are dependent on divalent metal cations for activity; PP2C requires Mg\text{2+}, and PP2B Ca\text{2+}, so this property can be used to distinguish them (Cohen et al, 1990).

OA is cell permeable, and has been successfully utilized in many cell types to give
insight into the importance of PPs and phosphorylation to a large number of physiological processes (Hardie et al, 1991, Table 1.1). In neurons, microtubule-associated proteins (MAPs), MAP2 and τ show increased phosphorylation in the presence of OA, changing their affinity for tubulin. This alters the extent of microtubule assembly, leading to changes in neural morphology (Arias et al, 1993). Pahan et al (1998) examined the effect of PP inhibitors, including OA, on the expression of iNOS and production of NO in astrocytes. The bacterial toxin LPS is thought to stimulate iNOS expression via NF-κB activation (Pahan et al, 1997a, b). OA stimulated the LPS-mediated activation of NF-κB in astrocytes, possibly by increasing the phosphorylation state of its inhibitor, IκBα (Beg et al, 1992). This was sufficient to cause LPS-mediated expression of iNOS and production of NO (Pahan et al, 1998). Other studies in astrocytes have shown that OA treatment dramatically increased mRNA content, gene transcription and secretion of nerve growth factor (NGF) from cultured astrocytes (Pshenichkin and Wise, 1995). Astrocytes stained with antibodies to the glial fibrillary acidic protein (GFAP) showed increased staining of GFAP, and changes in cell morphology from a flat to a stellate appearance with longer processes when treated with 250 nM OA (Arias et al, 1993). A similar change in astrocyte morphology is characteristic of the effect of β-amyloid protein, however this change was prevented in cells treated with OA at a concentration of 25 nM and the cells retained their flat morphology with short processes, although it had no effect at 5 nM (Salinero et al, 1997).

There are many intracellular processes regulated by reversible phosphorylation in astrocytes including the liberation of AA, a major precursor of eicosanoids, from membrane phospholipids in response to extracellular stimuli. The process is thought to involve Ca^{2+}-induced phosphorylation of cPLA_2 and activation of MAPK and PKC (Lin et al, 1993, Qui et al, 1993, 1994, Ambs et al, 1995, Chen and Chen, 1998). The PPs involved in this regulation are unknown although PP1 and PP2A are the main PPs that dephosphorylate the substrates of PKC (Seki et al, 1995). The mechanisms of regulation
of this pathway are important since AA production provides the precursor for synthesis of eicosanoids including prostanoids, which are potent inflammatory mediators released by astrocytes.

PKC is also involved in the regulation of receptor-stimulated hydrolysis of membrane inositol phospholipids to form inositol phosphates. The activation of PKC by phorbol esters results in a decrease in agonist-induced PI turnover (Labarca et al, 1984, 1987, Orellana et al, 1985, Vicentini et al, 1985, Watson and Lapetina, 1985, Monaco and Mufson, 1986, Fisher et al, 1992, Pearce et al, 1988). This pathway provides the link between purinergic receptor stimulation and release of the second messenger Ca^{2+}, which is involved in the regulation of a large number of intracellular processes. In astrocytes, certain PKC subtypes (ie. α, δ and θ) were found to be involved in the regulation of the purinoceptor/G protein/PLC pathway, leading to a reduction in P_{2} receptor-mediated PI turnover (Chen and Chen, 1996). Other sites for control of phosphoinositide turnover by reversible phosphorylation may also be important, such as its inhibition by PKA (Robertson et al, 1990, Tertyshnikova and Fein, 1998, Bugrim, 1999).

The aims of the present study were to measure the activities of PP1, PP2A, PP2B and PP2C in astrocytes since identification and quantitation of individual PP activities in these cells has not previously been performed. Profiles have been constructed to show the relative contributions of each astrocyte PP acting on a particular substrate, and these have been compared to similar profiles in hepatocytes, human lung mast cells (HLMC) and whole rat brain. The PP activities were measured both in mature astrocytes and at various developmental stages during their growth in culture to find out whether the activity of any or all of the PPs changed during this time. The effect of OA on AA release from, and IP production by cultured astrocytes as described above was also determined to show whether PPs are important in the regulation of these processes.
3.2 Results

3.2.1 Phosphorylase phosphatases in astrocytes

Liberation of $^{32}$P-labelled phosphate from phosphorylase-a by phosphatase activity in astrocytes after 21 days in culture was linear over 6 min of incubation, the total rate of phosphate release was $0.41 \pm 0.11$ nmoles /min/mg protein (Figure 3.1).

The most marked inhibition in phosphatase activity was by 2 µM OA which reduced the reaction rate to $0.02 \pm 0.01$ nmoles phosphate released/min/mg protein. This showed that the majority of phosphatase activity in the astrocyte extracts capable of dephosphorylating phosphorylase-a was OA sensitive, i.e. PP1 or PP2A. The lower concentration of OA (1nM), reduced the reaction rate to $0.21 \pm 0.04$ nmoles phosphate released/min/mg protein. At nM concentrations OA inhibits nearly all PP2A but not PP1 (Cohen et al, 1989) so it can be assumed that the decrease in activity seen in the presence of 1 nM OA was largely due to an inhibition of PP2A activity. Another way of separating PP1 and PP2A activity is to inhibit only PP1 using inhibitor-1. In this case, inhibitor-1 produced a 55 % decrease in phosphatase activity to $0.18 \pm 0.09$ nmoles phosphate released/min/mg protein. Taken together, these data suggest that approximately 50-55 % of total phosphorylase phosphatase activity in astrocytes is PP1. Since Inhibitor-1 concentration cannot be measured, it was used at a final dilution sufficient to achieve maximal inhibition.

In figure 3.2A, the mean rates of reaction in the presence of inhibitors are represented as percentages of total activity in each assay. 2 µM OA is known to inhibit both PP1 and PP2A, while 1 nM OA inhibits only PP2A. The inhibition seen in each case can then be used to calculate the contribution of PP1 and PP2A to the total phosphorylase phosphatase activity seen in astrocyte homogenates (Figure 3.2B). Thus PP2A was calculated as that proportion of total activity inhibited by 1 nM OA.
Figure 3.1  Time course of dephosphorylation of phosphorylase a by astrocyte phosphatases

Phosphorylase phosphatase activity was assayed in extracts of astrocytes after 21 days in culture by the dephosphorylation of $^{32}$P-labelled phosphorylase a as described in Materials and Methods (section 2.7.5). A time course of $^{32}$P-phosphate release is shown for assays performed in the absence of inhibitors (total) or in the presence of inhibitor-1, or OA at the concentrations shown. Each value is the mean ± sem. of 3 determinations.
Figure 3.2 Phosphorylase phosphatase profile in cultured astrocytes

Phosphorylase phosphatase activity was assayed in extracts of astrocytes after 21 days in culture by the dephosphorylation of $^{32}$P-labelled phosphorylase a as described in Materials and Methods (section 2.7.5). **A.** Dephosphorylation reaction rates are shown as a percentage of the total activity (i.e., in the absence of inhibitors) for assays containing either inhibitor-1 or OA at the concentrations indicated. **B.** Relative proportions of each PP present were calculated from the data in A on the basis of OA inhibition. Each value is the mean ± sem. of 3 determinations.
Figure 3.3 Phosphorylase phosphatase profile in two other cell types

The two other cell types were assayed in the same way as astrocytes for phosphorylase phosphatase activity in the presence or absence of OA, then calculated as individual PP activities as described in Figure 3.2. Hepatocytes, isolated as described by Seglen (1976) and Berry et al (1991) (A.) and human lung mast cells (HLMC), isolated as described by Peachell and Munday (1993) (B.), were assayed for phosphorylase phosphatase activity by the dephosphorylation of $^{32}$P-labelled phosphorylase a as described in Materials and Methods (section 2.7.5). The relative proportions of each PP present were calculated on the basis of inhibition by OA. Each value is the mean of 3 observations ± sem.
The activity resistant to 2 μM OA was then subtracted from the activity remaining in the presence of 1 nM OA to calculate the contribution of PP1 to total phosphorylase activity. It can be seen in Figure 3.2B that PP1 accounts for 43.2 ± 2.0 % of total phosphorylase phosphatase activity and PP2A for 54.2 ± 2.2 %. A small proportion (3.2 ± 1.7 %) of phosphorylase phosphatase activity was resistant to inhibition by 2 μM OA.

When the proportions of PP1 and PP2A activity were calculated using the inhibition produced by inhibitor-1 rather than OA, a slightly different profile was seen. Inhibitor-1 blocked 60.0 ± 6.8 % of total PP activity and therefore 36.8 % was due to PP2A, after subtracting the activity resistant to 2 μM OA. It is not clear what caused this discrepancy. Inhibitor-1, being a physiological inhibitor of PP1 and having no effect on other PPs should be more specific than OA. The data, however, suggest that the amounts of phosphorylase phosphatase activity in astrocytes due to PP2A and PP1 are similar.

In order to put the findings of the PP composition of astrocytes into perspective, the same experiments were conducted in two other cell types, hepatocytes and human lung mast cells (HLMC), see Figure 3.3A and 3.3B, respectively. PPs have several roles in metabolic processes in the liver (Cohen, 1989, Shenoliker and Nairn, 1991) so the profile of PPs in hepatocytes was determined as an example of a cell type whose function is largely metabolic. Hepatocytes contained about twice as much PP2A (62.7 ± 5.2 %) as PP1 (27.2 ± 5.4 %), determined using OA inhibition, which is different to the proportion in astrocytes where it is approximately equal. However, Cohen et al (1989) showed equal proportions of PP1 and PP2A in rat liver, which consists largely of hepatocytes. In the same study, rabbit whole brain was found to have about 40 % PP1 and 60 % PP2A activity, which is similar to the present finding in astrocytes.

OA and other PP inhibitors have been shown to inhibit the release of histamine and leukotriene C4 by HLMC (Peachell and Munday, 1993) which suggests an important role
for PPs in HLMC function, so these cells were chosen to determine the PP profile found in an inflammatory cell type. There was a different profile of PP activity in HLMC compared to both astrocytes and hepatocytes. Using OA to measure the relative proportions of PP1 and PP2A, HLMC contained PP1 (71 % ± 3) and PP2A (15 % ± 3) (Figure 3.3B). If PP2A predominates in a cell with high metabolic activity and PP1 predominates in a cell with inflammatory function, then perhaps an equal distribution of PP1 and PP2A in astrocytes, which exhibit both functions, is not surprising.

### 3.2.2 Casein phosphatases in astrocytes

Casein phosphate is a substrate for PP2A and PP2C, so this was used to look at the relative amounts of these two enzymes in astrocytes. In the presence of Mg^{2+}, both PPs dephosphorylate casein phosphate and since PP2C is resistant to OA inhibition (Cohen et al., 1990), this was used to determine the proportion of each PP present in the cells. Figure 3.4 shows a time course of dephosphorylation of casein phosphate by astrocyte phosphatases in the absence and presence of two OA concentrations. It can be seen from the graph that casein phosphatase activity was linear over 8 min incubation, and that a very small but insignificant difference exists between the rates of dephosphorylation at 1 nM and 2 μM OA concentrations confirming that the activity of PP1 against this substrate was negligible. The graph shows that the majority of astrocyte phosphatase acting on casein phosphate is sensitive to 1 nM OA and therefore is PP2A.

Since there was no significant difference between activity measured in the presence of nM or μM OA, a concentration of 2 μM OA was used to differentiate between PP2A and PP2C. Figure 3.5A shows that the presence of 2 μM OA inhibited total casein PP activity by 77.9 ± 5.6 % and this was taken to represent the proportion of PP2A whilst that remaining in the presence of 2 μM OA (22.1 ± 5.6 %) was taken to be PP2C (Figure 3.5B). PP2C activity has not been previously determined in astrocytes, so this data cannot
be compared to the work of others.

Hepatocytes are a cell type in which PP2C has recognised properties in regulating the enzymes controlling cholesterol synthesis (Ingebritsen et al, 1983a, Moore et al, 1991). The casein PP profile in these cells was determined as a comparison with that in astrocytes. The hepatocytes were assayed in the presence or absence of OA at 2 µM as described (Figure 3.6) and the relative amounts of PP2A and PP2C determined. Hepatocyte PP2A comprised 72.2 ± 2.2 % of total and PP2C 27.8 ± 2.2 % of total activity. This casein phosphatase profile measured in hepatocytes was quite similar to that measured in astrocytes (Figure 3.5B, Figure 3.6).

3.2.3 Dephosphorylation of the PKA R² subunit peptide

It is clear from Figure 3.7A that the phosphorylated R² peptide can be used as a substrate for calcineurin, and that calcineurin requires Ca²⁺/calmodulin for activity. Assay of calcineurin in the absence of Ca²⁺ or presence of EGTA, which chelates Ca²⁺, significantly reduced phosphatase activity (Figure 3.7A). The inhibition of phosphatase activity seen when commercially obtained calcineurin was incubated with 0.5 µM OA (Figure 3.7A) was surprising. It is known that calcineurin is sensitive to high concentrations of OA (Bialojan and Takei, 1988), however 0.5 µM OA would not be expected to inhibit calcineurin (Fruman et al, 1992), since half-maximal inhibition of the enzyme normally occurs at 5 µM OA (Bialojan and Takei, 1988). Two possible explanations for this inhibition suggest themselves: the calcineurin may be contaminated by PP1 or PP2A which would then be inhibited by 0.5 µM OA; or the inhibitory effect of OA on calcineurin is dependent on relative concentrations of both the inhibitor itself and the enzyme, which is present here at a low concentration of 0.87 µg/ml. In their study, Fruman et al (1992) used 100 nM calcineurin, which if the molecular weight of bovine brain calcineurin is approximately 80 kDa (Klee et al, 1998), is equivalent to
Figure 3.4  Time course of dephosphorylation of casein-phosphate by astrocyte phosphatases

Casein phosphatase activity was assayed in extracts of astrocytes after 21 days in culture by the dephosphorylation of $^{32}$P-labelled casein phosphate in the presence of 20 mM MgCl$_2$ as described in Materials and Methods (section 2.7.6). A time course of casein phosphatase activity is shown for assays performed in the absence of inhibitors (total) or in the presence of OA at the concentrations shown. A typical rate of total casein phosphatase activity is 0.12 nmoles phosphate released/min/mg protein. Each value is the mean ± sem. of 3 determinations.
Figure 3.5  Casein phosphatase profile in cultured astrocytes

Casein phosphatase activity was assayed in extracts of astrocytes after 21 days in culture by the dephosphorylation of \(^{32}\)P-labelled casein phosphate in the presence of 20 mM MgCl\(_2\) as described in Materials and Methods (section 2.7.6). A. Dephosphorylation reaction rates are shown as a percentage of the total activity (ie. in the absence of inhibitor) for assays in the presence or absence of 2 µM OA. B. The relative proportions of each PP present were calculated from the data in A: activity inhibited by 2 µM OA was plotted as PP2A, PP2C being the activity resistant to OA inhibition. Each value is the mean ± sem. of 8 determinations.

![Figure A](image1.png)

![Figure B](image2.png)
Figure 3.6  Casein phosphatase activity in Hepatocytes

Hepatocytes, isolated as described by Seglen, 1976 and Berry et al, 1991 were assayed for phosphorylase phosphatase activity by the dephosphorylation of $^{32}$P-labelled casein phosphate as described in Materials and Methods (section 2.7.6). The relative proportions of each PP present were calculated on the basis of inhibition by 2 μM OA. Each value is the mean ± sem. of 4 determinations.
Phosphatase activity capable of dephosphorylating the PKA R\textsubscript{II} subunit peptide was assayed by the dephosphorylation of \textsuperscript{32}P-labelled R\textsubscript{II} peptide as described in Materials and Methods (section 2.7.7) in the presence or absence of 1 mM CaCl\textsubscript{2}, 0.5 \textmu M calmodulin (CaM), 5 mM EGTA or 0.5 \textmu M okadaic acid (OA) where indicated. The sources of PP activity assayed were: A. A commercial preparation of calcineurin (0.87 \textmu g/ml), B. Whole rat brain extract at a protein concentration of 0.1 mg/ml. Each value is the mean ± sem. of 3 determinations.
about 8.0 μg/ml, and this is nine times the dilution required for linear activity in the present study. If the inhibition of calcineurin by OA is dependent on the relative concentrations, it may be possible that 0.5 μM OA is able to inhibit calcineurin at this low enzyme concentration.

Calcineurin is known to comprise 1 % of total brain protein, and calcineurin activity was measured in whole brain homogenates. Figure 3.7B shows that linear Rf peptide phosphatase activity was measured over 10 min incubation, but that the inclusion of 0.5μM OA reduced the activity at 10 min to 24.5 ± 4.5 % of the total. It is possible therefore that this peptide is a substrate for PPl and PP2A as well as calcineurin, which should not be inhibited by this concentration of OA. The remaining activity in the presence of OA was further reduced by the addition of EGTA (to chelate all Ca²⁺ present) to just 5.5 ± 1.5 % of the total PP activity at 10 min which suggests that Ca²⁺-dependent, OA-resistant PP activity, ie. calcineurin, is present, comprising 19 % of the total activity.

Since calcineurin comprises 1 % of total brain protein, which was at a concentration of 0.1 mg protein/ml, then calcineurin will have been present at a concentration of 1 μg/ml, as a rough estimate. This is therefore similar to the amount of calcineurin assayed in Figure 3.7A (0.87 μg/ml). It is possible that some of the activity of calcineurin in the brain extract (Figure 3.7B) may have been masked by OA inhibition as it appears to have been for the commercial preparation of calcineurin (Figure 3.7A), if in fact the calcineurin was uncontaminated with other PPs. It is difficult to see how the situation could have been remedied to definitely isolate the individual PP activities using OA inhibition since concentrations of less than 0.5 μM OA which would not inhibit calcineurin would not have inhibited PP1 activity either. One solution was to use a combination of 1 nM OA to inhibit PP2A together with inhibitor-1 to inhibit PP1, as calcineurin would not be inhibited by 1 nM OA or inhibitor-1. Brain extract was assayed on one occasion in the
Figure 3.8  Dephosphorylation of the PKA R1 subunit peptide by astrocyte phosphatases: comparison of protein phosphatase inhibitors

Phosphatase activity capable of dephosphorylating the PKA R1 subunit peptide was assayed in extracts of astrocytes after 21 days in culture using a protein concentration of 0.1 mg/ml by the dephosphorylation of 32P-labelled R1 peptide as described in Materials and Methods (section 2.7.7) in the presence or absence of 1 mM CaCl2, 0.5 μM calmodulin (CaM), 5 mM EGTA and of two different inhibitors: A. used 0.5 μM OA to inhibit OA-sensitive phosphatases and B. a combination of 1 nM OA to inhibit PP2A and inhibitor-1 to inhibit PP1. Each value is the mean ± sem. of 3 (A) or 6 (B) determinations.
presence of the two inhibitors, which together reduced dephosphorylation of the phosphorylated R₁ peptide to 35% of the total activity in the presence of 1 μM Ca²⁺. The exclusion of Ca²⁺ and the addition of EGTA further reduced the activity to 12% of total, so using this method, calcineurin comprised 23% of total activity in a single assay (not shown). This is comparable to the 19% shown in Figure 3.7B. It is likely that the concentration of calcineurin was not too low for the use of 0.5 μM OA, and that the R₁ peptide is a substrate for OA-sensitive phosphatases.

Astrocytes were assayed for R₁ peptide phosphatase activity. Calcineurin activity was separated from other PP activity using 0.5 μM OA or the combination of 1 nM OA and inhibitor-1. Figure 3.8A shows the assay performed in the presence or absence of 0.5 μM OA, which reduced PP activity to 20.3 ± 1.2% of the total activity in the presence of 1 μM Ca²⁺ and 13.0 ± 5.0% in the presence of EGTA after 10 min incubation. Thus 7% of the total activity at 10 min was OA-insensitive/Ca²⁺-dependent PP activity, i.e. calcineurin.

The astrocyte R₁ peptide phosphatase assays were repeated using 1 nM OA and inhibitor-1 which caused less inhibition of PP activity after 10 min incubation (Figure 3.8B). In the presence of Ca²⁺, activity was inhibited to 29.0 ± 7.5% of the total compared to 14.7 ± 4.0% in the absence of Ca²⁺. This was a larger proportion (14%) of Ca²⁺-dependent PP activity than that seen in Figure 3.8A. In 5 of the 6 observations, however, at both time points, the activity was greater in the absence of EGTA, so it appears that a small amount of calcineurin activity is present, but it is small and obscured by variability between samples.
3.2.4 Developmental study of protein phosphatases in primary astrocytes

Figure 3.9A shows total phosphorylase phosphatase activity in astrocytes over the first 21 days growth in culture. Phosphatase activity capable of dephosphorylating phosphorylase a rapidly increased from $2.04 \pm 1.03$ nmoles $^{32}$P-phosphate released/ min/mg protein at postnatal day 7 to a peak of $18.08 \pm 5.77$ at 10 days before falling to $13.78 \pm 6.55$ nmoles $^{32}$P-phosphate released/ min/mg protein at day 21. The maturation of astrocytes is completed between 2-3 weeks after culturing, the progress of which is reflected by the rate of DNA synthesis (Figure 3.9C). The rapid increase in phosphorylase phosphatase activity seen between days 7 and 10 slightly precedes the period of maximal DNA synthesis (Figure 3.9C) which occurs at days 7-14.

At day 7 total casein phosphatase activity ($1.38 \pm 0.25$ nmoles $^{32}$P-phosphate released/ min/mg protein) (Figure 3.9A) was similar to total phosphorylase phosphatase activity (Figure 3.9B). From day 10 onwards the total phosphorylase phosphatase activity exceeded casein phosphatase activity. Maximum phosphatase activity was reached with each substrate between day 7 and day 10 and had begun to decrease by day 21. Phosphorylase phosphatase activity increased by 786 % between day 7 and day 10, while casein phosphatase activity increased by 160 % over the same period. At day 21, phosphorylase phosphatase activity had fallen to 675 % of the day 7 value, while casein phosphatase activity had fallen to just 80 % of the day 7 value.

Figure 3.9C shows the changes in DNA content in cultured astrocytes over 21 days growth. The amount measured at day 5 ($1.41 \pm 0.02 \mu g/well$) increased to $2.07 \pm 0.03 \mu g/well$ at day 7. This was followed by a steep rise in DNA content up to a maximum of $20.00 \pm 0.56 \mu g/well$ at day 18. The steepest part of the curve, corresponding to the period over which the most rapid growth occurred was between day 7 and day 14.
Figure 3.9  Total phosphatase activity in astrocytes during development

Astrocyte phosphatase activity against phosphorylase a and casein-phosphate was measured using the methods described in section 2.7.5,6 over 21 days growth in culture. A. Total phosphorylase phosphatase activity, and B. Total casein phosphatase activity, at various time points was plotted against the length of time in culture. C. DNA content was determined as described in section 2.13 and expressed per well of a 6 well plate. Each value is the mean ± sem. of 3 (A, B) or 6 (C) determinations.
It can be seen from Figures 3.10A and B that the peak of total phosphorylase phosphatase activity seen in Figure 3.9A did not correspond to an increase in an individual phosphatase, since the relative proportions were fairly constant over growth in vitro. Figure 3.10A shows the relative proportions of PP1:PP2A over growth to 21 days assessed using OA inhibition, while the same graph is plotted in Figure 3.10B based on inhibitor-1 inhibition of PP1 activity, calculated as described. The two methods have produced similar PP profiles for PP1:PP2A, which also agree with the findings in Figure 3.2 that the activities of PP1 and PP2A are similar in day 21 astrocytes.

Figure 3.10C shows the relative proportions of the casein phosphatases in astrocytes during growth in vitro. In this case there is a pronounced difference in the relative activities of PP2A and PP2C. There is a trend of increasing activity of PP2C from 0.49 ± 0.18 nmoles phosphate released/min/mg protein at day 7 to 1.17 ± 0.08 nmoles phosphate released/min/mg protein at day 14, while PP2A activity increased from 0.92 ± 0.08 nmoles phosphate released/min/mg protein at day 7 to 2.65 ± 0.8 nmoles phosphate released/min/mg protein at day 10. The activity of PP2A was similar at day 14, but fell again by day 21. At day 21 the activity of PP2C was 0.39 ± 0.12 nmoles phosphate released/min/mg protein, or 33 % of total activity, which is close to the value seen previously for day 21 astrocytes (Figure 3.4) of 22.1 ± 5.6 % of total activity. The ratio of PP2A:PP2C increased from day 7 to 10 (from 65 % to 76 %) then fell to 69 % at day 14 and 33 % at day 21.
Cultured astrocytes were assayed for phosphorylase and casein phosphatase activity as described in Materials and Methods (section 2.7.5.6) during growth to 21 days in culture. Relative proportions of PP1:PP2A activity dephosphorylating phosphorylase a are represented as estimated by (A.) 1 nM and 2 μM OA inhibition or (B.) Inhibitor-1 inhibition. Relative amounts of PP2A:PP2C dephosphorylating casein during development to 21 days in culture are shown in C. Each value is the mean ± sem of at least 3 determinations.
3.2.5 The effect of OA on AA release from astrocytes \textit{in vitro}.

Astrocytes were prelabelled with $^3$H-AA, washed and incubated at 37°C for 60 min in the presence or absence of the calcium ionophore A23187 and the release of $^3$H-AA was determined over 60 min. It can be seen from Figure 3.11 that 10 μM A23187 increased $^3$H-AA release. This effect was visible within 15 min incubation when 3.82 ± 0.18 % of the total $^3$H-AA present was released in the control experiment and 5.87 ± 0.48 % of the total $^3$H-AA released in the presence of the ionophore. After 60 min the cumulative amount of $^3$H-AA released was 7.66 ± 0.72 % in the control assay and 14.40 ± 0.80 % total $^3$H-AA released in the presence of A23187. Rates of release were calculated as 0.08% ± 0.014 and 0.20 % ± 0.013 of the total released/min for control and A23187 stimulated cells, respectively.

Figure 3.12 shows that the stimulation of $^3$H-AA release induced by A23187 was concentration-dependent, but saturable. The $K_a$ for A23187 was 1.5 μM, and the $V_{\text{max}}$ was 19.2 % of total $^3$H-AA released. This shows that the concentration of A23187 (10 μM) used in these assays was enough to ensure maximum stimulation of $^3$H-AA release.

The effect of 1 μM OA on $^3$H-AA release over time was examined (Figure 3.13). OA clearly increased the rate of $^3$H-AA release above control values. The release from control cells was notably lower than that observed in Figure 3.11, because the cells were from different populations. Thus a quantitative comparison of the effects of A23187 and OA was not possible using these data. The effects of A23187 and OA were directly compared in the same astrocyte cultures (Figure 3.14). 1 μM OA was able to produce stimulation of AA release above control, this was about half that achieved by 10 μM A23187. The percentage $^3$H-AA released above control was 105.5 ± 13.3 % by A23187 and 48.7 ± 3.9 % by OA. In combination the two agents produced an increase in $^3$H-AA
Figure 3.11  Time course of A23187-stimulated $^3$H-AA release from cultured astrocytes

Cultured astrocytes were prelabelled for 18 h with 0.2 μCi/ml $^3$H-AA as described in Materials and Methods (section 2.5), washed then incubated in the presence or absence of 10 μM A23187 for 60 min. Radiolabel released into the incubation medium was calculated as a percentage of the total radiolabel incorporated into the cultures. Results are means ± sem. from 3 determinations. Values that differ significantly from controls at the same time point by Student’s t-test are shown (P< 0.01*).
Figure 3.12  Concentration-dependence of A23187-stimulated $^3$H-AA release

Cultured astrocytes were prelabelled for 18 h with 0.2 μCi/ml $^3$H-AA as described in Materials and Methods (section 2.5), washed then incubated in the presence of A23187 at the concentrations indicated for 30 min. Radiolabel released into the incubation medium was calculated as a percentage of the total radiolabel incorporated into the cultures. Results are means of duplicate determinations.
Figure 3.13  Time course of OA-stimulated $^3$H-AA release from cultured astrocytes

Cultured astrocytes were prelabelled for 18 h with 0.2 $\mu$Ci/ml $^3$H-AA as described in Materials and Methods (section 2.5), washed then incubated in the presence or absence of 1 $\mu$M OA for 60 min. Radiolabel released into the incubation medium was calculated as a percentage of the total radiolabel incorporated into the cultures. Results are means ± sem. from 3 determinations.
Figure 3.14  Release of $^3$H-AA from cultured astrocytes in response to A23187 and OA

Cultured astrocytes were prelabelled for 18 h with 0.2 μCi/ml $^3$H-AA as described in section 2.5, washed, then incubated in the presence or absence of 10 μM A23187 and/or 1 μM OA for 30 min. Radiolabel released into the incubation medium was calculated as a percentage of the total radiolabel incorporated into the cultures. Data are presented as the % above the control (no additions) amount of AA released in 30 min. Results are mean ± SEM from 6 determinations.
release of 148.3 ± 4.0 % above control. This is similar to the sum of the individual stimulation caused by the two agents, suggesting an additive effect of OA and A23187 on AA release from astrocytes.

The fact that $^3$H-AA release was increased by OA and A23187 in an additive manner suggests that they exert this effect by different mechanisms. Both agents were studied to see if they required influx of extracellular Ca$^{2+}$ to increase $^3$H-AA release (Figure 3.15). A23187 produced marked stimulation of $^3$H-AA release, but only in the presence of extracellular Ca$^{2+}$, as would be expected. The stimulation produced by incubation with OA was the same in the presence or absence of Ca$^{2+}$, so OA appears to exert its effect independently of Ca$^{2+}$-influx.

The results presented in Figure 3.16 demonstrate the effect of OA on ATP stimulated $^3$H-AA release from cultured astrocytes. Incubation with 300 μM ATP for 30 min caused an increase of 25.8 ± 3.1 % of total $^3$H-AA release over control. OA caused a greater increase in $^3$H-AA release, 48.7 ± 3.9 % of total. The combined effect of OA and ATP was to increase the $^3$H-AA release to 72.7 ± 9.8 % of total, approximately the sum of the individual effects.
Figure 3.15  Dependence of A23187- and OA- stimulated $^3$H-AA release on extracellular calcium

Cultured astrocytes were prelabelled for 18 h with 0.2 μCi/ml $^3$H-AA as described in Materials and Methods (section 2.5). Astrocytes were either washed in normal buffer or Ca$^{2+}$-free buffer containing 0.2 mM EGTA, then incubated in that buffer in the presence or absence of 10 μM A23187 or 1 μM OA for 30 min. Radiolabel released into the incubation medium was calculated as a percentage of the total radiolabel incorporated into the cultures. Results are means ± sem. from 3 determinations.
Figure 3.16 Effect of OA on ATP stimulated AA release from cultured astrocytes.

Cultured astrocytes were prelabelled for 18 h with 0.2 µCi/ml $^3$H-AA as described in section 2.5, washed, then incubated in the presence or absence of 300 µM ATP and/or 1 µM OA for 30 min. Radiolabel released into the incubation medium was calculated as a percentage of the total radiolabel incorporated into the cultures. Data are presented as the % above the control (no additions) amount of AA released in 30 min. Results are mean ± SEM from 6 determinations.
3.2.6 The effect of OA on NA-induced intracellular IP accumulation in astrocytes \textit{in vitro}.

$^3$H-IP accumulation was measured in cultured astrocytes in response to agonist stimulation in the presence and absence of the PP and PK inhibitors OA and staurosporine, as described in Materials and Methods (section 2.6). Figure 3.17 shows that NA caused a large increase in IP accumulation. The inclusion of OA with NA lead to a 50\% reduction in the ability of NA to induce IP accumulation.

Staurosporine is a protein kinase inhibitor which is most effective against PKC ($IC_{50}$ 2.7 nM), but also inhibits other protein kinases including PKA ($IC_{50}$ 8.2 nM, Tamaoki, 1991). Inhibition of protein kinases by staurosporine prevented the partial block of NA-induced IP accumulation by OA. A simple explanation of this phenomenon would be that OA inhibition allows a protein kinase to phosphorylate targets in the absence of PP antagonism and this diminishes the response to NA. Inhibition of the PK (possibly PKC or PKA) by staurosporine removes the challenge to the NA response.
Figure 3.17  Effects of OA and staurosporine on NA-induced IP accumulation in cultured astrocytes.

$^3$H-IP accumulation was measured in cultured astrocytes in response to agonist stimulation in the presence and absence of OA and staurosporine, as described in Materials and Methods (section 2.6). Results are presented as $^3$H-IP (dpm)/$10^4$ dpm in lipids. Results are mean ± SEM from 5 determinations.
3.3 Discussion

The results presented here show that cultured astrocytes possess the four most well known protein serine/threonine phosphatases: PPl, PP2A, PP2B (calcineurin) and PP2C. PP1 and PP2A activities were compared by dephosphorylation of 32P-glycogen phosphorylase (Figure 3.1). Inhibitor-1 produced an inhibition of PPl, which suggested a slightly higher proportion of PPl (60.0 ± 8 % of total phosphorylase phosphatase activity) than was suggested by OA inhibition (43.2 ± 2.0 % of total phosphorylase phosphatase activity). A possible explanation for this slight discrepancy is that it is not only the concentration of inhibitor that determines to what extent each phosphatase is inhibited, but also the concentration of each phosphatase present in the cell extract. Low enzyme concentrations, especially of PP2A may be more completely inhibited by a given concentration of OA than higher enzyme concentrations (Cohen et al, 1989, Hardie et al, 1991, Bialojan and Takai, 1988). Although the total protein concentration in the cell extracts was known, the individual PP concentrations were not.

In this study the relative amounts of PPl and PP2A activity in primary astrocytes appear to be similar (Figure 3.2). However, PP1 and PP2A have discrete cellular functions and different substrate specificities. Ingebritsen and Cohen (1983) showed that PPl has more affinity for phosphorylase than PP2A does. This may mean that in absolute terms there is a little more PP2A present and a little less PPl than dephosphorylation of phosphorylase suggests (Figure 3.1).

Using similar methods Cohen et al (1989) reported that 57 % of total rabbit brain PPs were PP2A and 40 % PPl. Sim et al (1994) measured about 80 % PPl activity and 20% PP2A in rat forebrain and Dudek and Johnson (1995) measured approximately 73 % of total phosphorylase phosphatase activity as PPl and 27 % PP2A in rat forebrain. These results may suggest a difference between species and/or in regional distribution within
the brain, and possibly in distribution between cell types. The brain contains several cell types of which astrocytes are only one. Pahan et al (1998) measured PP activity in astrocytes after 30 min incubation with increasing concentrations of OA. PP activity decreased rapidly at first, from 0 to 4 nM OA, after which the fall in activity was more gradual, suggesting inhibition of PP2A was complete at 4 nM OA and only PP1 was active in the presence of higher OA concentrations. Although PP1 and PP2A are clearly present in astrocytes according to several studies using PP inhibitors (Arias et al, 1993, Pschenichkin and Wise, 1995, Vinadé and Rodnight, 1996, Salinero et al, 1997, Pei et al, 1997, Pahan et al, 1998) this is the first demonstration that they are present in approximately equal quantities.

Astrocytes perform a large range of functions within the brain including maintenance of homeostasis of the neuronal environment, recovery of nutrients from the bloodstream to supply the brain, signalling to neurons and other astrocytes by the release of various mediators. PP1 and PP2A are likely to have important roles in these functions. The study by Pahan et al (1998) demonstrated a role for OA-sensitive PPs in the cytokine-induced signal transduction pathway for the induction of iNOS. OA stimulated the LPS- and cytokine-mediated production of NO as well as expression of iNOS protein and mRNA in astrocytes. Arius et al (1993) treated GFAP-positive astrocytes with 250 nM OA, which induced a change in morphology from flat with short processes to stellate with extended processes. PP1 in astrocytes is responsible for the dephosphorylation of GFAP in immature hippocampal slices (Vinadé and Rodnight, 1996). GFAP is phosphorylated in vitro by PKA, PKC, CaMPK II and the cdc2 kinase (Harrison and Mobley, 1991, Yano et al, 1994, Tsujimura et al, 1994). This phosphorylation appears to regulate the dynamic equilibrium between the polymerised and depolymerized states of GFAP and is particularly observed during mitosis (Inagaki et al, 1994, 1996).

PP1 and PP2A have well known roles in the liver in regulation of glycogen metabolism,
and PP2A in the regulation of glycolysis, gluconeogenesis and fatty acid and cholesterol metabolism (Ingebritsen and Cohen 1983). Astrocytes are metabolically active (Edmond et al, 1987, Edmond, 1992, Hertz and Peng, 1992, Peuchen et al, 1996). Astrocytes are able to take up glucose directly from the bloodstream and store it in the form of glycogen as a metabolic buffer for the brain, unlike neurons which contain neither glycogen (Ibrahim, 1975) nor glycogen phosphorylase (Pfeiffer et al, 1990). Astrocytes also oxidise fatty acids and produce ketone bodies (Blázquez et al, 1999). It is possible that all the above processes are regulated in astrocytes by the same protein phosphatases as in hepatocytes. Hepatocytes, a largely metabolic cell type, contain more PP2A (62.7 ± 5.2 %) than PP1 (27.2 ± 5.4 %) (Figure 3.3A).

HLMC are an inflammatory cell type and contain only 15 ± 3 % PP2A but 71 ± 3 % PP1, which is almost the reverse of that seen in hepatocytes. A similar profile was seen in another inflammatory cell type, basophils, in which 79 % of PP activity was PP1 and only 17 % PP2A (Peirce et al, 1996). It has been proposed that PP1 plays an important role in regulating IgE-mediated histamine release in HLMC (Peachell and Munday, 1993) and basophils (Pierce et al, 1996). Astrocytes do secrete a variety of substances including inflammatory mediators, particularly when they are in a reactive state or in culture (Eddleston and Mucke, 1993, McMillan et al, 1994, Ridet et al, 1997). Astrocytes are a major source of eicosanoids in the brain (Seregi et al, 1987, Murphy et al, 1988) including leukotrienes (Hartung and Toyka, 1987) and prostaglandins such as PGD₂ (Gebicke-Haerter et al, 1988). HLMC have a similar role in the lungs, in fact one main function of mast cells is to release histamine and other inflammatory mediators such as PGD₂ and leukotrienes in response to an IgE-mediated influx of Ca²⁺ (Bach, 1982). Thus it is possible that the Ca²⁺/calmodulin dependent PP2B is important in these cells (Peachell et al, 1995, Triggiani et al, 1989), although the high levels of PP1 and sensitivity of the process to OA has implicated PP1 in IgE-mediated histamine release (Peachell and Munday, 1993, Pierce et al, 1997).
It is interesting that the inflammatory cell types HLMC and basophils contain more PP1 and hepatocytes, which have a homeostatic/metabolic function contain more PP2A while astrocytes, which perform both functions, contain similar amounts of both. If PP1 is associated with an inflammatory function and PP2A is associated with a metabolic function, then the fact that astrocytes contain similar activities of PP1 and PP2A may indicate that both of these two functions are important in astrocytes in culture.

The activity of PP2A in astrocytes is similar to that of PP1 but exceeds that of PP2C. PP2A accounted for 73.8 ± 6.6% of the total casein phosphatase activity, while PP2C accounted for only 26.2 ± 6.6% (Figure 3.5). It is thought that PP2C may be involved in signal transduction in the brain by catalysing the dephosphorylation of autophosphorylated CaM kinase II (Fukanaga et al, 1993). This was demonstrated in neurons, but CaM kinase II has also been shown to phosphorylate GFAP in cultured astrocytes (Yano et al, 1994) so CaM kinase II is one known PP2C substrate present in astrocytes.

Other roles for PP2C have been suggested by studies in yeast. PP2C inhibits an osmosensing PBS2/HOG1 MAPK cascade in yeast, which responds to osmotic and heat shock (Maeda et al, 1994, Shiozaki and Russell, 1995). Astrocytes are resistant to damage by heat shock; they have been shown to produce heat shock protein hsp70 (Nishimura et al, 1988, 1991, Narasimhan et al, 1996) in response to temperature elevations of 6-8°C. Recently, human PP2C has been demonstrated to negatively regulate the stress-activated protein kinases (SAPK) p38 and JNK (Takekawa et al, 1998), which become activated by extracellular stress, including osmotic shock, oxidative stress, protein synthesis inhibitors, UV radiation and anti-cancer drugs (Kyriakis and Avruch, 1996). SAPK is present at very high levels in the brain, compared to other tissues such as liver (Hu et al, 1997). Astrocytes are cells that respond to stress in the brain by transforming to a reactive state in which they express surface molecules and produce
various neurotrophic factors and cytokines (Eng et al. 1992). It is not known how protein phosphorylation and dephosphorylation are involved in this response. The SAPK pathway is present in astrocytes, in which it becomes activated by cytokines such as TNF-α and IL-1, UV light, cycloheximide, heat shock and mechanical injury (Zhang et al. 1996); it may be another target for PP2C.

In liver, PP2C dephosphorylates and activates HMG-CoAR, and dephosphorylates and inactivates its upstream regulator, AMPK. In this way PP2C reverses inhibition of cholesterol and fatty acid synthesis resulting from an elevated AMP/ATP ratio (Moore, 1991) found during conditions of stress such as heat shock and anoxia (Corton et al. 1994). AMPK and HMG-CoAR are known to be present in astrocytes, (see chapter 4, Langan et al. 1987). The proportion of PP2A:PP2C activity in astrocytes (72.2 ± 2.2% : 27.8 ± 2.2 %) is very similar to that in hepatocytes (73.8 ± 6.6 % : 26.2 ± 6.6 %). There are therefore two ways in which PP2C could possibly restore cellular function in astrocytes once a stress has passed, by suppressing the SAPK pathway and by removing the inhibition of the energy-consuming biosynthetic processes of cholesterol and fatty acid synthesis.

Calcineurin activity was measured in astrocytes and in whole brain using the peptide derived from the regulatory subunit of PKA as a substrate. Figure 3.7A shows that the method did demonstrate calcineurin activity, since commercially available calcineurin produced de-phosphorylation of the peptide which was significantly reduced in the absence of Ca^{2+}. When the assay was carried out using extracts of astrocytes and whole brain however, OA was able to abolish most peptide phosphatase activity (Figure 3.7B, Figure 3.8). Some of the remaining activity was Ca^{2+}-dependent, but considering calcineurin comprises 1 % of total brain protein (Stemmer and Klee, 1991), this activity was unexpectedly low, particularly in the whole brain.
Until recently it was believed that astrocytes did not contain calcineurin (Billingsley et al. 1994, Dawson et al. 1994, Usuda et al. 1996). However, Vinadé et al. (1997) found a low level of calcineurin in primary cultures of hippocampal astrocytes, detectable using immunoblotting of both the A and B subunits, but too low to be detected using immunocytochemistry. The calcineurin Aβ isoform was more recently shown by immunofluorescence to be present in gerbil hippocampal activated astrocytes, appearing between days 4-7 after transient ischaemia (Hashimoto et al., 1998), which suggests that the amount of calcineurin present in astrocytes may increase in response to stress stimuli. Calcineurin A and B have also been shown in cultured rat astrocytes (Matsuda et al., 1998).

The present study was able to detect a low level of calcineurin activity in astrocytes (see section 3.2.3, Figure 3.8). When 0.5 μM OA was used to inhibit PP1 and PP2A only 7% of the remaining activity could be attributed to calcineurin (Figure 3.7, 3.8A). OA is known to inhibit calcineurin at higher concentrations than this (0.5 mM), however, 0.5 μM may have been enough in this case to partially inhibit the enzyme if the calcineurin concentration was very low (see section 3.2.3). When a combination of inhibitor-1, to inhibit PP1 and 1 nM OA, to inhibit PP2A was used slightly more of the remaining activity was Ca^{2+}-dependent (14 %), but was still relatively low. Matsuda et al (1998) have recently shown the presence of calcineurin A, probably the Aβ_{2} isoform, and the B subunit in cortical astrocytes using both immunoblotting and immunofluorescence at about 5-10 % of the level seen in whole rat brain. It is possible then that the low levels of calcineurin measured in the present study do reflect the fact that calcineurin is only present at very low levels in astrocytes.

Calcineurin regulates transmitter release (Nichols et al., 1994), growth factor transcription (Klee et al., 1998), several proteins such as the nerve terminal phosphoprotein dynamin I (Liu et al., 1994), ion channels (Armstrong, 1989, Chen et al., 1995), adenylate cyclase
The RII subunit of PKA (Blumenthal et al., 1986) and protein kinase C substrates (Seki et al., 1995). So these properties may represent possible roles for calcineurin in astrocytes. Matsuda et al. (1998) showed a role for calcineurin in Ca\(^{2+}\) paradox-like injury in cultured cortical astrocytes, which is considered to be an *in vitro* model of ischaemia/reperfusion injury, possibly implying a pathological role for calcineurin in these cells. Recently a potential role for calcineurin in the pathogenesis of brain ischaemia and traumatic injury has been described in which it appears to have both protective and toxic actions in neurons (Morioka et al., 1999). While inhibition of calcineurin by FK506 and cyclosporin A reduce glutamate neurotoxicity (Dawson et al., 1993) and delay neuronal death (Drake et al., 1996, Ide et al., 1996), substrates of calcineurin such as the NMDA receptor, Ca\(^{2+}\)-channel and IP\(_3\) receptor have a protective role when dephosphorylated by calcineurin (Morioka et al., 1999). It is likely that calcineurin in astrocytes is also involved in these processes as part of their response to injury. Other calcineurin substrates present in astrocytes include the microtubule-associated proteins MAP2 and tau (Arius et al., 1993); NOS (Galea et al., 1992, Hu et al., 1995) and the RII subunit of PKA (Massa et al., 1991).

Changes in total PP activity over 21 days growth in culture (Figure 3.9 A,B) suggest that protein phosphatases may be involved in the development of astrocytes. Peak activity of PP1, 2A and 2C occurs around day 10 in culture. This is when DNA concentration in the cells is increasing most rapidly, i.e. when there is most rapid growth (Figure 3.9C). A uniform increase in all three PPs is observed, since the relative proportions are basically unchanged (Figure 3.10) over the first 21 days of growth. Although little is known about changes in astrocyte PPs during development, Dudek and Johnson (1995) have studied the changes in PP1 and 2A in rat forebrain from postnatal day 1 to adult. They found PP1 activity, mirrored by the immunoprecipitated protein, was at a constant level throughout the study. In contrast, in the present study PP1 activity in cultured astrocytes peaked at day 10 (Figure 3.10 A,B). PP2A activity in forebrain was highest from days 1 to 3, after
which it decreased to adult levels of activity and protein expression by day 21 (Dudek and Johnson, 1995). In astrocytes PP2A activity peaked at about day 10 of growth in culture and then fell (Figure 3.10). It must be remembered, however, that cultured astrocytes are in a different environment to astrocytes in situ, and the properties and rate of maturation of these cells may be different.

During postnatal development major changes occur in the cytoskeleton of both neurons and astrocytes, especially in the microtubules and microtubule-associated proteins (MAPs), whose phosphorylation state regulates their ability to bind to and stabilize microtubules (Brugg and Matus, 1991). PP activities were measured by Dudek and Johnson (1995) in microtubule preparations from rat forebrain at day 3 and adult. In the microtubule fraction PP1 activity at day 3 was double that measured in the adult and PP2A was slightly less at day 3 than adult, both of which are different patterns to those seen in whole brain. This suggests that targeting of PPs to the cytoskeleton changes during development. The binding of PP1 to its microtubule-associated targeting subunit, tau (Liao et al, 1998) allows dephosphorylation of microtubules, resulting in increased microtubule assembly and stability. If PP1-mediated dephosphorylation and assembly of microtubules was an early event in brain development this would explain the changes in microtubule-associated PP1 activity. A role for PP2A is not excluded since various regulatory subunits of PP2A have been identified of which one or more may be cytoskeletal (Zolnierowicz et al, 1994, Dudek and Johnson, 1995).

In astrocytes the levels of PPs peak during the period of greatest cytoskeletal changes, day 10-14 in culture. The maturation of astrocytes includes the addition of intermediate filament proteins including GFAP to a primary cytoskeletal structure of microtubules. The expression of GFAP increases rapidly during days 10-14 (Sancho-Tello et al, 1995). GFAP may be a target of the increased PP activity found in astrocytes during this period as PP1 is known to dephosphorylate GFAP (Vinadé and Rodnight, 1996).
Production of inflammatory lipid mediators, the eicosanoids, is dependent on the availability of the precursor, free AA. This is because AA is not stored but produced as required from cleavage of membrane phospholipids by a phospholipase, usually cytosolic phospholipase A₂ (cPLA₂). The 85 kDa cPLA₂, demonstrated in grey matter astrocytes by Stephenson et al (1994), is activated by phosphorylation and is dependent on Ca²⁺ (Lin et al, 1992, Qui et al, 1993, Kramer et al, 1993). An increase in intracellular calcium concentration, [Ca²⁺]ᵢ, causes cPLA₂ translocation and binding to the nuclear or ER membrane (Channon et al, 1990, Clark et al, 1991, Nalefski et al. 1994). The relative importance of phosphorylation and [Ca²⁺]ᵢ in the activation of cPLA₂ varies according to the stimulus, and the cell type being studied (Qui et al. 1998). It has been reported that the only protein kinase whose phosphorylation of cPLA₂ is known to increase its activity is MAPK (Lin et al, 1993). In rat striatal astrocytes, AA release was stimulated by the combination of A23187 and the PKC activator PMA, the effect was reversed by pretreatment with a non-specific inhibitor of PLA₂, mepacrine (Zanassi et al, 1998). Thus it appears that PKC is also involved in the process of AA release by the action of PLA₂ in astrocytes, either by direct phosphorylation of cPLA₂ or indirectly (Stella et al, 1997, Chen and Chen, 1998, Jeremy et al, 1987).

In astrocytes, entry of external Ca²⁺ via the Ca²⁺ ionophore A23187 stimulates tyrosine phosphorylation of p42 MAPK (Chen and Chen, 1998), this is downstream of activation of PKCα, which is not a tyrosine kinase. Following this phosphorylation of MAPK, the characteristic gel mobility shift of cPLA₂ is seen, indicating phosphorylation of cPLA₂ on serine-505 and consequent activation, leading to the release of AA from membrane phospholipids (Figure 3.11, Lin et al, 1993, Chen and Chen, 1998). Chen and Chen (1998) found that the release of AA from cerebellar astrocytes induced by the Ca²⁺ ionophore A23187 was only partially inhibited (40 %), even though MAPK activation was completely blocked, by the down-regulation of PKC, which is known to be activated and translocate to the membrane on stimulation by calcium ionophore (Chen, 1994).
Hence the A23187-stimulated AA release observed in this study (Figures 3.11, 3.12) is likely to be the result of PKC and MAPK mediated phosphorylation and activation of cPLA₂ and a PKC- and MAPK-independent mechanism. The latter may involve Ca²⁺-stimulated translocation of cPLA₂ or a different protein kinase phosphorylation of cPLA₂ (Nemenoff et al., 1993, Kang et al., 1997, Chen and Chen, 1998). Toumier et al. (1997) found that in astrocytes H₂O₂ activation of the MAPK isoform ERK and c-Jun NH₂-terminal kinase (JNK) induced phosphorylation of cPLA₂ and stimulated AA release. Both basal and H₂O₂-induced phosphorylation of cPLA₂ were unaffected by treatment of astrocytes with GF109203X, a PKC inhibitor (Toullec et al., 1991). In monocytes or macrophages no mobility shift of cPLA₂ could be detected in response to A23187 and its stimulation of AA release was MAPK- and PKC-independent (Xu et al., 1994, Ambs et al., 1995, de Carvalho et al., 1996). NA stimulated AA release in rabbit aortic smooth muscle cells has also been attributed to a PKC-independent pathway in which CaM kinase II is implicated (Muthalif et al. 1996).

Figure 3.14 shows that the stimulations of AA release by OA and A23187 were additive, indicative of their different mechanisms. The increase in AA release produced by A23187 was dependent on the influx of extracellular Ca²⁺ (Figure 3.15), while OA stimulation of AA release was independent of extracellular Ca²⁺. An increase in [Ca²⁺]ᵢ in response to A23187 alters the phosphorylation state of proteins by its activation of PKC and Ca²⁺/CaM-dependent PKs. OA inhibits PP1 and PP2A, which are known to be responsible for opposing many of the effects of PKC (Seki et al., 1995) as well as other PKs. Furthermore, the stimulation of AA release seen during OA treatment of astrocytes (Figure 3.13 to 3.15) may be mediated by its activation of MAPK (Haystead et al., 1990). This activation is probably via the inhibition of PP2A which itself would otherwise inhibit both MEK, by dephosphorylating 2 serine residues (Alessi et al., 1995), and MAPK, by dephosphorylating threonine-183 (Anderson et al., 1990, Alessi et al., 1995).
ATP can be released into the extracellular fluid as a result of cell lysis, selective membrane permeabilisation (such as occurs during hypoxia) or exocytosis of secretory granules from neurons of the central and peripheral nervous system (Motte et al., 1995). ATP acts on astrocytic P2Y purinergic receptors to promote eicosanoid release in vitro (Bruner and Murphy, 1990, Pearce et al., 1989, Chen and Chen, 1998). Extracellular ATP stimulates influx and accumulation of Ca\(^{2+}\) in astrocytes (Pearce et al., 1989, Neary et al., 1988) which leads to increased phosphorylation of some cellular proteins (Neary et al., 1991). The means by which ATP stimulates AA release in astrocytes (Figure 3.16) is the same as that of A23187, via Ca\(^{2+}\)-activation of protein kinases such as PKC or CaM kinase II, leading to MAPK activation and cPLA\(_2\) phosphorylation and activation (Chen and Chen, 1998). Since ATP increases AA release via activation of PKs, the effect of OA is additive to that of ATP (Figure 3.16). PP1 and/or PP2A must normally dephosphorylate proteins whose phosphorylation promotes AA release in astrocytes.

Where AA release survives down-regulation of PKC\(\alpha\), \(\delta\) and \(\theta\) by 24 h TPA treatment, a possible role for PKC\(\eta\) emerges, which can be translocated, but not down-regulated by TPA (Chen and Chen, 1996, Chen et al., 1997). Kang et al., 1997 studied the activation of MAPK by P2Y receptor activation in astrocytes. An inhibitor of Ca\(^{2+}\)-dependent PKCs, Gö 6976, only slightly reduced ATP-evoked MAPK activation, while an inhibitor of both Ca\(^{2+}\)-dependent and -independent PKCs, Ro 31-8220, almost completely blocked ATP-evoked MAPK activation. Kang and co-workers concluded that P2Y receptor activation was coupled to a MAPK signalling pathway in which a calcium-independent PKC isoform was upstream of MAPK, which they suggested was PKC\(\delta\). Astrocytes are known to contain PKC\(\alpha\), \(\delta\), \(\theta\), \(\eta\) and \(\zeta\) (Chen et al., 1995, Chen and Chen, 1996).

AA release from astrocytes in the presence of A23187 was four times greater than that seen with ATP in the present study (Figure 3.14, 3.16), which is similar to the result obtained by Chen and Chen (1998). ATP, however, is a physiological promoter of AA
release in astrocytes via P2Y purinergic receptors (Bruner and Murphy, 1993, Sorg et al. 1995). ATP stimulated AA release has a role in providing energy substrates for astrocytes by stimulating glycogenolysis (Sorg et al. 1995) and increasing glucose uptake (Yu et al, 1993). The activation of glycogen phosphorylase by AA may be via a general mechanism common to polyunsaturated free fatty acids as seen in hepatocytes (Gomez-Munoz et al, 1991) or possibly via AA induced Ca\(^{2+}\) release (Delumeau et al, 1991).

Upstream of the increase in intracellular Ca\(^{2+}\) and PKC activity that stimulates AA release is PLC. In the CNS, stimulation of various cell surface receptors leads to the hydrolysis of inositol phospholipids via the action of PLC to produce the second messengers DG and IP\(_3\), which releases Ca\(^{2+}\) from intracellular stores (Berridge, 1993). Several isoforms of PLC exist, the most abundant in glial cells is PLC\(\delta\) (Mizuguchi, 1991). The accumulation of \(^{3}\text{H}\)-inositol phosphates was measured in response to NA, OA and staurosporine.

NA stimulation of astrocytes caused a large increase in IP accumulation above basal levels (Figure 3.17). This was halved in the presence of OA, even though OA alone caused a slight stimulation. This may mean that a PK, allowed by OA to act without PP antagonism, phosphorylates and antagonises NA-induced inositol phosphate production in astrocytes. The effect of OA on NA-stimulated IP accumulation was opposed by the presence of staurosporine, (Figure 3.17) although staurosporine alone had no effect on NA-induced IP accumulation. Staurosporine, which is a relatively non-specific protein kinase inhibitor is most potent in the inhibition of PKC and to a lesser extent PKA. It is possible that OA was allowing a protein kinase to have an inhibitory effect on NA-induced \(^{3}\text{H}\)-IP accumulation by restoring protein(s) to a phosphorylated state. The actions of PKC are known to be reversed by at least 3 protein phosphatases in the brain, including PP1 and PP2A, which are OA-sensitive (Seki, 1995).
Previous studies on regulation of the inositol phospholipid signalling pathway in astrocytes have shown that acute phorbol ester treatment inhibits agonist-induced inositol phospholipid breakdown (Pearce et al., 1988), which is thought to be due to receptor regulation by PKC (Pearce and Murphy, 1993). Accordingly, down regulation of PKC by prolonged phorbol ester treatment caused a 233% increase in NA-induced IP accumulation (Pearce and Murphy, 1993), which was in agreement with the theory that PKC normally regulated the pathway by negative feedback of the receptor. It would be expected that inhibition of PKC by staurosporine would cause the reverse of acute phorbol ester treatment, which was the case; staurosporine induced a slight increase in basal and no increase in NA-induced IP accumulation (Figure 3.17).
CHAPTER 4

THE AMP-ACTIVATED PROTEIN KINASE AND ACETYL-CoA CARBOXYLASE IN RAT BRAIN AND CULTURED ASTROCYTES
AMP-activated protein kinase (AMPK) was first identified as the kinase responsible for the phosphorylation and inactivation of both acetyl-CoA carboxylase (ACC) and 3-hydroxy, 3-methylglutaryl CoA reductase (HMG-CoAR), the enzymes that catalyse the rate-limiting steps in fatty acid and cholesterol synthesis, respectively (Carling et al, 1987). AMPK is a heterotrimeric enzyme consisting of catalytic (α) and regulatory (β and γ) subunits. All three subunits are required for activity. The enzyme is activated by phosphorylation on the α subunit, while AMP binds to the γ regulatory subunit (Cheung et al, 2000). The β subunit acts as a scaffold for the complex, interacting with the catalytic α subunit and the activating γ subunit (Hardie et al, 1998). Multiple isoforms of all three subunits have been identified, raising the possibility that a large family of AMPK complexes exists (Stapleton et al, 1996). Two isoforms of AMPK α subunit have been identified; α1 and α2 (Stapleton et al, 1996). Liver contains about 50 % α1, 50 % α2, while brain contains about 75 % α1, 25 % α2 (Cheung et al, 2000). Three γ subunit isoforms have been identified, liver contains about 90 % γ1, 10 % γ2, while brain contains 36 %, 36 %, 27 % γ1, γ2, γ3, respectively (Cheung et al, 2000).

AMPK is activated by a rise in the intracellular AMP concentration, or rather an increase in the AMP/ATP ratio (Corton et al, 1994). This constitutes a stress, which the enzyme responds to by phosphorylation and inhibition of ACC and HMG-CoAR, thereby switching off the nonessential and energy-utilising pathways of fatty acid and cholesterol synthesis to preserve the cell’s energy status. AMP activates the kinase in two ways, firstly by a direct allosteric effect on AMPK itself (Carling et al, 1987), and its upstream kinase, known as AMPK kinase (AMPKK), which phosphorylates and further activates AMPK upon stimulation by a high AMP/ATP ratio (Hawley et al, 1996) and secondly binding of AMP to AMPK renders it a better substrate for AMPKK and a worse substrate for PP2C, its inactivating phosphatase (Davies et al, 1995).
AMPK activity is regulated by the AMP/ATP ratio (Corton et al, 1994), which, in some tissues, is itself influenced by the PCr/Cr ratio, so a possible link between these two systems was investigated. It has been proposed that AMPK activity is inhibited by a high PCr/Cr ratio, and that AMPK phosphorylates and inactivates the muscle-specific isoform of CK (MM-CK) (Ponticos et al, 1998). AMPK is sensitive to any stress stimulus that lowers the energy status of the cell causing a drop in PCr/Cr and a rise in AMP/ATP, and responds by switching on ATP-generating systems (eg. fatty acid oxidation) and initiating energy-saving measures such as inhibition of fatty acid and sterol synthesis (Hardie and Carling, 1997).

Verhoeven et al (1995) demonstrated that mRNA for AMPK was present in brain at a lower proportion of total RNA than in liver or mammary gland. However, mRNA level does not necessarily reflect the expression or specific activity of the enzyme in any tissue. Two groups have measured AMPK activity in brain homogenates, as either 11 % (Davies et al 1989) or 50 % (Stapleton et al 1996) of that measured in liver. Phosphorylation of SAMS, which is a synthetic peptide substrate for AMPK was used by both authors to estimate AMPK activity in tissues. The template for this peptide is a short section of ACC including serine-77 and serine-79, the major sites of phosphorylation by AMPK and by PKA. The peptide has been modified by the replacement of the serine phosphorylated by PKA with alanine so it is a specific substrate for AMPK (Davies et al, 1989, see section 1.10.2). No information was available about the regional or cellular distribution of AMPK in brain. However, two locations are particularly likely: oligodendrocytes, since AMPK is a regulator of ACC, which is enriched in these cells; and astrocytes, which respond to various types of stress to the brain (Norenberg, 1994), including ischaemia (Hori et al, 1994). The existence of multiple isoforms of AMPK subunits suggests there may be tissue specific distribution, regulation and function. Cheung et al (2000) report different AMPK subunit composition in brain compared to liver, which could mean altered properties of AMPK in brain compared to liver.
New substrates of AMPK are still emerging; the muscle-specific isoform of CK (MM-CK) (Ponticos et al, 1998), PFK-2 (Marsin et al, 2000), intermediate filaments in hepatocytes (Velasco et al, 1998b), endothelial NO synthase (eNOS) in rat hearts (Chen et al, 1999), Raf-1 in cytosolic extracts of NIH-3T3 cells, and possibly in intact CHO cells (Sprenkle et al, 1997). The Raf-1 kinase has been implicated in apoptosis (Wang et al, 1996), a process recently shown to be inhibited by AICAR in rat thymus cells (Stefanelli et al, 1998) and in astrocytes (Blázquez et al, 2001), implicating a role for AMPK in regulation of gene expression, signalling pathways and in apoptosis. ACC remains the best substrate for AMPK, perhaps because of its central role in energy metabolism. Not only does it regulate the rate of fatty acid synthesis, but its product malonyl-CoA is an allosteric inhibitor of fatty acid oxidation. Phosphorylation and inactivation of ACC by AMPK has the dual effect of inhibiting the energy-utilising fatty acid synthesis and activating the energy-producing fatty acid oxidation.

Brain has a high lipid content, largely due to the long-chain fatty acids which are major constituents of the myelin sheath that insulates neurons. The process of myelination occurs during a few weeks starting at postnatal day 10-12 (P10-12) in the rat. The maximal rate of myelination occurs at P18-20 (Norton and Poduslo, 1973) in a specialised type of glial cell, the oligodendrocyte. Long-chain fatty acids, which are required in huge quantities during this short time frame, can be synthesised via two different pathways: chain elongation of shorter chain fatty acids or de novo synthesis of long-chain fatty acids (Brady, 1960, Aeberhard et al, 1969). ACC catalyses the rate-limiting step in the de novo synthesis of fatty acids: the conversion of acetyl-CoA to malonyl-CoA. The change in activity of this enzyme during development closely parallels the rate of lipogenesis in various brain regions (Gross and Warshaw, 1974, Patel and Tonkonow, 1974, Yeh et al, 1983). It has been known for some time that ACC is present in brain (Gross and Warshaw, 1974, Patel and Tonkonow, 1974), and is enriched in oligodendrocytes, the site of myelination (Tansey and Cammer, 1988, Moore and Brophy, 1994).
4.2 RESULTS

4.2.1 A comparison of the properties of AMPK from rat liver and brain

AMPK was partially purified from rat brain and liver as described in materials and methods (section 2.12.3). The brain enzyme had a lower specific activity than the liver enzyme (0.41 ± 0.09 nmol \(^{32}\)P-phosphate into SAMS peptide/min/mg protein in brain; 1.67 ± 0.85 nmol \(^{32}\)P-phosphate into SAMS peptide/min/mg protein in liver), but showed a similar profile of sensitivity to increasing concentrations of AMP (Figure 4.1). The \(K_a\) for AMP determined in each case using the Wilkinson Derivation plot in the presence of 0.2 mM ATP was 1.61 ± 0.41 \(\mu\)M for the brain and 3.37 ± 0.57 \(\mu\)M for liver. The \(K_a\) is dependent on the AMP/ATP ratio, Carling et al (1989) measured the \(K_a\) for more extensively purified rat liver AMPK at both 0.2 mM ATP (1.4 \(\mu\)M) and 2.0 mM ATP (14 \(\mu\)M), which is a more physiological ATP concentration.

Brain and liver AMPK were assayed in the presence of increasing concentrations of PCr up to 200 mM (Figure 4.2). Dose-dependent inhibition of AMPK from both sources was observed in the absence of AMP but this dependence was different for brain and liver. Liver AMPK was 60% inhibited by 50 mM PCr while brain AMPK was virtually unaffected. Estimated EC\(_{50}\) for PCr for the liver enzyme was 35 mM and for brain AMPK 75 mM.

Ponticos et al (1998) reported that inhibition of antibody-purified liver AMPK by PCr occurred in the presence or absence of AMP. This was also true in the present study for the liver enzyme (Figure 4.2B). However, addition of 150 \(\mu\)M AMP to the brain enzyme greatly increased the sensitivity of AMPK to PCr inhibition so that the EC\(_{50}\) decreased to 32 mM PCr whereas in the absence of AMP it was 75 mM PCr (Figure 4.2A). In the presence of 150 \(\mu\)M AMP the sensitivity of brain AMPK to inhibition by PCr was
AMPK was purified to the post mono-Q stage as described in materials and methods (see section 2.12.3) from both rat brain and liver. The two preparations were assayed for AMPK activity by the incorporation of $^{32}$P-phosphate into SAMS peptide over 5 min incubations as described in materials and methods (see section 2.8.2), over a range of concentrations of its allosteric activator, AMP. ATP was present at 0.2 mM in each assay. Activity was calculated as percent of control (no AMP). **A.** Brain AMPK activity, $100\% = 0.41 \pm 0.09$ nmol phosphate into SAMS peptide/min/mg protein. **B.** Liver AMPK activity, $100\% = 1.67 \pm 0.85$ nmol phosphate into SAMS peptide/min/mg protein. Each value is the mean of two or three determinations.
Figure 4.2 Effect of phosphocreatine on AMPK activity of rat brain and liver

AMPK was purified to the post mono-Q stage as described in materials and methods (see section 2.12.3) from both rat brain and liver. The two preparations were assayed for AMPK activity by the incorporation of $^{32}$P-phosphate into SAMS peptide as described in materials and methods (see section 2.8.2) over a range of concentrations of phosphocreatine (PCr) in the presence or absence of 150 μM AMP. Activity was calculated as percent of control (no PCr). A. Brain AMPK activity, in the absence of AMP, average control value 0.75 ± 0.04 nmoles phosphate into SAMS/min/mg protein, and in the presence of 150 μM AMP. B. Liver AMPK activity, in the absence of AMP, average control value 2.46 ± 0.89 nmoles phosphate into SAMS/min/mg protein, and in the presence of 150 μM AMP. Each value is the mean ± sem, where appropriate, of at least three determinations.
indistinguishable from liver AMPK (Figure 4.2). The EC$_{50}$ for PCr inhibition of liver AMPK measured by Ponticos et al (1998) was approximately 15 mM PCr. The discrepancy between this value and the values reported here may be due to the greater purity of AMPK used in the studies of Ponticos et al (1998) as this had been further purified past the DEAE sepharose step by immunoprecipitation.

Figure 4.3 shows that the sensitivity of liver AMPK to activation by increasing AMP concentrations is unchanged by the presence of 100 mM PCr (Figure 4.3B). However, brain AMPK appears to show a slight increase in sensitivity to AMP in the presence of 100 mM PCr (Figure 4.3A).

From its initial discovery, AMPK was known to be reversibly phosphorylated and activated (Carling et al, 1986). Subsequent insensitivity to OA and experiments with purified phosphatases have led to the conclusion that PP2C is the physiological PP that dephosphorylates liver AMPK (Davies et al, 1995). Figure 4.4 shows that brain AMPK is also regulated by reversible phosphorylation, in this experiment it was dephosphorylated and inactivated (80%) by incubation with PP2C.

To make some comparison of the substrate specificity of brain and liver AMPK a range of peptide substrates were assayed with each enzyme. The consensus sequence of AMPK has been found by various groups to include a hydrophobic residue at -4 or -5 from the serine to be phosphorylated (Weekes et al, 1993, Toomey et al, 1995, Dale et al, 1995), and hydrophobic at +4 or +5 (Weekes et al, 1993, Dale et al, 1995), and a basic residue at -3 or -4 (Sullivan et al, 1994, Weekes et al, 1993, Dale et al, 1995, Toomey et al, 1995). The primary amino acid sequence surrounding serine-79 of ACC (HMRSSMSGGLHVKKRR) contains the hydrophobic met at -5, a basic arg at -4, and leu and val at +4 and +5, so it is a very good substrate for AMPK.
AMPK was purified to the post mono-Q stage as described in materials and methods (see section 2.12.3) from both rat brain and liver. The two preparations were assayed for AMPK activity by the incorporation of $^{32}$P-phosphate into SAMS peptide as described in materials and methods (see section 2.8.2) over a range of concentrations of AMP in the presence or absence of 100 mM phosphocreatine (PCr). Activity was calculated as percent of control (no AMP). A. Brain AMPK activity, control value $0.41 \pm 0.09$ nmoles phosphate into SAMS/min/mg protein. B. Liver AMPK activity, control value $1.67 \pm 0.85$ nmoles phosphate into SAMS/min/mg protein. Values are from two determinations.
Figure 4.4 Dephosphorylation and inactivation of brain AMPK by PP2C

AMPK was purified from rat brain as far as the DEAE step, as described in materials and methods (section 2.12.3). AMPK was then incubated at 37°C over 30 min in the presence of recombinant PP2C in the presence of MgCl₂ (PP2C will be active), and the absence of MgCl₂ (PP2C will remain inactive). At time points throughout this incubation, aliquots were removed and diluted into buffer containing 50 mM NaF (to inhibit active phosphatase) and assayed for AMPK activity by the incorporation of $^{32}$P-phosphate into SAMS peptide as described in section 2.8.2. Results are shown as pmol $^{32}$P-phosphate incorporated into SAMS /min from a single determination.
Table 4.1 Specificity of brain and liver AMPK for synthetic peptide substrates.

Peptides containing a modified consensus sequence of the residues surrounding serine 79 of ACC were synthesised by N Flinn at the School of Pharmacy, University of London (Toomey et al, 1995). Phosphorylation by DEAE purified brain or liver AMPK of each synthetic peptide shown was assayed by the incorporation of $^{32}$P-phosphate into each peptide as described in materials and methods (see section 2.8.2). Peptides were used at a final sub-saturating concentration of 40 μM in the assay and only initial rates of phosphorylation (over the first 5 min) were measured. Peptides are referred to in the text by the four letter code shown in colour. Serine-79 is underlined. Activity is expressed as a percentage of the rate of phosphorylation of the parent peptide, HMRSSMSGLHVKRR. Values are mean ± sem. for 3 observations, 5 for SAMS.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Brain</th>
<th>Liver</th>
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<tbody>
<tr>
<td>HMRSSMSGLHVKRR</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>AMRSSMSGLHVKRR</td>
<td>295 ± 31</td>
<td>445 ± 155</td>
</tr>
<tr>
<td>WMRSSMSGLHVKRR</td>
<td>434 ± 61</td>
<td>444 ± 147</td>
</tr>
<tr>
<td>HFRSSMSGLHVKRR</td>
<td>250 ± 36</td>
<td>208 ± 26</td>
</tr>
<tr>
<td>HMRSASMSGLHVKRR</td>
<td>77 ± 13</td>
<td>290 ± 45</td>
</tr>
<tr>
<td>HARSSMSGLHVKRR</td>
<td>24 ± 15</td>
<td>7 ± 5</td>
</tr>
<tr>
<td>HMAASSMSGLHVKRR</td>
<td>15 ± 15</td>
<td>3 ± 3</td>
</tr>
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The peptides prepared by Toomey et al (1995) were based on this sequence from ACC, with alterations of the first three residues, and these were used to assay brain and liver AMPK. Both brain and liver AMPK activity increased when the his at -6 was replaced with an uncharged ala or a hydrophobic trp group, and when the met at -5 was replaced with the bulky, hydrophobic phe (Table 4.1). However, the arg at -4 was vital to AMPK from both tissues. Its replacement with an uncharged ala virtually abolished AMPK activity, as did replacing met at -5 with ala. The data presented in Table 4.1 shows that the substrate specificity of AMPK in liver and brain is very similar. One important exception to this is that brain AMPK phosphorylates SAMS to a similar extent to SSMS, but liver AMPK phosphorylates SAMS four times better than the brain enzyme (Table 4.1). This is hard to explain, if it is due to contaminating kinases these cannot include PKA because neither brain nor liver AMPK phosphorylated HARSSMS to any great extent (see section 1.9.1), and the other peptides are phosphorylated with similar ratios by both brain and liver AMPK. The use of SAMS as a peptide substrate is not invalidated by these data because at 200 μM SAMS the specific activities of brain and liver AMPK were equivalent. At the sub-saturating concentration of 40 μM, brain AMPK has less affinity for SAMS than liver AMPK.
4.2.2 AMPK and ACC in brain regions

Tissue samples from four brain regions designated as neocortex, sub cortex, pons and cerebellum (see introduction, section 1.1) were assayed for AMPK activity by incorporation of $^{32}$P-labelled phosphate into SAMS in the presence or absence of AMP. Figure 4.5A shows a time course of SAMS phosphorylation by AMPK in rat sub cortex. The rate of phosphorylation was increased from $0.32 \pm 0.03$ to $0.58 \pm 0.09$ nmoles phosphate incorporated into SAMS/min/mg protein by the presence of AMP. These values are slightly higher than the basal or AMP-stimulated AMPK activity reported by other groups for whole brain ammonium sulphate fractions (Davies et al, 1989, Stapleton et al, 1996). However, a more purified sample was used in the present study than that used by the other groups.

The AMPK activities for all four regions are summarised in Figure 4.5B. AMPK was present in all four areas of rat brain, but it had a slightly higher basal level in sub cortex and cerebellum. In all 4 regions it could be activated 2-3 fold by AMP to achieve a similar level of activity.

ACC is recognised as the best substrate for AMPK. It is present in brain, where lipid biosynthesis is very important, for example in myelination. The activity of ACC was measured in the same four regions of rat brain to see if there were parallels in the distribution and expression of ACC activity with those of AMPK in brain. ACC activity was found at similar levels in all four regions of rat brain, much like AMPK activity. Neocortex appeared to have slightly higher ACC activity, but this is unlikely to be significant (Table 4.2).
Figure 4.5 AMPK activity in rat brain regions.

AMPK was purified from 4 regions of rat brain as far as the DEAE step, as described in materials and methods (section 2.12.3). A. A time course of $^{32}$P-phosphate incorporation into SAMS peptide by sub-cortical AMPK in the presence or absence of 200 μM AMP. Each value is the mean ± sem. of five determinations. B. AMPK Activity in each of the four brain regions in the presence or absence of 200 μM AMP. Results are shown as nmol $^{32}$P-phosphate incorporated into SAMS /min /mg DEAE purified protein and are means ± sem. for the number of animals shown in brackets.

A

![Graph showing time course of $^{32}$P-phosphate incorporation into SAMS peptide by sub-cortical AMPK in the presence or absence of 200 μM AMP.]

B.

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<thead>
<tr>
<th>Brain Region</th>
<th>- AMP</th>
<th>+ AMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neo Cortex</td>
<td>0.21 ± 0.03 (8)</td>
<td>0.64 ± 0.05 (8)</td>
</tr>
<tr>
<td>Sub Cortex</td>
<td>0.32 ± 0.03 (5)</td>
<td>0.58 ± 0.09 (5)</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>0.33 ± 0.09 (8)</td>
<td>0.61 ± 0.15 (8)</td>
</tr>
<tr>
<td>Pons</td>
<td>0.18 ± 0.03 (6)</td>
<td>0.62 ± 0.08 (6)</td>
</tr>
</tbody>
</table>
Table 4.2 ACC activity in brain regions

ACC was partially purified from 4 regions of rat brain by 6 % PEG precipitation step, as described in materials and methods (section 2.9), ACC activity was assayed for 90 s at 37°C as the transfer of $^{14}$C from NaH$^{14}$CO$_3$ to malonyl-CoA at 10 mM citrate (saturating citrate concentration) as described in materials and methods (section 2.9). Values are expressed as nmoles $^{14}$C into malonyl-CoA/min/g wet weight tissue, and are mean (± sem where appropriate) for the number of determinations shown in brackets.

<table>
<thead>
<tr>
<th>Brain region</th>
<th>ACC activity</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Neocortex</td>
<td>12.29 ± 1.92</td>
<td>1.92</td>
<td>(3)</td>
</tr>
<tr>
<td>Sub Cortex</td>
<td>9.54 ± 0.12</td>
<td>0.12</td>
<td>(3)</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>6.44</td>
<td></td>
<td>(2)</td>
</tr>
<tr>
<td>Pons</td>
<td>8.34</td>
<td></td>
<td>(2)</td>
</tr>
</tbody>
</table>
4.2.3 AMPK and ACC in whole brain during development to 21 days

The first 21 post-natal days in the rat represent a period of rapid growth and development, including myelination. Therefore patterns of change in AMPK and ACC activity over this period were examined. Figure 4.6A shows day 1 brains as an example of the assay which showed linear incorporation of $^{32}$P-phosphate into SAMS over 3 min. Basal activity was $0.13 \pm 0.01$ nmoles phosphate incorporated into SAMS/min/mg protein and this was activated to $0.27 \pm 0.01$ nmoles phosphate incorporated into SAMS/min/mg protein in the presence of 200 µM AMP. This was a two-fold increase comparable to that seen in adult rat brain regions (Figure 4.5B). The specific activity at day 1 was only about 50% of that measured in adult brain regions but this could reflect a higher total protein content of day 1 brain as much as it could reflect a lower expression of AMPK.

The developmental changes in AMPK activity in whole brain are presented in Figure 4.6B. The basal level of activity remained constant during the growth period, there appeared to be a slight increase at P4, but this was not significant. AMP-stimulated activity also remained fairly constant throughout the 21 days. Increased AMP activation was observed at P14 but this was not significant compared to other ages.

Figure 4.7 shows the changes in ACC activity during development of rat brain to P21, measured at 10 mM citrate to ensure maximum activation. Figure 4.7A shows a drop in activity from P1 to P2 followed by a 180% increase to a maximum at P14. These activity changes are expressed per gram wet weight of whole brain tissue and are thus influenced by changes in cell size, lipid content etc. In order to examine changes in the cellular content of ACC activity, ACC activity was expressed per mg DNA (Figure 4.7B). The concentration of DNA is roughly proportional to the number of cells present The profile obtained in this way was in fact remarkably similar to Figure 4.7A.
Figure 4.6 AMPK in rat whole brain over development to 21 days.

AMPK was purified from rat brain as far as the DEAE step from rat brain, as described in materials and methods (section 2.12.3) and its activity measured by the incorporation of $^{32}$P-phosphate into SAMS peptide as described in materials and methods (section 2.8.2) in the presence or absence of 200 μM AMP. **A.** Time course of $^{32}$P-phosphate incorporation into SAMS peptide by AMPK from postnatal day 1, each value is the mean ± sem. of five determinations. **B.** AMPK activity, measured at postnatal days 1 to 21 as shown. Each value is the mean ± sem. for the number of determinations shown in brackets.
ACC activity in adult rat brain regions ranged from 6.4 to 12.3 nmoles $^{14}$C incorporated into malonyl-CoA/min per g wet weight of tissue (Table 4.2) the maximum of 27.96 ± 3.13 nmoles $^{14}$C incorporated into malonyl-CoA/min per g wet weight of tissue seen at day 14 of development (Figure 4.7B) must correspond to an increased demand for fatty acid synthesis during early brain development and myelination. The increased activity of ACC at this time of development could be due to increased expression of ACC or it could be due to post-translational modification such as phosphorylation.

ACC was assayed over a range of concentrations of its allosteric activator, citrate, in PEG pellets from whole rat brains over a range of ages from postnatal day 1 to 21 (P1 to P21). Brain ACC was shown to be citrate-dependent. Typical citrate curves are shown in Figure 4.8 for P1 and P14. There was far more ACC activity at 0 mM citrate in the P14 brains than P1, suggesting citrate-independent activity exists at the later stage.

ACC can be further characterised using the Michaelis Menten parameters, $V_{\text{max}}$ and $K_a$ for citrate. A summary of these, calculated using the Wilkinson Derivation plot for the 6 age points studied is presented in Table 4.3. $V_{\text{max}}$ was unchanged for the first 6 days of development but increased by approximately 50 % from P6 to P14 and P21. $K_a$ was unchanged for the first 4 days of development but decreased approximately 50 % from P14 to P21. The pattern of $V_{\text{max}}$ seen over development (Table 4.3) is similar to that of ACC activity at 10 mM citrate over development as presented in Figure 4.7. The enzyme activity appears from the data presented in Table 4.3 to reach a maximum rate and a maximum citrate sensitivity at approximately P21. It has been speculated that citrate-independent activity is due to dephosphorylated and polymerised ACC (Munday and Hemingway, 1999). While the changes in citrate sensitivity are consistent with decreased ACC phosphorylation, Figure 4.6 reveals no decrease in AMPK activity over time. It is possible that an increase in PP2A might be involved.
Figure 4.7 Developmental changes in ACC activity in rat brain.

6 % PEG pellets were prepared from rat brains of various postnatal ages. ACC activity in PEG pellets was assayed by the transfer of $^{14}$C from NaH$^{14}$CO$_3$ to malonyl-CoA as described in materials and methods (section 2.9) at 10 mM citrate. Activity is expressed as nmol/ min/ g wet weight of tissue (A) and per mg DNA (B). Each value is the mean ± sem. of four determinations.
Figure 4.8  Citrate dependence of Acetyl-CoA carboxylase (ACC) activity in PEG pellets prepared from whole brain.

6% PEG pellets were prepared from rat brains of various postnatal ages. ACC activity in PEG pellets was assayed by the transfer of $^{14}$C from $\text{NaH}^{14}\text{CO}_3$ to malonyl-CoA over a range of citrate concentrations as described in materials and methods (section 2.9). A. Postnatal day 1, each value is the mean of five observations with sem. represented by vertical bars. B. Postnatal day 14, each value is the mean ± sem. of four determinations.
The protein phosphatases responsible for dephosphorylation of AMPK and ACC, PP2C and PP2A, respectively, were assayed over postnatal rat brain development. There was a slight tendency towards a decrease in the activity of PP2A, while PP2C activity remained constant (Figure 4.9). While no change in PP2C activity is consistent with no change in AMPK activity (Figure 4.6), a downward trend in PP2A activity is not consistent with the increased $V_{\text{max}}$ and decreased $K_{s}$ for citrate observed for ACC (Table 4.3).

4.2.4 AMPK and ACC in cultured astrocytes

AMPK activity had not been assayed in astrocytes prior to this study. It was not possible to detect any AMPK activity in a crude extract of cultured astrocytes, so AMPK was partially purified by PEG precipitation and DEAE cellulose chromatography as described in materials and methods (section 2.8.2). AMPK activity was demonstrated in astrocytes both in PEG pellets and in PEG and DEAE purified protein (Figure 4.10). PEG purification gave quite low activity, which showed linear incorporation of $^{32}$P-phosphate over 3 min (Figure 4.10A). The basal rate was $0.17 \pm 0.02$ nmoles $^{32}$P-phosphate into SAMS/min/mg PEG-purified protein, and was only activated 80% by 200 $\mu$M AMP to $0.31 \pm 0.03$ nmoles $^{32}$P-phosphate into SAMS/min/mg PEG-purified protein (Figure 4.10C).

Further purification of the astrocyte enzyme by DEAE cellulose chromatography not only increased the specific AMPK activity (basal activity was $0.61 \pm 0.03$ nmoles $^{32}$P-phosphate incorporated into SAMS/min/mg protein) but also increased its stimulation by AMP to nearly 3-fold activation (Figure 4.10B and 4.10C).
Table 4.3  Changes in kinetic parameters of ACC during rat brain development

Using the Wilkinson Derivation plot on the Enzpak computer program, $K_a$ for citrate and $V_{\text{max}}$ were determined for each age and are presented as means ± sem. for the number of animals shown in brackets.

<table>
<thead>
<tr>
<th>Age/days</th>
<th>$K_a$ (mM)</th>
<th>$V_{\text{max}}$ (nmol $^{14}\text{C}$ into malonyl-CoA/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.63 ± 0.47</td>
<td>0.75 ± 0.05 (5)</td>
</tr>
<tr>
<td>2</td>
<td>2.56 ± 0.52</td>
<td>0.67 ± 0.06 (4)</td>
</tr>
<tr>
<td>4</td>
<td>2.71 ± 0.42</td>
<td>0.70 ± 0.08 (4)</td>
</tr>
<tr>
<td>6</td>
<td>1.64 ± 0.53</td>
<td>0.73 ± 0.16 (4)</td>
</tr>
<tr>
<td>14</td>
<td>1.37 ± 0.21</td>
<td>1.01 ± 0.09 (4)</td>
</tr>
<tr>
<td>21</td>
<td>1.32 ± 0.14</td>
<td>1.04 ± 0.11 (5)</td>
</tr>
</tbody>
</table>
Figure 4.9  Changes in PP2A and PP2C activity during rat brain development

PP2A and PP2C activities were assayed in extracts of rat whole brain at the postnatal ages shown by the dephosphorylation of $^{32}$P-labelled casein phosphate in the presence and absence of OA and of 20 mM MgCl$_2$ as described in Materials and Methods (section 2.7.6). Relative proportions of PP2A:PP2C activity during development to 21 days are shown. Each value is the mean ± sem of the number of determinations shown in brackets.
It has been known for some time that ACC was present in whole brain (Patel and Tonkonow, 1974, Thampy and Koshy, 1991) and especially enriched in oligodendrocytes (Tansey and Cammer, 1988, Moore and Brophy, 1994), the glial cells responsible for myelination. The presence of ACC in astrocytes was unconfirmed. As a positive control for comparison, samples were also prepared from lactating rat mammary gland, which is a rich source of ACC (Mackall and Lane, 1977).

Citrate-dependent ACC activity was evident in PEG pellets and ammonium sulphate precipitates prepared from lactating rat mammary gland (Figure 4.11C, D) but no activity was detectable in samples prepared from astrocytes (Figure 4.11A, B). This is despite the fact that the protein concentrations of the astrocyte samples used in the ACC assay were several-fold higher than those of the mammary gland samples.
AMPK was partially purified from cultured astrocytes by PEG precipitation and DEAE cellulose chromatography, as described in materials and methods (sections 2.7.4, 2.8.2). AMPK activity was measured as $^{32}$P-phosphate incorporation into SAMS peptide in the presence or absence of 200 μM AMP as described in section 2.8.2. Time courses of $^{32}$P-phosphate incorporation into SAMS peptide are shown for AMPK in PEG pellets (A) and following DEAE chromatography (B). AMPK activity as a rate of incorporation is shown (C). Each value is the mean ± sem. of 3 determinations.
A putative substrate of AMPK in astrocytes is the astrocyte-specific GFAP, a major component of the glial cell cytoskeleton. GFAP contains the sequence PGTRLS\(^{58}\)LARMP near its N terminus. This has hydrophobic pro at -5, basic arg at -2, and hydrophobic met and pro at +4 and +5, so that serine-38 is potentially a substrate for AMPK provided this sequence is accessible \textit{in vivo}. This sequence is also one of a number of possible substrates for PKA, which is known to phosphorylate GFAP (McCarthy \textit{et al}, 1985, Mobley and Combs, 1992). A commercially available pure human GFAP sample was incubated with \([\gamma^{-32}P]\) MgATP and either the pure C subunit of PKA from bovine heart or AMPK purified as far as the DEAE step from rat liver. Parallel incubations with purified rat liver ACC (a known substrate for these protein kinases) were run for comparison.

The 265 kDa band of ACC is visible in lanes 1 and 4 (Figure 4.12A) and the 51 kDa band of GFAP is visible in lanes 2 and 5 (Figure 4.12A). The autoradiograph shows that GFAP is heavily phosphorylated by PKA (lane 5, Figure 4.12B) and is clearly a better substrate for PKA than ACC (lane 4, Figure 4.12B). Autophosphorylation of the 41kDa PKA band is also observed in lanes 4 to 6. Phosphorylation of GFAP by AMPK was also apparent (lane 2, Figure 4.12B) and while \(^{32}P\) incorporation may not have been as high as that observed with PKA, it was equivalent to the incorporation of \(^{32}P\) into ACC by AMPK. This may be very significant as ACC is recognised as the best substrate for AMPK.

This preliminary data suggests that GFAP may indeed be a substrate for AMPK. Further work is suggested which includes prior treatment of GFAP with a phosphatase to ensure minimal basal phosphorylation, identification of the phosphorylation site on GFAP, investigation of the specificity of AMPK for GFAP as a substrate compared to other known substrates and identification of GFAP as a substrate compared to other known substrates and identification of GFAP as a target \textit{in vivo} eg. in response to astrocyte treatment with AICAR.

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Figure 4.11 ACC activity in astrocytes.

ACC activity was assayed in 6% PEG pellets (A) and 40% ammonium sulphate precipitates (B) prepared from astrocytes, as described in materials and methods (section 2.9). As a positive control, ACC was also assayed in PEG pellets (C) and ammonium sulphate precipitates (D) prepared from lactating rat mammary gland. Data are the result of a single determination except for C, which shows the mean ± sem. of 3 determinations.
Human GFAP or rat liver ACC were incubated with $[\gamma^{32}\text{P}]-\text{MgATP}$ in the presence of the C subunit of PKA or 200 µM AMP and AMPK purified as far as the DEAE step from liver. After 30 min at 37°C SDS sample buffer was added and 10% SDS PAGE gels run. Coomassie stained (A) and then dried and autoradiographed (B). Lanes: 1-ACC plus AMPK, 2-GFAP plus AMPK, 3-AMPK alone, 4-ACC plus PKA, 5-GFAP plus PKA, 6-PKA alone.
4.3 Discussion

AMPK partially purified from rat brain had a lower activity than that from liver, which could be due to different amounts of contaminating proteins in the two preparations or differences in phosphorylation status of AMPK. The sensitivity of AMPK to activation by AMP at 0.2 mM ATP in the two tissues differs slightly, but not significantly, with $K_a$ for AMP $1.61 \pm 0.41 \mu M$ for the brain and $3.37 \pm 0.57 \mu M$ for liver. It is thought that the $\alpha$ and $\gamma$ subunits govern sensitivity to AMP (Cheung et al, 2000). There is a considerable difference between the subunit composition of AMPK in the two tissues. Brain AMPK contains 75% $\alpha_1$ compared to 50% $\alpha_1$ in liver, brain contains the three $\gamma$ subunits in similar amounts, while liver AMPK contains 90% $\gamma_1$ and no $\gamma_3$ (Cheung et al, 2000). Assay of AMPK complexes containing different combinations of $\alpha$ and $\gamma$ subunits demonstrated that complexes containing $\alpha_2$ were more AMP-sensitive than those containing $\alpha_1$, and complexes containing $\gamma_2$ were more AMP-sensitive than those containing $\gamma_1$ or $\gamma_3$ (Cheung et al, 2000). Brain may be more sensitive to AMP due to its higher content of $\gamma_2$, however it also contains less $\alpha_2$ than liver AMPK.

It is thought that the substrate specificity of AMPK is influenced by its $\alpha$ subunit composition (Woods et al, 1996, Michell et al, 1996). AMPK from liver has equal amounts of $\alpha_1$ and $\alpha_2$, while brain has 75% $\alpha_1$ and only 25% $\alpha_2$. It is possible that AMPK has a different set of substrates in brain than it does in liver, for which a greater proportion of $\alpha_1$ is necessary. The AMPK specificity for its substrate consensus sequence was very similar for brain and liver enzyme, with the unexpected exception that SAMS was a poorer substrate for brain AMPK. The only explanation for this appears to be the different isoform profile of AMPK in the two tissues. Woods et al (1996) report differences in $\alpha_1$ and $\alpha_2$ substrate specificity, but none of the differences they showed can be applied to the set of peptides used in the present study. There has been one report of reduced phosphorylation of SAMS by AMPK $\alpha_2$, Michell et al (1996) reported an increase in $K_m$
for SAMS and a reduced \( V_{\text{max}} \) for the \( \alpha_2 \) isoform compared to \( \alpha_1 \). However, this does not explain the current data as the brain contains less \( \alpha_2 \) than liver AMPK.

AMPK activity is regulated by the AMP/ATP ratio (Corton et al., 1994), which is itself governed in tissues with rapidly fluctuating energy requirements such as brain by the phosphocreatine (PCr)/creatine (Cr) ratio, under the control of creatine kinase (CK) (Wallimann et al., 1992). PCr was found to cause dose-dependent inhibition of both rat liver and rat brain AMPK in the presence or absence of AMP (Figure 4.2). There were differences in the response of the two preparations to PCr in the absence of AMP. Brain AMPK was insensitive to PCr below 50 mM PCr whereas this concentration had already produced a 60% inhibition of liver AMPK. Estimated EC\(_{50}\) for PCr for the liver enzyme was 35 mM and for brain AMPK 75 mM. This difference could be due to the different subunit composition of AMPK in the two tissues, but it is not known which subunit is involved in PCr inhibition. The fact that the difference in response to PCr is abolished by the presence of AMP suggests AMP induces a conformational change in the enzyme which removes these differences.

Ponticos et al. (1998) measured the EC\(_{50}\) for PCr of antibody-purified rat liver AMPK as 20 mM PCr in the presence or absence of AMP, which is a little lower than the present study, but is for a more pure enzyme. Since physiological PCr concentrations in resting brain are reported to range from 4-6 mM (Erecinska and Silver, 1989), it is difficult to see how this allosteric regulation of AMPK by PCr could have any physiological significance unless there is compartmentalisation and localised areas of high PCr concentration.

Protection from ischaemia and hypoxia was afforded to liver cells of transgenic mice (modified to express B-CK in the liver) by the elevated production of PCr generated by CK causing buffering of ATP levels and intracellular pH (Miller et al., 1993). In the normal liver there is no CK and no detectable PCr, and the onset of hypoxia is closely
followed by a drop in ATP. The over-expression of CK in liver produced a basal level of PCr which fell following the onset of ischaemia to zero after 40 min. The ATP level under these conditions fell very slowly and was maintained at above half its normal level for 40 min (Miller et al, 1993). This shows the advantage the brain, which contains B-CK, has in that PCr acts as a buffer, prolonging ATP levels.

AMPK is only activated in pathological conditions when the AMP/ATP ratio rises, which only occurs once PCr has been exhausted. When the tissue begins to recover from, for example, hypoxia, AMPK is active due to the presence of AMP. Recovery will bring a rise in the PCr/Cr ratio. If this occurs while AMP/ATP is still high, according to Figure 4.2C, PCr would be capable of inhibiting AMPK before the AMP level fell, causing inactivation of the enzyme.

Glial cells express far higher levels of B-CK mRNA than neurons (15-fold higher in astrocytes than neurons, and 17-fold higher in oligodendrocytes than neurons, Molloy et al, 1992). Activity of B-CK has been reported to be four-fold higher in oligodendrocytes than astrocytes (Manos et al, 1991). B-CK may have a protective role in astrocytes during hypoxia; treatment of human U87 glioblastoma cells with prostaglandin E1 (PGE1), prostaglandin E2 (PGE2) or cholera toxin (an activator of Go, proteins) increased B-CK transcription in a PKA-dependent manner (Kuzhikandathil and Molloy, 1995). Hypoxia and ischaemia can induce the release of prostaglandins in the CNS (Coceani and Pace-Asciak, 1976) which presumably could lead to increased transcription of B-CK. PGE1 has a cytoprotective effect in normothermic hepatic ischaemia (Helling et al, 1994) and protects rat hippocampal cells against hypoxic injury (Otsuki et al, 1994). Although astrocytes are the major site for release of prostanoids in the CNS (Hartung and Toyka, 1987, Seregi et al, 1987, Murphy et al, 1988), they also have receptors for many prostaglandins including PGE1 and PGE2 (Murphy and Pearce, 1987). These receptors are positively linked to adenylate cyclase, and treatment of astrocytes with PGE1 and PGE2
increases cAMP accumulation (Evans et al, 1987). It is therefore possible in astrocytes that hypoxia or similar stress causes release of PGs that increase B-CK transcription via cAMP-coupled receptors, resulting in increased PCr/Cr ratio that antagonises activated AMPK in a down-regulatory response.

In conclusion brain AMPK has properties in common with liver AMPK such as regulation by phosphorylation and AMP, sensitivity to PCr and substrate specificity, with some subtle differences; $K_a$ for AMP, $EC_{50}$ for PCr, SAMS phosphorylation.

AMPK has not been assayed in separate regions of brain before this study. Other groups have demonstrated AMPK activity in ammonium sulphate pellets of whole rat brain with similar or slightly lower activity than that seen here (Stapleton et al, 1996, Davies et al, 1989). The enzyme is clearly present in all four regions of rat brain at fairly similar activity levels. The activity in sub cortex is significantly higher than that in neocortex or pons ($P<0.05$, Student’s t-test), other comparisons between regions showed no significant difference using Student’s t-test. However, in the presence of AMP the enzyme is activated to the same level in all four regions.

It is possible that some activation of AMPK by phosphorylation may have occurred as a result of hypoxia during isolation of the tissue, causing an increase in the AMP/ATP ratio. In liver, this is avoided by freeze-clamping the tissue in liquid nitrogen when the animal is deeply anaesthetized but the blood is still flowing. Brain cannot be freeze-clamped, but it does contain PCr to protect ATP levels and slow the activation of AMPK. Hypoxia was minimised by rapid dissection in ice-cold buffer followed by freezing in liquid nitrogen as quickly as possible. This was also performed for all ACC analysis where phosphorylation and inactivation by AMPK could be a problem (as it is in dissected rather than freeze-clamped livers).
ACC is a major substrate of AMPK and the greatest ACC activity was in neocortex, followed by subcortex, while cerebellum had the lowest ACC activity. This pattern does not mirror the pattern of AMPK activity as may have been expected. However, it is interesting that a reciprocal pattern was seen in the PKA activity ratio in brain regions, as shown in Figure 5.2C. PKA activity ratio was highest in cerebellum and lowest in neocortex. PKA does cause phosphorylation and some inactivation of ACC in vitro, but not to the same extent as AMPK (Munday et al, 1988a). The activation of PKA by cAMP causes phosphorylation of ACC by AMPK by an unknown mechanism (Haystead et al, 1990b). ACC activity is also influenced by other factors such as citrate concentration and dephosphorylation by PP2A, and the concentration of the enzyme itself. The pattern of ACC activity may simply reflect the amount of ACC protein per gram tissue in each region.

There was little variation in AMPK activity in whole brain during postnatal development. No other developmental study of AMPK exists to compare this data to. It seems unlikely that AMPK would have a specific role during brain development, since it is thought to become active only in pathological conditions such as hypoxia. However, this is a condition that does occur in the neonate, and AMPK is present at an adequate level throughout development to respond to such a condition just as it would respond to stress in the adult animal.

Various studies of ACC activity, expression, and mRNA levels over development have been produced over the years. Gross and Warshaw (1974) measured ACC activity in whole brain supernatants partially purified by filtration through a sephadex G-25 column. ACC activity gradually increased from birth to a maximum at P13, after which it declined. This pattern of ACC activity over rat brain development was the most similar to the data of the present study.
It has been reported that mouse ACC 265 kDa isoform mRNA levels are at a maximum at P5, from which they steadily decrease to 20% of maximum by P20 in mouse brain (Garbay et al., 1997). This is a very similar pattern to that reported by Spencer et al. (1993) for ACC protein expression in rat brain as determined by enzyme-linked immunosorbent assay (ELISA) using 2 different ACC 265-specific antibodies. Enzyme content fell slightly from birth (P1) to P5, then rose to a maximum at P9, after which it rapidly fell from a maximum value of about 0.030 to about 0.003 ng ACC protein/μg total protein (Spencer et al., 1993). Both of the above methods have the limitation that absolute amounts of mRNA and protein are not recorded, only the proportion present compared with total cellular mRNA or protein, and these do not necessarily represent ACC activity.

Tansey and Cammer (1988) reported that four independent studies over 14 years had agreed that ACC activity in rat brain decreased from a maximum at day 10 or earlier. However only one of these studies used whole brain (Gross and Warshaw, 1974, who actually report a maximum at day 13), two studies were in forebrain and brain stem and Patel and Tonkonow (1974) measured ACC activity in rat brain cortex slices. In the latter study the cortical slices were preincubated for 30 min with 20 mM MgCl₂ and 20 mM citrate to measure total activity. The ACC activity assayed by Patel and Tonkonow (1974) was at its maximum at P6 after which it fell to less than half-maximum by P20. This is different from the profile illustrated in Figure 4.7, measured at 10 mM citrate, where after a slight drop between P1 and P2 activity increased to P14 and P21. The maximal rate of myelination in the rat brain is found at day 20 (Norton and Poduslo, 1973), so it would make sense for ACC activity to remain high until this age.

The maximum activity of ACC at saturating citrate concentrations in 6% PEG pellets from rat lactating mammary gland (Figure 4.11C) is approximately 10-fold higher than that seen in 6% PEG pellets from the immature rat whole brain (Figure 4.8). This presumably reflects a lower concentration of ACC protein in brain. Spencer et al. (1993) found 50-100
fold lower levels of ACC polypeptide per μg cytosolic protein in brain tissue than in adult liver.

The protein phosphatases PP2C and PP2A both result in dephosphorylation and activation of ACC; PP2A directly and PP2C via its inactivation of AMPK, which phosphorylates and inactivates ACC. Therefore the increase in ACC activity from P2 to P20 could in part be due to increased PP2C or PP2A. In astrocytes, PP activity increased as the cells developed (see Figure 3.9). However, PP2C did not change over brain development and this parallels the lack of change of AMPK. PP2A activity does not change over development, therefore the increase in ACC activity from P2 to P20 may be due to some other factor such as a change in ACC concentration.

AMPK activity was confirmed in astrocytes. Basal AMPK activity in brain regions varied from 0.21 to 0.33 nmoles $^{32}$P-phosphate incorporated into SAMS/min/mg DEAE purified protein, whereas in astrocytes activity was 0.61 ± 0.03 nmoles $^{32}$P-phosphate incorporated into SAMS/min/mg DEAE purified protein. Both show similar 3 to 4-fold activation by the inclusion of AMP. These data show that AMPK activity is approximately 2.5-fold higher in cultured cortical astrocytes than in the brain as a whole, which is consistent with the role of astrocytes in response to stress in the brain (Norenberg, 1994) and the role of AMPK in the response to stress in other cells (Hardie and Carling, 1997). From these results it does seem plausible that a major site of AMPK expression in the brain is in the astrocytes, and that the enzyme plays a part in the complex response of these cells to injury.

AMPK is also highly expressed in neurons in the developing rat brain according to Culmsee et al (2001) who report that catalytic and noncatalytic subunits of AMPK are present at high levels in embryonic hippocampal neurons in vivo and in cell culture. AICAR protected hippocampal neurons against death induced by glucose deprivation,
chemical hypoxia, and exposure to glutamate and amyloid beta-peptide, suggesting that AMPK can protect neurons against metabolic and excitotoxic insults (Culmsee et al, 2001).

A recent study by Turnley et al, (1999) measured AMPK subunit expression in astrocytes and neurons of each brain region. The cortex was the only region in which they could not measure AMPK isoforms in astrocytes; the present study shows this was not due to an absence of AMPK. The \( \alpha_2 \) subunit was detected at far higher levels throughout the CNS than \( \alpha_1 \), in contrast with the findings of Cheung et al (2000) who reported 75% \( \alpha_1 \) in the brain. These differences may reflect the different antibodies used by the two labs. An important finding of Turnley et al (1999) was that AMPK is up-regulated in activated astrocytes, and that they contain the \( \alpha_2, \beta_2 \) and presumably the \( \gamma_3 \) subunits since they could not detect \( \gamma_1 \) or \( \gamma_2 \), although there may have been a problem with the \( \alpha_1 \) antibody used in this study.

Having ascertained the presence of AMPK in astrocytes, the cells were assayed for one of its known substrates, ACC. In this study it was not possible to detect any ACC activity in cultured astrocytes. However, it is still possible that the enzyme is present, although it may be at a very low concentration or at a very low activation state. Recently, Blázquez et al, (1998) have reported measuring ACC activity in cultured astrocytes, measuring incorporation of [1-\(^{14}\)C]acetyl-CoA into fatty acids in a reaction coupled to the fatty acid synthase reaction (Bijleveld and Geelen, 1987), rather than the classical method of bicarbonate fixation used in the present study.

This group propose that astrocytes are similar to hepatocytes in the following ways: cultured astrocytes are capable of producing ketone bodies at rates similar to those of hepatocytes, (Auestad et al, 1991, Blázquez et al, 1998), under the control of AMPK (Blázquez et al, 1999) ie. in conditions of ischaemia/hypoxia when glucose is limited

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Both astrocytes and hepatocytes show a preference for fatty acids over glucose as their primary metabolic fuel (Edmond, 1992, Zammit, 1994). Control of ketogenesis by carnitine palmitoyltransferase-I (CPT-I) is consistently high in both astrocytes and hepatocytes (Blázquez et al, 1998, Spurway et al, 1997), in fact CPT-I of rat liver and that of rat astrocytes appears to be the same enzyme in that both have a low sensitivity to malonyl-CoA (Brown et al, 1997, McGarry and Brown, 1997) and similar sensitivity to the synthetic inhibitor of CPT-I, TDGA (Guzmán and Geelen, 1992). CPT-I activity relative to total cell protein is similar in liver and astrocytes (Guzmán and Geelen, 1992, Velasco et al, 1997a,b, Blázquez et al, 1998). Thus astrocytes and hepatocytes share an intermediate level of CPT-I activity, between the low activity of lipogenic tissues such as white adipose tissue and lactating mammary gland and the high CPT-I activity of oxidative tissues such as skeletal muscle and heart.

CPT-I is regulated by inhibition by malonyl-CoA, the product of the reaction catalysed by ACC. According to Blázquez et al (1998), the properties of ACC are similar in astrocytes and hepatocytes in two ways: the activity of ACC relative to total cell protein is similar in astrocytes and hepatocytes (Blázquez et al, 1998, Bijleveld and Geelen, 1987, Velasco et al, 1997a), and therefore malonyl-CoA levels are also similar (Blázquez et al, 1998, Guzmán and Geelen, 1992, Guzmán et al, 1995). The ACC isoform pattern in astrocytes (Blázquez et al, 1998) is similar to that in hepatocytes (Bianchi et al, 1990, Guzmán et al, 1995). Both cell types contain predominantly ACC-265; 90-95 % of total astrocyte ACC was ACC-265, and 5-10 % ACC-280, compared to liver which contains 75-80 % ACC-265 (Bianchi et al, 1990, Guzmán et al, 1995). In fact it appears that the ACC isoform profile of astrocytes is closer to that of both neurons (Blázquez et al, 1998) and white adipose tissue (95-100 % ACC 265, Bianchi et al, 1990) than hepatocytes.

The method used by Blázquez et al (1998) to assay ACC in astrocytes was not the classical method used in the present study. The latter method successfully measured
NaH\(^{14}\)CO\(_3\) incorporation into malonyl CoA by ACC in homogenised brain and mammary tissue, and in homogenised primary cultured hepatocytes in our lab, but was unable to measure any ACC activity in homogenised cultured astrocytes. Blázquez et al (1998) used digitonin permeabilisation of astrocytes and thus performed the assay without removing the cells from the plate on which they were grown. This would avoid unnecessary stress to the cells, and may provide the key to why ACC could be detected in astrocytes by the latter method, since conditions of stress cause activation of AMPK and phosphorylation and inactivation of ACC.

Possible substrates of AMPK in astrocytes include ACC, which is implied by the regulation of ketone body production in astrocytes by AMPK shown by Blázquez et al (1999), HMGCoAR, a substrate of liver AMPK which is known to be present in astrocytes (Langan et al, 1987), Raf-1, a substrate of AMPK (Sprenkle et al, 1997) implicated in apoptosis (Wang et al, 1996), a process recently shown to be inhibited by AICAR in astrocytes (Blázquez et al, 2001), and GFAP, which, whose expression is increased in periods of stress, when AMPK is activated, which contains a potential consensus sequence for AMPK (Strausberg, 2001), and appears to be a substrate for AMPK in vitro comparable with ACC.
CHAPTER 5

THE cAMP-DEPENDENT PROTEIN KINASE (PKA) IN RAT BRAIN AND CULTURED ASTROCYTES DURING DEVELOPMENT
5.1 Introduction

Many neurotransmitters exert their biological effects via activation of receptors that generate second messengers. The best known of these second messengers is cAMP, which causes phosphorylation in response to an extracellular signal by its activation of PKA. The structure, regulation and functions of this enzyme are described in detail in section 1.9 of the introduction.

The brain is rich in PKA (Miyamoto et al, 1969, Corbin et al, 1975). Enzyme activity phosphorylating kemptide has been measured throughout the different regions of the brain (Ventra et al, 1996) and expression of PKA protein is found in neurons, astrocytes and oligodendrocytes (Stein et al, 1987). Substrates of PKA in the brain include receptors, ion channels, enzymes and structural proteins (Dudai, 1987). The effects of PKA on CNS receptors occurs at the level of gene transcription and/or translocation of receptors, as in the case of the GABA receptor (Thompson et al, 2000), as well as direct phosphorylation of the receptor protein such as the NMDA and α-2-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) glutamate receptors (Feliciello et al, 1999, Banke et al, 2000). K⁺ channels in neurons are regulated either positively or negatively by PKA phosphorylation, depending on their type, and voltage gated Ca²⁺ channel conductance is increased by PKA phosphorylation (Greengard, 1987). PKA is also implicated in the control of neuronal growth (Beyer and Karolczak, 2000) and glial cell proliferation and differentiation (Bhat et al, 1995), and in the development of long term memory (Linden and Ahn, 1999).

Roles of PKA in astrocytes include phosphorylation of GFAP and vimentin (McCarthy et al, 1985, Mobley and Combs, 1992) Figure 4.12 shows that GFAP is an excellent substrate for PKA; PKA in astrocytes stimulates glycogen turnover (Cambray-Deakin et al, 1988), stimulation of the expression of Na⁺-dependent glutamate/aspartate
transporters, GLUT-1 and GLAST (Schlag et al, 1998) and suppression of iNOS induction (Feinstein et al, 1993).

PKA regulatory subunits are localised in different areas within the cell: the R\textsubscript{I} subunit is primarily cytosolic (Rubin et al, 1972, Corbin et al, 1978), while the R\textsubscript{II} subunits are found in the plasma membrane, cytoskeleton, secretory granules or the nuclear membrane (Corbin et al, 1975, Salvatori et al, 1990, Leiser et al, 1986, Joachim and Schwoch, 1990). R\textsubscript{II} subunits are localised to these structures via AKAPS, thus ensuring that PKA is targeted close to its substrates (Scott and McCartney, 1994). In hypothalamus, cerebellum and brainstem R\textsubscript{II}\alpha and R\textsubscript{II}\alpha mRNA predominates, while in the neocortex and corpus striatum the major component is R\textsubscript{II}\beta (R\textsubscript{II}\beta was not investigated, Ventra et al, 1996). Neurons and astrocytes contain predominantly (more than 80%) R\textsubscript{II} subunits, of mainly the β form while oligodendrocytes contain equal amounts of R\textsubscript{I} and R\textsubscript{II}α (Stein et al, 1987). R\textsubscript{II}\beta protein co-localizes with AKAP150 protein in rat which amplifies the transmission of cAMP signals to the nucleus (Paolillo et al, 1999).

The means of association of C with R\textsubscript{I} and R\textsubscript{II} differ, because R\textsubscript{I} has a pseudosubstrate site, while R\textsubscript{II} has an autophosphorylation site (Hofmann et al, 1975, Walter et al, 1977). Autophosphorylated R\textsubscript{II} has reduced affinity for C, allowing dissociation at a lower concentration of cAMP than R\textsubscript{I} (Hofmann et al, 1975). This means that the PKA activity ratio, a measure of the amount of free C subunits compared to undissociated holoenzyme, for each type may differ, and could be an indication of the isoform composition in a given tissue. Studies of the levels of each subunit during development of rat and human brain revealed no significant changes in activity (Lohmann et al, 1978, Schmidt and Sokoloff, 1973, Schmidt et al, 1980).

PKA containing R\textsubscript{I} is thought to be important in cell differentiation and proliferation,
whilst PKA containing $R_{II}$ appears to be the major enzyme in the developed, non-proliferating cell. Expression of PKA$_{II}$ occurs predominantly in normal, non-proliferating tissues and growth arrested cells, whereas enhanced levels of PKA$_{I}$ are detected steadily in tumour cells and transiently in normal cells exposed to mitogenic stimuli (Cho-Chung et al, 1995). The same group showed that the growth of human neuroblastoma cells was inhibited by over-expression of PKA$_{II}$ (Kim et al, 2000). However, a different group have reported a role for PKA$_{II}$ in cell differentiation of human trophoblast cells in vitro (Keryer et al, 1998a) and in HeLa cells. At the onset of mitosis, cyclin-dependent kinase I (CDK1) phosphorylates $R_{II}\alpha$, and this may alter its subcellular localization (Keryer et al, 1998b). Another isoform-specific function of PKA is memory: using knockout mice, it has been shown that hippocampus-mediated spatial learning and memory functions were associated with type I, while $R_{II}\beta$-knockout mice had defective motor learning (Brandon et al, 1998). This was confirmed by Woo et al (2000) who showed that distinct patterns of synaptic activity induced different forms of hippocampal LTP by recruiting distinct isoforms of PKA.
5.2 Results

5.2.1 PKA activity in four regions of adult rat brain

Figure 5.1A shows a time course of PKA activity in a crude extract of rat neocortex. Linear activity was seen over 12 min incubation. In the presence of 10 μM cAMP the reaction rate was 4.93 ± 0.44 nmoles phosphate incorporated into kemptide/min/mg protein; without cAMP it was 0.99 ± 0.22 nmoles phosphate incorporated into kemptide/min/mg protein. The activity ratio, a measure of the degree of dissociation of the C-subunit, was calculated for this assay by dividing the initial activity by the activity in the presence of added cAMP, to give a value of 0.24 ± 0.01 for the data in Figure 5.1A (an activity ratio of 0.00 would represent the undissociated holoenzyme and 1.00 the fully dissociated subunits). The activity ratio represents the level of PKA dissociation in the tissue upon isolation. The phosphodiesterase inhibitor IBMX was included in all buffers to prevent the destruction of cAMP (Palmer et al, 1980). Published values for the activity ratio of PKA in brain include 0.43 for PKA$_i$ and 0.24 for PKA$_{II}$ (Corbin et al, 1975). Of the tissues studied, liver activity ratios were the closest to brain (0.19 and 0.25 for PKA$_i$ and PKA$_{II}$, respectively), while heart activity ratios were 0.07 for PKA$_i$ and 0.15 for PKA$_{II}$. It is interesting that, according to the same study, brain PKA is mainly type II, and the activity ratio obtained in Figure 5.2 is equivalent to the PKA$_{II}$ activity ratio measured by Corbin et al (1975).

Figure 1B shows the degree of inhibition of PKA by its specific inhibitor PKI. Total kemptide kinase activity (i.e. without PKI) was 3.49 ± 0.06 nmoles phosphate incorporated into kemptide/min/mg protein while the presence of PKI reduced the rate to 0.26 ± 0.08 nmoles phosphate incorporated into kemptide/min/mg protein. Therefore PKI caused a 92% inhibition of rat neo cortical kemptide kinase activity. This reflects the high specificity of PKI as a PKA inhibitor. Thus PKA activity is taken as kemptide kinase activity that is sensitive to PKI.
Figure 5.1 Time course of PKA activity in rat brain neo cortex

PKA activity was assayed in extracts of adult rat neo cortex by the incorporation of $^{32}$P-phosphate into kemptide in the presence or absence of 10 μM cAMP ± 50 units/μl PKI. Incubations were for 12 min at 37°C, as described in materials and methods (section 2.8.1) A. A time course of PKI-sensitive PKA activity in the presence and absence of cAMP is shown B. A time course of PKA activity in the presence of cAMP, showing the inhibition produced by PKI. In both figures, each value is the mean of 4 observations, with sem. represented by vertical bars.
Figure 5.2A shows that the initial PKA activity was similar in each of four brain regions, ranging from $1.01 \pm 0.09$ nmoles phosphate into kemptide/min/mg protein in neocortex to $1.61 \pm 0.01$ nmoles phosphate into kemptide/min/mg protein in the sub cortex. In each case a large activation was produced by the addition of 10 μM cAMP (Figure 5.2B), which was most pronounced in the sub cortex ($5.80 \pm 0.10$ nmoles phosphate into kemptide/min/mg protein) and least in the cerebellum ($2.42 \pm 0.22$ nmoles phosphate into kemptide/min/mg protein). The sub cortex had the highest initial PKA activity and the highest total PKA activity. The PKA activity ratios for pons, neocortex and sub cortex were all comparable ranging from $0.19 \pm 0.40$ (neocortex) to $0.31 \pm 0.01$ (pons) but the PKA activity ratio in cerebellum was significantly higher than in the other regions ($p < 0.05$) (Figure 5.2C). Cerebellum had the lowest total PKA activity but a larger proportion of this activity was dissociated into C subunit compared to other regions.

The cerebellum proved to have a different PKA R subunit isoform composition than the other regions of rat brain. Figure 5.3 shows a typical FPLC trace for each region. Separation over a salt gradient revealed peaks of PKA activity associated with $R_1$ (PKA$_1$) and $R_{II}$ (PKA$_{II}$), which eluted at approximately 0.13 M and 0.27 M NaCl, respectively. Previously published elution profiles for PKA separation report elution from DEAE at about 0.1 M (PKA$_1$) and 0.2 M (PKA$_{II}$) (Corbin et al, 1975, Walter et al, 1978). The area under each peak was taken as a measure of the activity of each isoform. Figure 5.3 clearly shows that in most of the brain, PKA$_{II}$ activity predominates, while in the cerebellum there is a greater proportion of PKA$_1$ (5.3A). The percentage of total PKA activity contributed by each isoform was calculated for each region of the brain (Table 5.1). Cerebellum contained $43.6 \pm 9.5\%$ PKA$_{II}$, but the others contained notably less in the order sub cortex, which contained $20.3 \pm 3.0\%$ PKA$_{II}$, Pons ($15.6 \pm 2.7\%$) and neo cortex ($12.3 \pm 4.1\%$ PKA$_1$). Thus, the majority of PKA activity in the brain is that of PKA$_{II}$, in line with other reports (Corbin et al, 1975, Hofmann et al, 1975).
PKA activity was assayed in extracts of the four regions of rat brain shown by the incorporation of $^{32}$P-phosphate into kemptide in the presence or absence of 10 μM cAMP. Incubations were for 12 min at 37°C, as described in materials and methods (section 2.8.1). Rates of $^{32}$P incorporation are shown in the presence, total activity (B) and absence, initial activity (A) of 10 μM cAMP. The degree of dissociation of the catalytic subunits is illustrated by the ratio of initial : total activity (C). Each value is the mean of 6 observations with sem. represented by vertical bars.
Figure 5.3 Separation of the PKA activity associated with the R₁ and R₉ isoforms in four regions of rat brain.

A crude extract of tissue from adult rat brain was applied to a DEAE column on FPLC, washed and eluted over a gradient of 0 to 0.6 M NaCl, as described in materials and methods, section 2.10. Fractions were collected and assayed for PKA activity in the presence of cAMP (10 μM) as described in materials and methods, section 2.8.1. The activity profile of the fractions is shown for the following regions: A. Cerebellum, B. Pons, C. Neo cortex, D. Sub cortex. The graphs show PKA activity (filled circles) and NaCl gradient (open circles). As only the ratio of PKA₁ and PKA₉ isoform activity was to be calculated, PKA activity is represented as cpm and not converted to nmoles ³²P-phosphate or normalised for protein loaded onto column.
R₁ and R₉ were present in similar proportions in rat cerebellum (Table 5.1). In order to test whether the high activity ratio seen in cerebellum (Figure 5.2C) is a property of the abundance of PKA₁ in this region, the two types of PKA were separated from a sample of rat cerebellum and assayed independently for cAMP sensitivity. The relative sensitivity of PKA₁ and PKA₉ to cAMP was determined by assaying the two peak fractions over a range of cAMP concentrations (Figure 5.4). At lower cAMP concentrations type I had a higher activity ratio than type II, but higher cAMP concentrations produced full activation of both types (Figure 5.4). Based on a range of possible activation from 0 to 1, the ED₅₀ for PKA₁ was about 10⁻¹⁰ M (0.1 nM) and for PKA₉ was about 10⁻⁷ M (0.1 μM). There are two possible causes of the higher activity ratio observed for PKA₁ at lower cAMP concentrations; either the isoform is more sensitive to cAMP, or PKA₁ from cerebellum tends to dissociate more easily for some other reason. The latter explanation seems more likely since in the absence of cAMP the activity ratios were 0.54 ± 0.09 for PKA₁ and 0.13 ± 0.04 for PKA₉. If the ED₅₀ is measured from these points, it makes them both about 10⁻⁷ M (0.1 μM). After elution the PKA holoenzymes were incubated with MgATP, buffer and kemptide for 15 min during the assay. This may be enough to cause dissociation of 50% of the C subunit from PKA₁. Early work on the properties of PKA₁ and PKA₉ from various tissues reported a similar effect. Preincubation for 5 min with the substrate histone resulted in almost complete dissociation of PKA₉, while PKA₉ was unaffected (except in the brain sample which showed some dissociation, but they thought this may be contaminated with C subunit). Preincubation for 5 min in the absence of substrate left PKA₁ less dissociated than PKA₉ (Corbin et al, 1975).

To check whether increased cAMP sensitivity or cAMP-independent dissociation was a common property of PKA₁, the cAMP sensitivity of PKA in a crude extract of rat heart was measured (Figure 5.5). Rat heart contains mainly PKA₁ (Corbin et al, 1975). The rat heart profile resembles the cerebellar PKA₉ profile and has the same ED₅₀ of 10⁻⁷ M
(0.1μM) as both PKA₁ and PKA₁ᵣ from cerebellum (Figure 5.4). To test whether the similarity between cerebellum PKA₁ᵣ and heart PKA₁ is a consequence of partial purification on DEAE, rat heart PKA₁ was separated on DEAE and its cAMP sensitivity was measured (Figure 5.5). The PKA₁ profile obtained was identical to the rat heart crude extract and cerebellum PKA₁ᵣ, therefore the increased cAMP sensitivity/ cAMP-independent dissociation is a property specific to PKA₁ᵣ in cerebellum. The latter is a more likely explanation since PKA₁ activity in cerebellum was 50% dissociated in the absence of cAMP, whereas rat heart PKA₁ was only 18% dissociated.

5.2.2 PKA activity in rat whole brain during development to 21 days

Whole brains from neonatal rat pups were removed at time points from postnatal day 1 (P1) to P21. Some changes were seen in initial PKA activity during the 21 days growth, with the exception of P4 there is a trend of increasing PKA initial activity from 0.45 ± 0.08 nmoles ³²P-phosphate incorporated into kemptide/min/mg protein at P1 to a maximum of 1.11 ± 0.13 nmoles ³²P-phosphate incorporated into kemptide/min/mg protein at P7 then a fall to 0.42 ± 0.06 nmoles ³²P-phosphate incorporated into kemptide/min/mg protein at P21. The activity at P7 was significantly greater than at P1 or P21 (p ≤ 0.0005), suggesting more free C-subunit was present upon isolation at this time (Figure 5.6A).
Table 5.1  Relative proportions of PKA₁ and PKA₂ in four regions of adult rat brain

The elution profiles of PKA activity eluted from DEAE were used to calculate the relative percentage of PKA₁ and PKA₂ in each extract. PKA₁ was defined as eluting between 5 and 11 ml, and PKA₂ between 14 and 20 ml. Percentage isoform composition is shown as means ± sem. for 3 determinations.

<table>
<thead>
<tr>
<th>Rat brain region</th>
<th>% type I</th>
<th>% type II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neocortex</td>
<td>12.3 ± 4.1</td>
<td>87.7 ± 4.1</td>
</tr>
<tr>
<td>Sub cortex</td>
<td>20.3 ± 3.0</td>
<td>79.7 ± 3.0</td>
</tr>
<tr>
<td>Pons</td>
<td>15.6 ± 2.7</td>
<td>84.4 ± 2.7</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>43.8 ± 6.7</td>
<td>56.2 ± 6.7</td>
</tr>
</tbody>
</table>
Figure 5.4  cAMP sensitivity of PKA$_I$ and PKA$_{II}$ from rat cerebellum

A crude extract of adult rat cerebellum was applied to a DEAE column, washed and eluted over a gradient of 0 to 0.6 M NaCl, as described in materials and methods, section 2.10. Fractions containing PKA$_I$ and PKA$_{II}$ were collected and assayed for PKA activity over a range of concentrations of cAMP as described in materials and methods, section 2.8.1. The degree of dissociation of the catalytic subunits for type I and II PKA is illustrated by the ratio of activity measured with and without exogenous cAMP (10 μM). Each value is the mean of 4 observations with sem. represented by vertical bars.
Figure 5.5  cAMP sensitivity of PKA₁ from rat heart

Rat heart contains mainly PKA₁, a crude extract of adult rat heart was assayed for PKA activity over a range of concentrations of cAMP as described in section 2.8.1. The assay was also performed using rat heart PKA₁, partially purified on DEAE as described in section 2.10. The degree of dissociation of the catalytic subunit is illustrated by the activity ratio, measured with and without exogenous cAMP added at the concentration indicated. Each value is the mean of 2 observations.
The total PKA activity showed a similar profile to the initial activity during 21 days growth, rising from P1 to P7 (1.32 ± 0.21 to 2.84 ± 0.28 nmoles phosphate incorporated into kemptide/min/mg protein, respectively, p< 0.001), then falling again to P21 (1.55 ± .020 nmoles phosphate incorporated into kemptide/min/mg protein, p< 0.01). In spite of these changes in total activity there was little change in the activity ratio of PKA during early postnatal development (Figure 5.6C). These remained within a small range (approximately 0.3 to 0.45), with a slight increase from P1 to P2 which was not significant. However there does seem to be a downward trend from P2 to P21. A larger range in activity ratio (0.2 to 0.5) was seen in adult brain regions (Figure 5.2). It appeared from cerebellum that a higher activity ratio corresponded with a greater concentration of PKA\text{I} (Figure 5.2, Table 5.1), perhaps due to cAMP-independent dissociation of the brain/cerebellum PKA\text{I} isoform. The observed downward trend in activity ratio seen during development from P2 to P21 may be due to a decreasing proportion of PKA\text{I}, as shown in Table 5.2.

Relative proportions of PKA\text{I} and PKA\text{II} changed over the 21 days growth. Figure 5.7 and Table 5.2 show that at P1 there was approximately 19% PKA\text{I} and 81% PKA\text{II}, although these are an average of only two sets of data. The proportion of PKA\text{I} increased to 34 ± 5% at day 10, before falling again to just 14 ± 2% at day 21. There may be more PKA\text{I} present during early development when it has a role in cell differentiation and proliferation, whilst PKA\text{II} appears to be the major enzyme in the developed, non-proliferating cell (Cho-Chung et al, 1995), so the proportion of PKA\text{II} may be expected to increase during development.
Figure 5.6 PKA activity in rat whole brain during development to 21 days

PKA activity was assayed in extracts of rat brain at the ages shown by the incorporation of $^{32}$P-phosphate into kemptide in the presence or absence of 10 μM cAMP. Incubations were for 12 min at 37°C, as described in materials and methods (section 2.8.1). Initial PKA activity measured in the absence (A) and total activity measured in the presence (B) of 10 μM cAMP is shown for each age group. The initial : total activity ratio is shown in (C). Values are presented as means ± sem. for the number of animals indicated in brackets.
A crude extract of rat brain was applied to a DEAE column on FPLC, washed and eluted over a gradient of 0 to 0.6 M NaCl, as described in section 2.10. Fractions were collected and assayed for PKA activity as described in section 2.8.1. The activity profile of the fractions is shown for the following ages: A. 1 day, B. 7 day, C. 10 day, D. 14 day, E. 21 day. Each shows a single, representative determination.
Table 5.2  Relative proportions of PKA<sub>i</sub> and PKA<sub>II</sub> in rat brain over development

The elution profiles of PKA activity from DEAE separation were used to calculate the relative percentage of each extract. PKA<sub>i</sub> was defined as eluting between 7 and 11 ml, and PKA<sub>II</sub> in the region between 15 and 21 ml. Percentage isoform composition is shown as means ± sem. for the number of determinations shown in brackets.

<table>
<thead>
<tr>
<th>Age of pups (days)</th>
<th>% type I</th>
<th>% type II</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14, 23</td>
<td>86, 77</td>
</tr>
<tr>
<td>7</td>
<td>22, 38</td>
<td>78, 62</td>
</tr>
<tr>
<td>10</td>
<td>34 ± 5 (3)</td>
<td>66 ± 5 (3)</td>
</tr>
<tr>
<td>14</td>
<td>27 ± 5 (3)</td>
<td>73 ± 5 (3)</td>
</tr>
<tr>
<td>21</td>
<td>14 ± 2 (4)</td>
<td>86 ± 2 (4)</td>
</tr>
</tbody>
</table>
5.2.3 PKA activity in cultured astrocytes

Astrocytes in culture become confluent at about 21 to 28 days in vitro. During this period of growth cells were harvested from each culture at 4 different time points and assayed for PKA activity (Figure 5.8). While there was no significant difference in total PKA activity during this time (Figure 5.8A), initial activity increased significantly from 0.11 ± 0.01 on day 7 to 0.17 ± 0.02 nmoles $^{32}$P-phosphate incorporated into kemptide /min/mg protein on day 10 (p< 0.05). The initial activity then fell to 0.10 ± 0.01 nmoles $^{32}$P-phosphate incorporated into kemptide /min/mg protein at day 14 in culture (p< 0.05), and 0.08 ± 0.02 nmoles $^{32}$P-phosphate incorporated into kemptide/min/mg protein at day 21 (Figure 5.8A). The activity ratio for PKA in astrocytes during growth in culture shows a similar pattern to that of its initial activity over this time (Figure 5.8C). The increase from day 7 to day 10 (0.35 to 0.42) and decrease to day 14 (0.35) were not significant, but the difference between day 10 and day 21 (0.21) is significant (P< 0.05).

The proportion of PKA$_i$ and PKA$_II$ in astrocytes in culture was measured at 3 time points. The proportions of PKA$_i$ to PKA$_II$ at day 7 (37% : 63%) and 14 (32% : 68%) in culture were similar (Figure 5.9). However, after 21 days in culture, when the cells were confluent, the proportion of PKA$_i$ had decreased to 16%. Although the astrocyte separation was only performed for a single culture at each time point, it is interesting to compare the trend in decreasing proportion of PKA$_i$ to the decreasing activity ratio in astrocytes (Figure 5.8). This correlation of higher activity ratio with higher proportions of PKA$_i$ was also seen in whole brain (Figure 5.6C, Table 5.2) and in brain regions (Figure 5.2, Table 5.1). Also interesting is the similarity between the PKA profile in day 21 astrocytes in vitro and the profile in adult neocortex (Figure 5.3C, Table 5.1), the tissue from which the astrocytes were isolated and P1 whole brain (Figure 5.7A, Table 5.2), the tissue from which the cortex was removed for culturing astrocytes. Data from cultured cells cannot be taken as an indicator of events in vivo, but in this case the data
shown from cultured cells confirms what has been shown for whole tissue, suggesting that the proportions of PKA_1 and PKA_II needed for cell proliferation and differentiation are the same whether the cell is growing in vivo or in vitro. Stein et al (1987) measured 82% R_II protein in astrocytes by immunoprecipitation, which is very similar to the 84% PKA_II activity shown in Figure 5.9C. Astrocytes in culture become confluent at about day 18 to 21, so by day 21 they will have stopped proliferating and will be fully differentiated. The enzymes required for those processes will no longer be needed in the same quantities, so the trend seen in the present study of decreasing proportion of PKA_1 with growth appears to support a role for PKA_1 in this process (Cho-Chung et al, 1995, Kim et al, 2000). PKA_II also has a role in cell differentiation (Keryer et al, 1998a,b), but perhaps has more important functions in fully formed and differentiated cells.
Figure 5.8 PKA activity in cultured astrocytes during development to 21 days

Cultured astrocytes were harvested at 7, 10, 14 and 21 days in culture and assayed for PKA activity. The cells from each culture were lysed and the supernatant assayed for PKA activity as described in materials and methods (section 2.8.1). Incubations were for 12 min at 37°C in the presence or absence of 10 μM cAMP ± 50 units/μl PKI. Results are presented as mean reaction rates in the absence (A) or presence (B) of 10 μM cAMP. The activity ratio at each time point was calculated and is shown in C Each value is the mean of 3 observations with sem. represented by vertical bars.
Figure 5.9  Separation of the PKA activity associated with the $R_i$ and $R_{ii}$ isoforms present in cultured cortical astrocytes

A crude extract of cultured cortical astrocytes was applied to a DEAE column on FPLC, washed and eluted over a gradient of 0 to 0.6 M NaCl, as described in materials and methods, section 2.10. Fractions were collected and assayed for PKA activity as described in materials and methods, section 2.8.1. The activity profile of the fractions is shown for the following number of days in culture: A. 7 days, B. 14 days, C. 20 days. Each figure represents a single determination.

A.

B.

C.
5.3 Discussion

PKA is distributed throughout the brain, with variations in activity (Walaas and Greengard, 1984) and isoform expression; R_\alpha and R_\beta are always present in a 1:1 ratio (Ventra et al, 1996), and are the major forms found in cerebellum, hypothalamus, thalamus and hippocampus. R_\beta is the predominant R subunit isoform in the brain, and is found at high levels in the neocortex and corpus striatum and at lower levels in the hypothalamus, hippocampus, basal ganglia, brainstem, forebrain and cerebellum (Ventra et al, 1996, Cadd and McKnight, 1989, Glantz et al, 1992). Approximately 70% of all PKA activity in the brain is associated with the particulate fraction (Erlichman et al, 1990, Lohmann et al, 1980, Stein et al, 1987, Bregman et al, 1989). Astrocytes and neurons both contain over 80% PKA_\beta, while oligodendrocytes contain similar amounts of PKA_\alpha and PKA_\beta (Stein et al, 1987). Primary cultured glial cells, unlike cultured neurons, contain mainly the \alpha forms of R subunit (Massa et al, 1991).

The R_\beta subunits of PKA are membrane bound via AKAPs and MAPs. Distribution of AKAPs follows the same pattern as that of R_\beta, localising them to the dendritic cytoskeleton, golgi apparatus, perikarya and postsynaptic densities, while MAP2 predominantly binds R_\alpha (Cadd and McKnight, 1989, Carr et al, 1992). The co-expression of AKAP150 and R_\beta helps to amplify the cAMP signal to the nucleus, suggesting that the PKA isoform composition of a cell influences its capability to respond to cAMP and to transmit signals to the nucleus (Paolillo et al, 1999). AKAP15 localizes PKA_\beta to brain sodium channels and probably other ion channels.
PKA activity is regulated by other proteins such as PKI, adenylate cyclase and cAMP-specific phosphodiesterase, so the distribution and types of each of these proteins will influence PKA activity. PKI\(\alpha\) is abundantly expressed in the brain, particularly in the cortex, hippocampus and hypothalamus, where it is mainly localised to neuronal cell bodies and dendrites, associated with the nucleus, microtubules and postsynaptic densities, all of these are areas where PKA is known to be localised, while PKI\(\beta\) is expressed in the cerebellum more than in other regions (Seasholtz et al, 1995, de Lecea et al, 1998). cAMP synthesis by adenylate cyclase is activated following agonist stimulation of the \(\beta\)-adrenergic receptor (\(\beta\)AR). PKA phosphorylates the \(\beta\)AR which uncouples adenylate cyclase. Recently, AKAP79/150 has been shown to co-precipitate with the \(\beta\)AR in cell and tissue extracts. The anchoring protein directly and constitutively interacts with the \(\beta\)AR and promotes receptor phosphorylation by PKA following agonist stimulation (Fraser et al, 2000).

PKA activity was found to be most abundant in the cortex and least in the cerebellum (Figure 5.2B). Cerebellar PKA also clearly exhibited a much higher activity ratio than PKA in the other regions (Figure 5.2C): the PKA activity ratio in crude extracts of cerebellum was 0.5 compared to 0.2 to 0.3 in the other regions. Compared to the other regions, cerebellum contained more than double the proportion of PKA activity associated with PKA\(_i\). Thus PKA\(_i\) and PKA\(_n\) were in almost equal proportions in cerebellum (Table 5.1). The observation that
cerebellum had the greatest proportion of PKA in an active state (Figure 5.2) could not have been predicted from its R subunit composition. The R₁ subunit of PKA binds to the C subunit via a pseudosubstrate site, while R₁₁ has an autophosphorylation site. Autophosphorylated R₁ has reduced affinity for C, so is more likely be in an active, dissociated state. Cerebellum contained the lowest proportion of R₁₁, and yet its PKA was the most fully activated on isolation. Corbin et al (1975) reported dissociation and increased activity ratio of PKA₁ but not PKA₁₁ from several different tissues by incubation with the substrate histone for 5 min; in the present study assays were incubated for 15 min in the presence of kemptide. This might suggest that PKA₁ is more easily dissociated independently of cAMP, it appears to be a property of both isoforms of PKA₁₁, since the tissues studied contain both isoforms.

Two possible explanations for the increased activity ratio in cerebellum would be:

1. Cerebellum has a higher cAMP concentration than other areas. This may be true, since Ventra et al (1996) found cerebellar adenylyl cyclase activity to be double that of cortical activity.

2. The activity ratio may be determined by the proportion of PKA₁ : PKA₁₁, which is highest in cerebellum.

Hofmann et al, (1975) reported a greater affinity of type I PKA (from skeletal muscle) for cAMP than type II (bovine heart), Kₜ 0.1 μM and 2.8 μM, respectively
\( K_b \) is the cAMP concentration required to bind 1 mole of cAMP to 1 mole of holoenzyme. However, this was only in the absence of \( \text{MgATP} \); in the presence of \( \text{MgATP} \), PKA was phosphorylated and there was little difference in \( K_b \).

PKA\(_1\) and PKA\(_II\) from cerebellum, separated on DEAE sepharose by FPLC, showed different cAMP dose response relationships (Figure 5.4). The \( K_d \) for cAMP was similar for both, but PKA\(_1\) was already 50\% dissociated after purification on FPLC, in the absence of cAMP (Figure 5.4). Only the R subunits of PKA bind to DEAE, so no free C subunits were bound to the column. No cAMP binds to the column, so the cAMP concentration in cerebellum was no longer a factor to cause dissociation.

To investigate whether incubation with substrate caused dissociation of cerebellar PKA\(_II\), as reported by Corbin et al (1975) for rat heart, or whether DEAE purification caused instability, the same experiment was performed on rat heart, which contains predominantly PKA\(_I\). Rat heart extract showed sensitivity to cAMP and a lack of initial dissociation that was comparable to PKA\(_II\) from cerebellum (Figure 5.5, 5.4). Rat heart PKA\(_I\) purified on DEAE showed a similar cAMP dose-response relationship to the crude extract, proving that neither FPLC purification, nor incubation with substrate causes dissociation of PKA\(_I\); it must therefore be a property of the cerebellar R\(_I\) subunit.

Cadd et al (1990) using recombinant techniques, prepared PKA holoenzymes containing combinations of C\( \alpha \), C\( \beta \), \( R_I \alpha \) and \( R_I \beta \). Holoenzymes containing C\( \beta \) had
similar activation properties to those containing Cα, but the R₁β holoenzyme was
activated at 4-7 fold lower cyclic nucleotide concentrations compared to
holoenzymes containing R₁α. Point mutations made in the pseudosubstrate site of
R₁β reduced its sensitivity to cAMP, but not enough to fully explain the difference.
It is not clear from Cadd et al (1990) whether the PKA holoenzyme activity was
measured in the absence of cyclic nucleotides; in the present study significant
dissociation of PKA₁ was seen in the absence of cAMP. Cerebellum contains R₁α
and R₉α, and some R₉β; R₁β has not been determined (Ventra et al, 1996). Rat
heart must also contain R₁α, since R₁β has been reported only in brain and
developing sperm cells (Clegg and McKnight, 1988). The cAMP-independent
activity of PKA₁ in cerebellum that is not observed for PKA₁ from rat heart may be
explained by a difference in R₁ subunit isoforms, but it is not known whether the
R₁β isoform exists in cerebellum.

Stellation of primary cultured astrocytes induced by cAMP occurred at much lower
cAMP concentrations in astrocytes cultured from cerebellum compared to
increased sensitivity of cerebellar PKA to cAMP-induced dissociation and a low
sensitivity of neocortical PKA to cAMP, which agrees with Figure 5.2C. They
suggest the presence of different isoforms may influence cAMP sensitivity; R₉β is
expressed at a high concentration in neocortex, and is bound to membranes and less
accessible to cAMP (Erlichman et al, 1990, Lohmann et al, 1980, Stein et al, 1987,
Bregman et al, 1989), while the cerebellum contains minimal R₉β and soluble,
cytosolic R₁α and cytoskeletal R₉α (Ventra et al, 1996). The large amount of free
C subunit present from cAMP-independent dissociation of the holoenzyme in the cerebellum may be regulated in part by PKIβ, which is highly expressed in cerebellum (Seasholtz et al., 1995).

The hypothesis that the activity ratio is partly dependent on the proportions of PKA<sub>i</sub>:PKA<sub>II</sub> is supported by the observation that cerebellum has the highest activity ratio and the highest proportion of PKA<sub>i</sub> while neocortex has the lowest of both (Figure 5.2, Table 5.1).

Whole brain PKA activity was variable over the 21 days it was measured (Figure 5.6). There was a general trend of increasing activity from day 1 to a maximum at day 7 followed by a fall to day 21. A similar study using microsomes prepared from rat brain showed no change in total activity from birth to 30 days of age, and a slight increase in initial activity which was not significant (Schmidt and Sokoloff, 1973). However, the basal cAMP level in rat brain increases 4-fold from birth to about day 20 when the adenylate cyclase system matures (Schmidt et al., 1970, Schmidt and Robison, 1972). The initial activity would be greater compared to total activity, which may explain the significant (P < 0.01) fall in activity ratio seen by day 30 (Schmidt and Sokoloff, 1973), which is the trend seen in the present study (Figure 5.6C). A later study using homogenates of rat and human cerebral cortex showed no change in total PKA activity from 3 to 30 months in rat and a slight decrease in human cortical total PKA activity from 2 days to 83 years of age (Schmidt et al., 1980).
The proportion of PKA₁ in whole brain is higher at the earlier time points; by day 21 it is just $14 \pm 2\%$, compared with $34 \pm 5\%$ at day 10. In adult brain, except for the cerebellum, the proportion of PKA₁ ranged from 12% to 20%, so adult values were reached by day 21 of development. This suggests a role for PKA₁ in early postnatal development which is over by day 21, such as in cell differentiation and proliferation (Cho-Chung et al, 1995, Kim et al, 2000).

In rat brain during development, PKA activity was five to tenfold higher than in mature cultured astrocytes (Figure 5.6, 5.8). Astrocytes must contribute to the total brain PKA activity to a relatively small extent compared to other cell types. Figure 5.9 shows the PKA₁ : PKAᵦᵧ proportions in astrocytes after 7, 14 and 21 days in culture. Interestingly, although these are single determinations, and in cells removed from their normal environment, the pattern of PKA₁ : PKAᵦᵧ expression over time in astrocytes reflects that seen in whole brain over time (Table 5.2). Stein et al (1987) report that both astrocytic and neuronal PKA holoenzyme contain more than 80 % $R_\text{I}$ subunit, while oligodendrocytes contain 50-60 % $R_\text{II}$. This agrees with Figure 5.9C for mature astrocytes in culture ($86 \pm 2\%$, day 21). Stein and co-workers used astrocytes isolated from 30 day old rats; the cells were not cultured, so a direct comparison cannot be made. However, similar proportions were seen in whole brain (Table 5.2).

Cultured astrocytes show a connection between $\%R_\text{I}$ and activity ratio; at days 7 and 14 activity ratios were high, 0.35 and 0.43 (Figure 5.8) and $\%R_\text{I}$ was 37 % and 32 %, respectively (Figure 5.9). At day 21, the activity ratio was 0.22 (Figure 5.8).
and the cells contained just 16% Rj. This theory doesn’t appear to work for the whole brain developmental study (Table 5.2); day 10 had the highest proportion of Rj (34%) and day 21 the lowest, 14% Rj, but their activity ratios are very similar (about 0.3). However Cβ1 is produced during P12-18 (Massa et al, 1991). Cβ1 is more sensitive to cAMP than Cα (Gamm et al, 1996), which may increase the activity ratio by day 21, so the two effects could cancel out.

Primary cultures of astrocytes from various brain regions exhibit differing sensitivity to cAMP (Won & Oh, 2000). Stellation induced by 8-CPT-cAMP, a cAMP analogue, was examined by Won and Oh (2000) in six brain regions. At a concentration of 25 μM 8-CPT-cAMP, 88% of cerebellar astrocytes were stellate, while astrocytes from the other brain regions (including cortical astrocytes, as used in the present study), were between 10% and 30% stellate (Won & Oh, 2000). The increased response to cAMP of cerebellar astrocytes may be explained in part by the strong gap-junction coupling found in astrocytes of this region, allowing signals to pass rapidly from cell to cell (Lee et al, 1994). However, the whole cerebellum also shows increased PKA dissociation/activation (Figure 5.2, Ventra et al, 1996). Neurons in this region must also be more sensitive to cAMP, since the cerebellum contains a large proportion of the brain’s neurons, many of which are generated, with the aid of astrocytes, during the first few postnatal weeks (Altman, 1972). The role of astrocytes during neurogenesis (see section 1.2) makes use of their ability to change morphology.
CHAPTER 6

GENERAL CONCLUSIONS
6. Conclusions

The roles of type I and II phosphatases, AMPK and PKA in the brain and astrocytes has been examined. The PPs are ubiquitous enzymes, and as might be expected, were all present in astrocytes. Specific roles of PPs in astrocytes are beginning to emerge (see table 1.1). In the present study a role for PP1 and/or PP2A has been suggested in the dephosphorylation of proteins whose phosphorylation promotes AA release in astrocytes and in the promotion of NA-induced IP accumulation. Changes in total PP activity over 21 days growth in culture suggest that protein phosphatases may be involved in the proliferation and differentiation of astrocytes. Peak activity of PP1, 2A and 2C occurs at about day 10 in culture, the time during which DNA concentration in the cells is increasing most rapidly, i.e. when there is most rapid growth. Further studies could seek to determine the role of OA-sensitive PPs in IP generation, which can lead to the stimulation of many cellular processes via Ca^{2+} release.

AMPK has been successfully demonstrated in rat brain and in cultured astrocytes. Its role in the regulation of fatty acid synthesis and oxidation is likely to be important in the brain, which has a high lipid content and high energy demand. Blázquez et al (1999) demonstrated its role in ketone body production in astrocytes, these function as an alternative fuel for neurons when glucose is limiting. Astrocytes are the principal cell type involved in the CNS response to injury and stress, and as such astrocyte AMPK is likely to play a role in energy conservation under these conditions. AMPK is involved in the regulation of glucose-activated
gene expression by phosphorylation of transcription factors (Russell et al, 1999, Lochhead et al, 2000, Woods et al, 2000). The role of AMPK in astrocytes may include regulation of gene expression, such as production of proteins involved in the astrocytic response to injury. GFAP, for example is expressed in large quantities in reactive astrocytes. AMPK is a possible regulator of its expression, being activated in times of stress. AMPK expression is up-regulated in activated astrocytes (Turnley et al, 1999).

AMPK activity was found at similar levels throughout the brain and changed little during brain development. The activity of ACC, a major substrate of AMPK, was highest in cortex and lowest in cerebellum. During postnatal development ACC activity peaked at day 14. This suggests that both AMPK and ACC have different roles in different areas of the brain and at different stages of brain development. ACC activity was undetectable in cultured astrocytes, but other groups have shown its presence in these cells using other methods (see section 4.3). GFAP was identified as a possible alternative AMPK substrate in astrocytes. There are many possibilities for the roles of AMPK in the brain and in each cell type of the brain, which will provide a fruitful area for future research.

A possible correlation between the proportion of PKA\(_1\):PKA\(_\alpha\) in a tissue or cell type and the activity ratio of PKA has been suggested by the present study. Cerebellar PKA had the highest activity ratio of the brain regions examined and a greater proportion of PKA\(_1\). However, PKA\(_1\) from cerebellum also exhibited significant cAMP-independent activity which was not observed in rat heart PKA\(_1\), suggesting
that the PKA\(_i\) isoform in cerebellum has different properties. A tendency for the PKA activity ratio to decrease through 21 days post-natal brain development and through 21 days astrocyte growth in culture, correlated with a tendency for a decrease in the ratio of PKA\(_i\):PKA\(_{ii}\) activity, providing further evidence for this hypothesis. The next step would be to identify the subunit isoforms of PKA present in the cerebellum and to immunoprecipitate R\(_i\) from cerebellum in order to compare it with R\(_i\) from other tissues.


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