A ROLE OF PROTEIN KINASE C (PKC) AND PHOSPHATIDYLINOSITOL 3-KINASE (PI 3-K) IN MOTONEURON DYSFUNCTION

by

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ABSTRACT

Amyotrophic lateral sclerosis (ALS), also known as motoneuron disease, is a neurodegenerative disorder of humans characterized by motoneuron loss in the spinal cord, brainstem, corticospinal tract neurons and other regions. Human postmortem data from spinal cord tissue of ALS patients revealed a significant increase in the activities of protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI 3-K) compared to control subjects. Protein levels of PKC, PI 3-K, protein kinase B (PKB) and p70 S6 kinase (p70 S6K) were also elevated in spinal cord tissue from ALS patients compared to controls. The activities and protein levels of PI 3-K, PKC, PKB, p70 S6K, Extracellular signal-regulated kinase (Erk)1 and Erk2 were not different in brain regions such as the motor cortex and visual cortex between ALS patients and control subjects. This data indicate that alteration in the activities of PKC and PI 3-K is present in the spinal cord tissue from ALS patients, an area that is pathologically affected in ALS.

One possible pathogenic mechanism of motoneuron death in ALS is the activation of glutamate receptors such as the NMDA receptor. To investigate the role of PKC in NMDA-mediated cell death in HEK 293 cells transfected with NR1A/NR2A subunits of the NMDA receptor, phorbol ester, a PKC agonist was added to the transfected HEK cells. Exposure to phorbol ester significantly augmented NMDA-mediated cell death and this augmentation could be antagonized by a specific PKC inhibitor (RO 320432) in the NR1A/NR2A transfected HEK cells. These data indicate that activation of PKC can potentiate neurotoxicity through NMDA receptor activation.

The pmn/pmn mouse is considered an animal model of motoneuron disease. PI 3-K was significantly reduced in spinal cord tissue of pmn/pmn mice compared to control mice at the age when these mice show significant impairment in locomotion and neurological function. In the cerebellum and brainstem, PI 3-K activities and protein levels were not significantly different between affected and control mice. The neurotrophic factor, BDNF was exogenously applied to these mice and caused a significant increase in PI 3-K activity in spinal cord tissue from both pmn/pmn and control mice, with a greater increase in the pmn/pmn mice. The role of PI 3-K and MEK inhibitors in the retrograde transport of fluorochromes from transected sciatic nerve was studied in pmn/pmn and control mice. The MEK inhibitor PD98059 had no effect on the number of labelled motoneurons in pmn/pmn and control mice. The PI 3-K inhibitor LY 294002 reduced the number of labelled motoneurons in control mice, but had no effect on the labelling of motoneurons in the pmn/pmn mice. Only in the presence of BDNF did LY 294002 inhibit retrograde labelling of motoneurons in the pmn/pmn mice. This data indicated that at least some of the retrograde labelling of motoneuron is PI 3-K-dependent in control mice. In the pmn/pmn mice, the reduced PI 3-K activity requires administration of BDNF to stimulate PI 3-K activity in order to improve motoneuron labelling. Thus, PI 3-K is important in the uptake and/or retrograde transport of substances by motoneurons and PI 3-K activity is altered in mice with motoneuron dysfunction.

Abnormal regulation of PI 3-K and PKC, together with processes such as excitotoxicity, could contribute to motoneuron death in ALS.

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LIST OF ABBREVIATIONS

AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
AMPA	α-amino-3-hydroxy-5-methyl-4 isoxazole proprionic acid
ATP	adenosine triphosphate
BDNF	brain-derived neurotrophic factor
BSA	bovine serum albumin
CNTF	ciliary neurotrophic factor
CPM	counts per minute
DAG	diacylglycerol
DMSO	dimethylsulfoxide
DTT	dithiolthreitol
EAA	excitatory amino acid
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid (disodium salt)
EGF	epidermal growth factor
EGTA	ethylene bis(oxyethylenenitrilo) tetraacetic acid
ERK1/2	extracellular signal regulated protein kinase 1/2
FRAP	FKBP-rapamycin associated protein
GAP	gtpase activating protein
GDNF	glial cell-derived neurotrophic factor
GDP	guanosine diphosphate
GEF	GTP/GDP exchange factor
GTP	guanosine triphosphate
Grb-2	growth receptor binding-2
GSK-3	glycogen synthase kinase-3
HEK	human embryonic kidney
HH1	Histone H1
HSP25/27	25/27 kDa heat shock protein
IGF-1	insulin-like growth factor-1
IP3	inositol (1,4,5) triphosphate

IRS-1	insulin receptor substrate-1
JNK	c-jun amino-terminal kinase
LPS	lipopolysaccharide
μg	microgram
μl	microlitre
μM	micromolar
mg	milligram
ml	millilitre
M	molar
MAPK/ERK	mitogen-activated protein kinase/extracellularly-regulated kinase
MAPKAP	mitogen-activated protein kinase activated protein kinase
MBP	myelin basic protein
MEK	MAPK/ERK kinase
MEKK	MAPK/ERK kinase kinase
MEM	minimum essential medium
MOPS	morpholinopropanesulfonic acid
NF	neurofilament
NGF	nerve growth factor
ng	nanogram
nm	nanometer
NMDA	N-methyl D-aspartate
PAK	p21-associated protein kinase
PAS	protein a sepharose
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
PDK1	3-phosphoinositide-dependent protein kinase-1
PI 3-K	phosphatidylinositol 3-kinase
PH	pleckstrin homology
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PKI	peptide inhibitor of protein kinase A

PKM	protein kinase M
PLCγ	phospholipase C gamma
PMA	phorbol 12-myristate 13-acetate
PMN	progressive motor neuronopathy
PMSF	phenylmethylsulfonyl fluoride
PP2A	protein phosphatase 2A
PS	phosphatidyl serine
PSS	physiological salt solution
rpm	revolutions per minute
SAPK	stress-activated protein kinase
SDS	sodium dodecyl sulphate
SEM	standard error of mean
SH2/SH3	src homology 2/3 domain
SOD	superoxide dismutase
SOS	son-of-sevenless
TBS	tris buffered saline
TOR	target of rapamycin
Tris	tris(hydroxymethyl)aminomethane
TTBS	TBS containing 0.05% Tween 20
Tween 20	polyoxyethylene-20-sorbitan monolaurate

LIST OF AMINO ACID CODES

Name	Three-letter code	One-letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glycine	Gly	G
Glutamic Acid	Glu	E
Glutamine	Gln	Q
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

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DEDICATION

I would like to dedicate this thesis

to my mother, Sientje Radjawane-Liem,
to my father, Nicolaas Radjawane,
and to my husband Tonny Wagey

Their love and encouragement have given me
confidence and strength to achieve my goals in life....

CHAPTER 1. GENERAL INTRODUCTION

1.1. Amyotrophic Lateral Sclerosis (ALS)

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease or motoneuron disease, is characterized by the gradual loss of motoneurons of the brainstem and spinal cord (lower motoneurons) and corticospinal tract neurons and other descending tracts (upper motoneurons) (Eisen and Krieger, 1998). It is a progressive disease typically having an onset starting in midlife, with an earlier onset usually being associated with a genetic factor. The clinical characteristics of ALS include hyperreflexia, muscle wasting and weakness, leading to paralysis and death within 3-5 years. ALS usually begins asymmetrically and distally in one limb, then spreads within the neuraxis to involve the other limb or adjacent segments. Sporadic ALS is more frequent in men, although the numbers of male: female ratio are variable. (reviewed in Eisen and Krieger, 1998).

Epidemiologically, there are three main forms of ALS. The most frequent form is the nonhereditary, so called sporadic form of ALS. Another form of ALS is associated with Parkinsonism and dementia, is found on the islands of Guam and Rota, and may be linked to potential neurotoxin(s) in the local environment. Some reports have proposed a genetic component combined with environmental factors for manifestation of ALS (Bailey-Wilson et al., 1993; Bains and Shaw, 1997; McGeer et al., 1997). The third form of ALS is a familial form, having autosomal dominant inheritance (FALS) which constitutes about 5-10% of all ALS cases. Clinically and pathologically, FALS patients are very similar to those with sporadic ALS. In a small proportion of FALS families, the disease was reported to have linkage to a locus on

chromosome 21q (Siddique et al., 1991). Approximately 15-20% of FALS patients have mutations in the Cu/Zn superoxide dismutase (SOD1) gene (Rosen et al., 1993).

1.1.1. Pathogenesis

ALS is likely a multifactorial disease and many pathogenic mechanisms have been hypothesized for this illness. Four likely mechanisms for the cause of ALS are briefly reviewed here and there is increasing evidence of crosstalk between these pathogenic pathways. The four mechanisms are excitotoxicity, oxidative stress, neurofilament abnormalities and autoimmunity.

i) Excitotoxicity

Excitotoxicity is the term originally introduced by Olney to describe the ability of excitatory amino acids to destroy neurons (Choi, 1992). Excitotoxicity results from imbalance of the release and uptake mechanisms of glutamate and other excitatory amino acids. Motoneurons possess N-methyl D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4 isoxazole proprionic acid (AMPA), kainate and metabotropic glutamate (mGlu) receptors (Eaton et al., 1993). Increased activation of the glutamate NMDA receptor subtype will cause an increase in intracellular calcium possibly leading to motoneuron death. There have been several reports indicating the involvement of abnormal glutamate metabolism in the plasma, cerebrospinal fluid and tissue from ALS patients (reviewed in Eisen and Krieger, 1998). The observation that the maximal velocity of glutamate transport was decreased in the spinal cord, as well as the motor and somatosensory cortices of ALS patients indicates impaired

glutamate reuptake in this disease (Rothstein et al., 1992; 1995). Rothstein and colleagues (1996) further found a loss of glutamate transporters, specifically of the astroglial subtype GLT-1 and the loss of this transporter may result in progressive limb weakness and motoneuron degeneration. Possibly a deficiency in the uptake of glutamate leads to increased extracellular glutamate which may be excitotoxic to motoneurons and other neurons contributing to cell death. The neuronal excitotoxicity leads to increased cytosolic calcium levels, which in turn could activate calcium-dependent enzymes, including proteases, xanthine oxidase and phospholipase A2, which are known to increase the levels of the superoxide anion.

ii) Oxidative stress

Oxidative stress reflects the presence of increased oxygen free radicals causing a disruption in cell homeostasis. Oxidative stress can induce cell death and has been implicated in the pathogenesis of ALS since some FALS patients have mutations in the gene encoding the cytosolic protein Cu/Zn SOD1 (Rosen et al., 1993). More than 60 mutations in the Cu/Zn SOD1 gene have now been identified (Siddique et. al., 1991; Rosen et al., 1993; Nakano et. al., 1994; Pramatarova et al., 1995; Cudkowiwics et al., 1997; Boukaftane et al., 1998). The Cu/Zn SOD enzyme is considered as an antioxidant agent as it detoxifies the superoxide anion to form hydrogen peroxide. Hydrogen peroxide is then converted to water by glutathione peroxidase. Superoxide anion can also be converted to peroxynitrite under normal condition by interacting with nitric oxide (Beckman et al., 1993). Earlier studies showed that the mutation in the SOD1 gene in FALS causes a change in activities of the enzyme (Bowling et al., 1993). However, this change does not occur from a

dominant negative interaction of the mutant and wildtype protein in the SOD1 dimers. The stability of the enzyme itself may be diminished causing a decrease in its half-life (Borchelt et al., 1994). The mechanisms proposed for neuronal death due to a mutation of Cu/Zn SOD1 may be attributed to the gain of a novel property of the enzyme that may cause neurotoxicity. Support for the hypothesis that a novel gain of function for mutant Cu/Zn SOD comes from studies of transgenic mice overexpressing high levels of mutant Cu/Zn SOD1 which develop clinical symptoms (eg. denervation, paralysis) similar to ALS patients (Gurney et al., 1994). Interestingly, the level of SOD1 activity in these mice is higher than normal. The high activity of SOD1 in these animals is unlikely to cause neuronal degeneration since mice overexpressing normal SOD1 do not develop motoneuron disease (Gurney et al., 1994). Other support for the novel gain of function hypothesis is the absence of true null mutations of Cu/Zn SOD1 in FALS.

These data generate hypotheses for the mechanisms causing cell death by the mutant SOD1. First, the instability of the enzyme itself may participate in forming toxic cytoplasmic aggregates. Second, mutations of SOD1 may cause alteration in its substrate affinity leading to formation of high levels of toxic products, such as increased affinity for hydrogen peroxide to generate hydroxyl radicals, or with peroxynitrite resulting in increased nitronium ions, which, in turn, catalyze nitration of tyrosine on neurofilaments and receptor-tyrosine kinase (Beckman et al., 1993; Brown, 1995). Third, the buffering capacity of Cu^{2+} or Zn^{2+} may be reduced due to instability of the mutant enzyme, which could lead to neurotoxocity from these metals, or the mutant enzyme itself and may be cytotoxic due to its decreased ability to detoxify superoxide anions. Some evidence for oxidative damage in ALS includes

increased protein carbonyl groups as markers of oxidative damage in frontal cortex from sporadic ALS patients (Bowling et al., 1993). Shaw and colleagues (1995) also showed that mean protein carbonyl level was increased by 119% in patients with motoneuron disease compared to control subjects. A study by Ferrante et al. (1997) provided both biochemical and histologic evidence for oxidative injury in sporadic and familial ALS.

iii) Neurofilament abnormality.

Neurofilament accumulation in cell bodies and proximal axons was reported by some groups as an early finding in sporadic and familial ALS patients (Hirano et al., 1984; Rouleau et al., 1996). Oxidative damage to neurofilaments due to increased tyrosine nitration could lead to cross linking causing cytoskeletal abnormalities and impaired axonal transport. Both cytoskeletal abnormalities and impaired axonal transport have been associated with ALS (Rouleau et al., 1996; Sasaki and Iwata, 1996). SOD1 is a ubiquitously expressed enzyme, however, the mutations in this enzyme specifically target motoneurons. This motoneuron specificity is discussed further in reviews by Rothstein (1996) and Brown (1995). Compared to other neurons, motoneurons have a larger size, long axons (up to 10,000:1, ratio of axonal length to cell width) and large numbers of neurofilaments. To maintain this special morphology, cytoskeletal proteins are essential and could be critical targets for injury (Brown, 1995). Figlewicz and colleagues (1994) have observed novel mutations in the carboxy domain of the heavy neurofilament protein (NF-H) in sporadic ALS patients which may alter the phosphorylation of the KSP site in the NF-H. It was reported that two other different mutations in the neurofilament heavy chain were

associated with two ALS patients (Julien, 1997). Further evidence of neurofilament abnormalities come from studies in transgenic mice. Mice that overexpress either normal or mutant neurofilaments develop a motoneuron disease phenotype (Cote et al., 1993; Xu et al., 1993), and mice that express modest levels of a point mutation in the neurofilament light chain reproduce aspects of human ALS pathology (skeletal muscle atrophy and abnormal neurofilament accumulation with a selective death of lower motoneurons) (Lee and Cleveland, 1994). As seen in human ALS, the larger motor axons with abundant neurofilaments were lost, while the smaller axons were spared (Cleveland, 1996). All of this evidence leads to the implication of abnormal neurofilament accumulation in the pathogenesis of ALS.

iv) Autoimmunity

A very brief citation of the role of autoimmunity in the pathogenesis of ALS is presented here, since autoimmunity will not be discussed in this thesis. Some studies have proposed that autoimmunity may be associated with ALS (Appel et al., 1994). Inoculation of purified bovine motoneurons into mice results in the presence of IgG immunoreativity within spinal motoneurons, indicating that a retrograde transport of IgG antibody specific to motoneurons had occurred (Englehardt, 1990). When antibodies to calcium channels from ALS patients are passively transferred to mice, the mice are observed to have selectively increased synaptic vesicle density in the presynaptic terminals of motoneurons. Appel and colleagues (1995) have found deposition of immunoglobulin and complement in spinal cord and motor cortex of ALS patients. Other studies have shown that antibodies to muscle L-type voltage-gated calcium channels were present in 38 out of 48 ALS patients (Smith et al.,

1992). However, the significance of the finding of the presence of Ca^{2+}-channel antibodies in a few ALS patients is unclear (Appel et al., 1995; Vincent and Drachman, 1996). The anti-Ca^{2+}-channel antibodies, together with abnormal glutamate metabolism, may contribute to the imbalance in calcium homeostasis, leading to cell death. However, the mechanism by which the antibody could alter calcium homeostasis is still unclear.

1.1.2 Treatment of ALS

Since only specific subpopulations of neurons are targeted in these diseases, the issues of selective vulnerability and the pathways leading to cell death could be important for potential therapeutic approaches. There is currently no effective treatment for ALS. Rothstein (1996) proposed that the slow degeneration of motoneurons in ALS is a multistep process that reflects an initiating event (eg. toxic free radicals), followed by a propagating series of cyclical cascades (eg. excitotoxicity).

The therapeutic approaches that have been applied to ALS include agents that could inhibit the excess release of glutamate, antagonize free radical damage and support neuronal growth, differentiation and survival. Until now, only one drug has been approved by the Food and Drug Administration (FDA) for the treatment of ALS. This drug, riluzole, a glutamate release inhibitor, has improved survival among ALS patients with specific clinical presentations. Survival was only marginally improved in limb-onset cases, whereas ALS patients with the bulbar-onset disease had better survival compared to controls (Bensimon et al., 1994). A recent study has examined the effect of riluzole on high-affinity glutamate uptake measured in rat spinal cord

synaptosome (Azbill et al., 2000). This study showed that riluzole can affect neurotransmission by enhancing the high-affinity glutamate uptake in rat spinal cord synaptosome. Besides riluzole, gabapentin, a glutamate synthesis inhibitor has also been shown to slow the decline in muscle strength (Miller et al., 1996). Antagonists for NMDA and non-NMDA receptors have also been proposed for ALS therapy. Antioxidants proposed for the treatment of ALS include vitamins E and C, β-carotene and N-acetylcysteine. Several growth factors have been tested in preclinical and clinical trials, including ciliary neurotrophic factor (CNTF), brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF) and insulin-like growth factor-1 (IGF-1). Some of these growth factors have failed to achieve improved outcomes during clinical trials. This may be due to aspects such as dose and delivery route. Several clinical trials are under way to test the delivery of BDNF directly into the cerebrospinal fluid to reduce the incidence of side effects due to systemic delivery. IGF-1 is available in the USA under an IND (investigational new drug) treatment protocol. These growth factors have also been tested intensively in different animal models of ALS (Sagot et al., 1997).

Based on current research in ALS, it is likely that motoneuron death in ALS is multifactorial. It is possible that in various patients oxidative injury, excitotoxicity and neurofilament dysfunction have some supporting role in the pathogenesis of the disease.

1.2 Signal transduction

Signals from the extracellular environment are transmitted from cell surface receptors through the plasma membrane to stimulate specific intracellular

biochemical pathways causing a specific response of the cell. The signals that mediate growth, survival, differentiation, proliferation or apoptosis of a cell are important processes that determine the regulation and fate of a cell. Many different signals are integrated which ultimately regulate gene expression through altering the phosphorylation state of various proteins. Reversible protein phosphorylation is a major mechanism of intracellular signaling that modulates a variety of enzyme activities and physiological process such as mitogenesis, cell survival, neurite outgrowth, and intracellular protein trafficking. There exist complex regulatory mechanisms that influence the activation of protein kinases and phosphatases to form a highly integrated network.

Extracellular signals such as growth factors, neurotransmitters and hormones bind to their cell surface receptors and the resulting signal will be transduced via protein signaling cascades. The receptors can associate with their specific effectors through interaction with various molecules such as small guanine nucleotide binding (G) proteins, adapter proteins, serine/threonine- or tyrosine-specific protein kinases, lipid kinases or phospholipases to relay the signal and target specific cellular function. To mediate protein-protein interactions, there are conserved protein modules that contain specific sequences which are recognized by other proteins. The recognition domains contain related sequences of 50-100 amino acids in length which are present in many signaling molecules which then build up a complex network of interacting proteins (Pawson, 1995). Some of these sequences include Src-homology-2 (SH2) and Src-homology-3 (SH3) domains which were found originally in the protein-tyrosine kinase src, a pleckstrin homology (PH) domain originally found in pleckstrin and C2 domains in protein kinase C (PKC). These domains fold into

functional modules independently of the rest of the protein. The SH2 domain recognizes specific phosphotyrosine peptide motifs and SH3 domains interact with proline-rich sequences in their target molecules (Anderson et al., 1990; Ren et al., 1991). PH domains are believed to associate with phospholipids and promote the association of signaling proteins with membranes (Harlan et al., 1994; Chan et al., 1999). C2 domains found in classical and novel PKC isoforms binds both Ca^{2+} and acidic phospholipids (Sutton et al., 1995).

There are different mechanisms of transmembrane signaling which use specific protein-protein interactions to recruit the targeted signaling molecules to an appropriate subcellular location where they interact with specific substrates and downstream effectors. For example, growth factor binding to specific receptor tyrosine kinases induces dimerization of the receptor and tyrosine-cross phosphorylation which in turn recruits binding sites for proteins with SH2, SH3, PH or C2 domains. Then the signal is relayed to its downstream effectors in the cytoplasm to activate a specific cellular response. The signaling pathways of phosphatidylinositol 3-kinase (PI 3-K), protein kinase B (PKB), p70 S6 kinase (p70S6K), protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) will be discussed (see Fig.1).

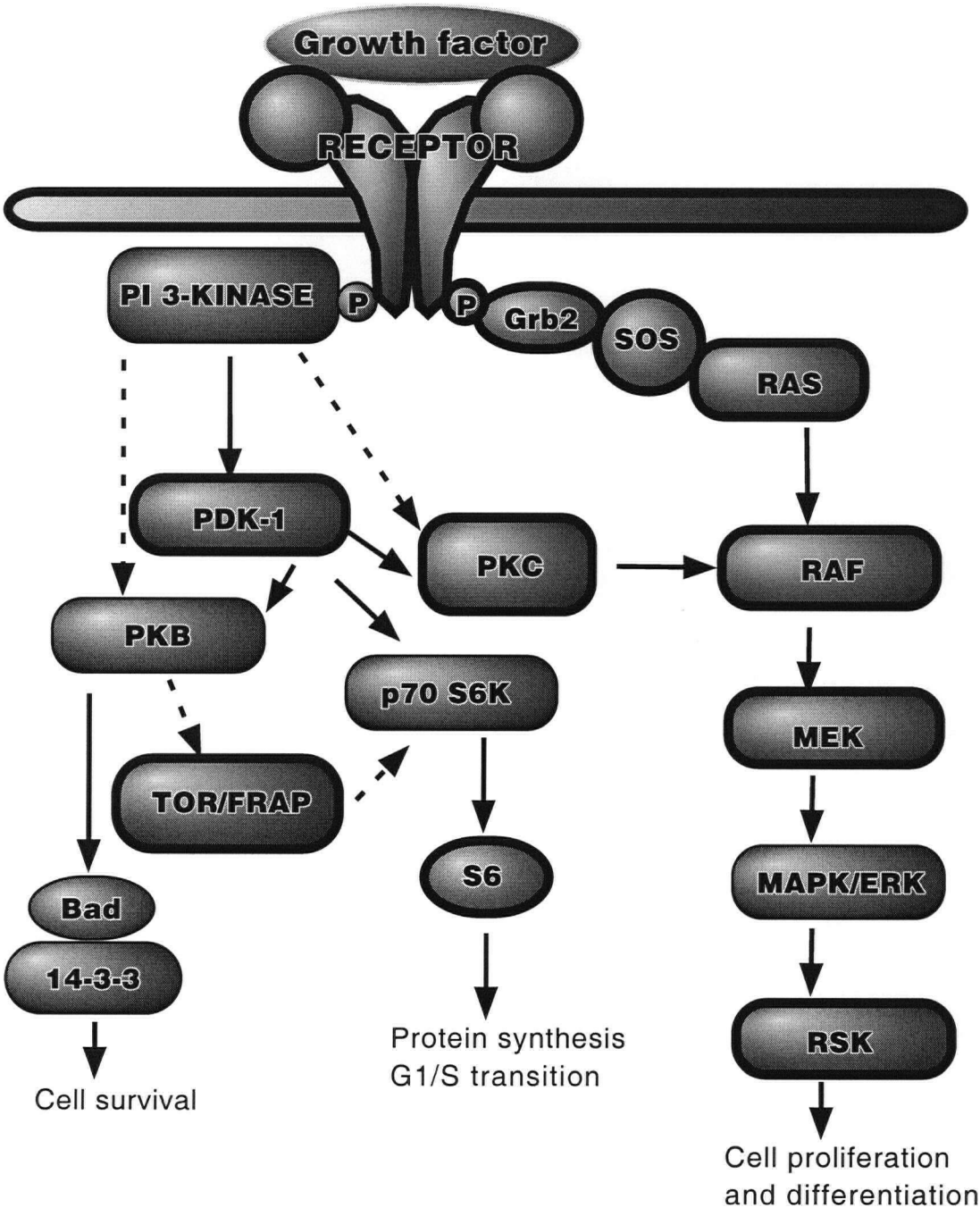

Fig.1. PI 3-K and MAPK signalling pathways. Binding of a growth factor to its receptor could activate various signalling pathways including the activation of PI 3-K, Ras and their downstream effectors.

1.2.1 Phosphatidylinositol 3-kinase (PI 3-K)

Studies of membrane lipids in signaling processes have significantly increased in recent years. Lipids are not only structural components of the membrane but also have important roles as intracellular messengers in cell regulation and survival. One of the more recently discovered lipid signaling pathways is that of a family of lipid kinases, namely PI 3-K. PI 3-K phosphorylates phosphoinositides at the D3 position of their inositol ring forming the lipid products phosphatidylinositol (3)-phosphate (PI 3-P), phosphatidylinositol (3,4)-bisphosphate (PI 3,4-P2) and phosphatidylinositol (3,4,5)-trisphosphate (PI 3,4,5-P3). These lipid products are not substrates for phospholipase C (PLC) and lie along a different pathway from the classical phosphoinositide pathway, which generates inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) (Kapeller and Cantley, 1994). Instead, the lipid products of PI 3-K are substrates for kinases and phosphatases that act on the inositol ring (Woscholski and Parker, 1997; Majerus et al., 1999), such as the recently discovered PI (3,5) P2, a product of the 5' phosphorylation of PI (3)-P (Jones et al., 1999). The phosphatases that target PI 3-K lipid products include the 5'-phosphatases (Stephens et al., 1993) and the 3'-phosphatases (Maehama and Dixon, 1999). SHIP (SH2 domain-containing inositol 5-phosphatase) is one of the members of the inositol polyphosphate 5 phosphatases (Scharenberg and Kinet, 1996; Chan et al., 1999). SHIP dephosphorylates Inositol (1,3,4,5) P4 and PI (3,4,5) P3. One of the 3'phosphatases that was discovered more recently is the tumor suppressor gene product, phosphatase and tensin homologue (PTEN), which converts PI (3,4) P2 to PI (4) P and PI (3,4,5) P3 to PI (4,5) P2 (Kandel and Hay, 1999).

The multiple isoforms of PI 3-K can be classified into 3 classes (Vanhaesebroeck et al., 1997; Vanhaesebroeck and Waterfield, 1999). Class IA is the PI 3-K mammalian enzymes consist of a catalytic subunit and an adapter/ regulatory subunit. In Class IA the catalytic subunits, p110α, p110β and p110δ form heterodimeric complexes with adaptor proteins, namely p85α, p85β and p55γ. The p110 subunits all contain a p85 or p55 binding domain, a ras binding domain, a PI kinase (PIK) domain and a C-terminal catalytic domain. The PIK domain is likely involved in substrate presentation. The p110α and β subunits are expressed ubiquitously, whereas the p110δ is expressed only in hematopoietic cells (Vanhaesebroeck and Waterfield, 1999). The smaller adaptor protein, p55, also binds to the three catalytic subunits. The p55 subunit is homologous to p85 but lacks an SH3 domain, one of the two proline-rich regions and the BH (break point cluster region homology) domain. The p85 subunits of PI 3-K, both have two SH2 domains separated by an inter-SH2 domain region which is also the interaction site of the adaptor to the p110 subunit. The SH2 domains of p85 bind phosphorylated tyrosine residues specifically within a pTyr-x-x-Met sequence. The p85 subunit contains an SH3 domain and a BH on its N-terminus. The BH domain has homology with the GAP (GTPase activating protein) of the BCR gene product (Musacchio et al., 1996). Two proline rich regions flank the BH domain raising the possibility that the p85 SH3 domain could interact with either of these polyproline motifs to cause an allosteric change in the adaptor protein (Kapeller and Cantley, 1994). There are two lipid binding domains present in PI 3-K, namely the FYVE (named after the first four proteins that contain this domain: Fab1p, YOTB, Vac1p, and Early Endosome Antigen 1) and PH domains. The FYVE and PH domains are important for

downstream signaling since they mediate protein-protein interaction. The FYVE domain exclusively binds PI (3)-P whereas the PH domain has a specificity for binding PI (3,4)-P2 and PI (3,4,5)-P3. The structure of these two lipid binding domain has been reviewed in Vanhaesebroeck and Waterfield (1999). Stimulation of almost every receptor that induces tyrosine kinase activity leads to the activation of Class IA PI 3-K. This protein-tyrosine kinase activation could be mediated through receptors with protein-tyrosine kinase activity (PDGF, EGF) or by nonreceptor-tyrosine kinases (JAK or Src family kinases).

Class IB PI 3-K enzymes consists of the catalytic subunit p110γ isoform. The p110γ subunit does not bind p85 and is stimulated by the G protein $\beta\gamma$-subunit. Otherwise the structure of the p110γ isoform is the same as other p110 subunits. It was reported that p110γ associates with the adaptor protein, p101, which does not have any homology with known proteins. The exact mechanism of the coupling of the adapter protein to the p110γ subunit is not clear (Vanhaesebroeck and Waterfield, 1999) . It has been reported that this class of PI 3-K can also responds to activation of the Gα subunit of the G-protein (Stoyanov et al., 1995; Murga et al., 1998). Class IB PI 3-K enzymes are restricted to leukocytes and appear to be present only in mammals.

Class II PI 3-Ks have been found to contain a C2 domain related to those of PKC family members (Virbasius et al., 1996). The mammalian class II enzymes consists of PI 3-K-C2 α, β, and δ isoforms (see Vanhaesebroeck and Waterfield, 1999). PI 3-K-C2 α and β are ubiquitously expressed, whereas the δ isoform is only present in the liver. The C2 domain of this class binds <u>in vitro</u> to phospholipids in a Ca^{2+} -independent manner (MacDougall et al., 1995; Arcaro et al., 1998). There is no

evidence for the binding of class II PI 3-K to any adapter protein, or Ras (Arcaro et al., 1998). Class II PI 3-K can be activated by insulin, EGF, PDGF, integrins and a chemokine called MCP-1 (Zhang et al., 1998; Brown et al., 1999). Unlike the members of class I PI 3-K enzymes which are mainly cytosolic, Class II PI 3-Ks are predominantly associated with the plasma membrane (Arcaro et al., 1998).

Class III PI 3-K enzymes have substrate specificity restricted to PI and are a homologue of the yeast vesicular protein-sorting protein, the Vps34p, the only PI 3-K in yeast. Both in yeast and in mammals, the single catalytic subunit of class III PI 3-Ks exists in a complex with a protein-serine/threonine kinase which is myristoylated at the N-terminal, namely Vps15p in yeast and the p150 in mammals. Not much is currently known about the regulation of this class of PI 3-K (Vanhaesebroeck and Waterfield, 1999).

PI 3-K also possesses intrinsic protein-serine/threonine kinase activity, beside its lipid kinase activity. The autophosphorylation of PI 3-K on its serine residue has been shown to down-regulate the lipid kinase activity of PI 3-K (Carpenter et al., 1993; Dhand et al., 1994).

The discovery of selective inhibitors of PI 3-K has improved our understanding of the function of PI 3-K, as well as the downstream signaling pathways activated by this family of enzymes. Wortmannin, one of the most commonly used PI 3-K inhibitors, is a fungal metabolite that binds covalently to the p110 subunit of PI 3-K and inhibits the enzyme activity at nanomolar concentrations (Yano et al., 1993). Another inhibitor of PI 3-K is LY 294002. This compound acts at micromolar concentrations by competitive inhibition of the ATP binding site of PI 3-K (Vlahos et al., 1994).

The activation of PI 3-K is an important step in receptor mediated signaling pathways. Ligand binding leads to activation of intrinsic receptor-tyrosine kinase activity or recruitment of associated protein-tyrosine kinase molecules. The phosphorylated tyrosine site within a specific consensus sequence of the receptor will serve as a docking site for the p85 subunit through the two SH2 domains. PI 3-K is activated through binding of the two SH2 domains of p85 with tyrosine phosphorylated proteins (Kapeller and Cantley, 1994). By translocating to the membrane, the enzyme is closer to its lipid substrate and the translocation also enables it to interact with GTP-bound Ras. The mechanism of p110γ subunit activation by G protein $\beta\gamma$ is less well understood. However, it was shown that in the presence of the p101 adaptor protein, there is a significant enhancement of the direct stimulation of p110γ by G$\beta\gamma$ (Domin and Waterfield, 1997).

The biological functions of PI 3-K have been discussed in reviews by Vanhaesebroeck and Waterfield (1996; 1999). PI 3-K plays a role in membrane trafficking and reorganization of the actin cytoskeleton (Brown et al., 1995). PI 3-K is important for membrane ruffling (Wennstrom et al., 1994) and actin rearrangement (Wymann et al., 1994). The role of PI 3-K in controlling the cytoskeleton in response to PDGF, insulin or IGF-1 (Wymann et al., 1994; Kotani et al., 1995) may be through its action in regulating the small GTP-binding proteins Rho and Rac (see Duronio et al., 1998). PI 3-K has also been implicated in cellular events such as mitogenesis, cell differentiation, cell survival and neurite out growth (reviewed in Vanhaesebroeck et al., 1996). In the nervous system, PI 3-K activity is important in regulating neurotrophin-mediated survival response in cerebellar, sympathetic, sensory, cortical, and motoneurons (reviewed in Kaplan and Miller, 2000).

The downstream targets of PI 3-K have been growing in numbers within the last five years. The 3-phosphoinositide-dependent kinase-1 (PDK-1) was discovered as a kinase that phosphorylates PKB in a PI 3-K dependent manner. PDK-1 can only phosphorylate Thr-308 of PKB in the presence of PI (3,4,5)-P3 (Alessi et al., 1997; Stokoe et al., 1997). This mechanism will be elaborated further in section 1.3.2 on PKB. PDK-1 is also an activator of the p70 S6K, p90 ribosomal S6 kinase (p90 Rsk) and the ACG family of kinases (PKA, PKC and PKG) (Cheng et al., 1999; Le Good et al., 1998; Chou et al., 1998; Pullen et al., 1998; Alessi et al., 1998; Richards et al., 1999). These kinases are activated through a similar mechanism as the phosphorylation of the equivalent residue Thr-308 of PKB. PI 3-K through the binding of its lipid products to PDK-1 can also activate the PAK (p21 activating kinase), the Tec family kinases (BTK) as well as GEFs (GTP/GDP exchange factor) and GAPs (GTPase activating proteins) for the Rac and ARF GTPases which play a role in vesicular membrane trafficking. There has also been evidence of the role of PI 3-K in the activation of the Erk (extracellularsignal-regulated kinase), a member of the MAPK (mitogen-activated protein kinase) family (reviewed in Vanhaesebroeck and Waterfield, 1999; Toker, 2000). Another downstream kinase in the MAPK family that has been shown to be activated by a membrane-targeted form of PI 3-K is the stress activated protein kinase (SAPK)/JNK, and a dominant negative mutant of PI 3-K can block EGF-induced activation of JNK (Logan et al., 1997).

1.2.2 Protein kinase B (PKB)

The protein-serine/threonine kinase PKB, also known as Rac (related to protein kinase A and protein kinase C), or Akt (cellular homologue of the retroviral

oncogene v-akt), is one of the downstream targets of PI 3-K. In mammalian tissues there are three main isoforms of PKB namely, PKB α, β and γ. PKB consists of a PH domain at its amino-terminus that binds phospholipids, a short glycine rich region, a catalytic domain and a small regulatory carboxy-terminus extension (reviewed in Kandel and Hay, 1999). The three isoforms of PKB have conserved serine and threonine residues (Thr-308 and Ser-473 in PKBα) which play a critical role in the activation of this enzyme. PKB activation is highly dependent on the activation of PI 3-K. Evidence for this originates from observations that both inhibitors of PI 3-K, Wortmannin and LY294002 diminishes PKB activation by growth factors (Burgering et al., 1995; Cross et al., 1995).

Activation of PKB was reported to be regulated by a PI-3-K dependent pathway (Franke et al., 1997). The lipid product of PI 3-K, PI (3,4,5)-P2, binds directly to the PH domain of PKB leading to dimerization and subsequent activation of the enzyme. Upon insulin-stimulation, PKB activity is also regulated by phosphorylation at Thr-308 and Ser-473. Work by Cohen's group indicated that both of these sites need to be phosphorylated to achieve full activation of PKB (Cohen et al., 1997). It has been shown that an intact PH domain which serves as a membrane-target module is required for the activation of PKB and may cause translocation of PKB to the plasma membrane where phosphorylation of PKB would lead to its full activation. The kinases responsible for phosphorylating PKB at Thr-308 and Ser-473 were the 3-phosphoinositide-dependent protein kinase-1 (PDK-1) and PDK-2, respectively (Alessi et al., 1997). PDK-1, a novel PH-domain containing protein, was reported to be a constitutively active kinase. Phosphorylation of PKB at Thr-308 is PI 3-K- dependent which is partly due to binding of PI (3,4,5)-P3 to the PH domain of

PKB, and not to that of PDK-1. PDK-1 will only phosphorylate PI (3,4,5)-P3-bound PKB, indicating that the access of PDK-1 to Thr-308 is normally restricted by the PH domain of PKB. A review by Downward (1998) proposed a model of PKB regulation as follows: The binding of PI (3,4,5)-P3 to the PH domain of PKB would cause PKB to translocate to the plasma membrane and expose the Thr-308 site for phosphorylation by PDK-1. Then Ser-473 of PKB is phosphorylated by PDK-2 which is probably regulated by PI (3,4,5)-P3. After being activated, PKB is believed to be removed from the plasma membrane and translocate to the cytosol and nucleus. The mechanism of phosphorylation of Ser 473 by PDK2 is still unclear. Recent observation by Balendran et al. (1999) showed that PDK-1 also interacts with the carboxy-terminal region of the PKC related kinase (PRK2) through its PIF (PDK-1 interacting fragment) region. This interaction converts PDK1 to act as a kinase that phosphorylates both Thr-308 and Ser-473. Thus, it is possible that PDK-1 can phosphorylate both residues depending on posttranslational conformational change or the interaction with another protein. Another study has showed that phosphorylation of Thr-308 triggers autophosphorylation of Ser-473 indicating that PDK-2 is likely not required for the activation of PKB or that there is probably not a separate PDK-2 for activating PKB (Toker and Newton, 2000; Toker, 1999). However, another group discovered that kinase-active ILK (Integrin-linked kinase) phosphorylates PKB on Ser-473 suggesting that ILK could be PDK-2 (Delcommenne et al., 1998).

PKB can be negatively regulated through several mechanisms (see Kandel and Hay, 1999). First, the PH domain in PKB can act both as a positive and negative regulator of this enzyme. In its inactive form, the PH domain hinders the accessibility

of PDK to phosphorylate Thr-308. Second, translocation of PKB to the nucleus (Andjelkovic et al., 1997) could also sequester this enzyme to prevent it from phosphorylating its targets in the cytosol. However, some of its targets, like forkhead proteins, are nuclear. Third, the action of specific phosphatases such as protein phosphatase 2A (PP2A), SHIP and PTEN could also negatively regulate PKB (reviewed in Kandel and Hay, 1999).

A known downstream target of PKB is glycogen synthase kinase-3 (GSK-3). Upon insulin stimulation, PKB phosphorylates GSK-3 on a serine residue in a PI 3-K-dependent manner (Cross et al., 1995). This event leads to inhibition of GSK-3 activity, resulting in the dephosphorylation of glycogen synthase and activation of several metabolic and gene -regulatory proteins involved in the insulin response. Another important action of PKB is to promote cell survival by inhibiting apoptosis. PKB phosphorylates Bad (a member of the Bcl-2 oncogene family, that promotes cell death) at Ser-136 in vitro and in vivo, and inhibits Bad-mediated death induction (Datta et al., 1997; del Peso et al., 1997). Phosphorylation of Bad leads to association of Bad with the tau form 14-3-3 proteins, thereby inhibiting its interaction with Bcl-xL (the death inhibiting member of the Bcl-2 family) that would promote cell survival (Zha et al., 1996). Another way that PKB could regulate cell death is by phosphorylation of caspase 9 which plays a role in apoptosis (Cardone et al., 1998). Moreover, phosphorylation of the forkhead transcription factor by PKB could also contribute to the prevention of cell death (Brunet et al., 1999; Kops et al., 1999; Guo et al., 1999). It has been shown that overexpression of one member of the forkhead transcription factor, FKHRL1, triggers apoptosis and this cell death could be prevented through FKHRL1 phosphorylation by PKB.

PKB has also been found to synergize with the Raf/Erk pathway to cause transformation of NIH 3T3 cells (Marte et al., 1997). Other downstream effectors of PI 3-K and PKB include a PI 3-K family member, the FKBP (FRAP/TOR), the elongation initiation factor binding protein, and 4E-BP1 (PHAS-1).

1.2.3 S6 Kinase (S6K)

Another target of PI 3-K is the p70 S6 kinase. This enzyme phosphorylates the S6 protein component of the 40S ribosomal subunit in response to various mitogenic stimuli. The p70S6K plays an important role in the progression of cells from the G1 to the S phase of the cell cycle and in cellular growth control mechanisms by regulating protein synthesis (Chung et al., 1992; Chou and Blenis, 1996). S6K also regulates cell size (Montagne et al., 1999) and ribosome biogenesis (reviewed in Dufner and Thomas, 1999).

Two isoforms of S6K, p70 S6K and p85 S6K are generated from the alternative splicing of mRNA arising from the same gene (Reinhard et al., 1994). The p85 S6K isoform is located in the nucleus, whereas, the p70 S6K is predominantly cytoplasmic. The p70 S6K contains a catalytic domain, an amino terminal acidic domain and a C-terminal domain that acts as a pseudosubstrate/autoinhibitory region by binding to the catalytic site (Banerjee et al., 1990). There are four proline-directed, mitogen-induced phosphorylation sites in the pseudosubstrate domain. The regulation and activation of p70S6K is a complex mechanism, requiring multiple phosphorylation events and interaction with other proteins. Pullen et al. (1998) showed that PDK-1 directly phosphorylates p70 S6K at Thr-229 and also phosphorylates PKB at Thr-308. Downward (1998) proposed the following model of

p70 S6K regulation. Proline-directed kinases such as Erk phosphorylate a number of phosphorylation sites at the autoinhibitory domain of p70 S6K. This event would open a conformational inhibition that could result from the interaction of the amino and carboxy-terminal portion of this enzyme. Thr-389 is then phosphorylated by a currently unidentified PI (3,4,5)-P3-dependent kinase. PKB was suggested as the kinase that phosphorylates Thr-389, however evidence for this is controversial (Burgering and Coffer, 1995; Conus et al., 1998). The conformational change and the phosphorylation events are both required to induce a further conformational change that exposes Thr-229 for phosphorylation by PDK1. Phosphorylation of both sites (Thr-229 and Thr-389) leads to strong synergistic activation of p70 S6K activity, as is seen with PKB phosphorylation. Phosphorylation of these sites is PI 3-K dependent and is reversed by a phosphatase indirectly activated by the inhibitor of p70 S6K, rapamycin. The molecular mechanism of activation of S6K was recently reviewed by Dufner and Thomas, (1999).

The immunosuppressant drug, rapamycin, is a bacterial macrolide that blocks the activation of p70 S6K by preventing the phosphorylation of specific sites including Thr-229, Thr-389, Ser-404 and Ser-411 (Ferrari et al., 1993; Pearson et al., 1995). These same sites are also inhibited by wortmannin (Han et al., 1995). One of the main sites that is targeted by rapamycin is Thr-389 (Dennis et al., 1996). It is believed that the inhibitory effect of rapamycin on S6K is produced either by blocking the activation of an upstream kinase of S6K or by activating a protein phosphatase (reviewed in Dufner and Thomas, 1999).

Several studies have implicated p70 S6K as a downstream effector of PI 3-K, as inhibition of PI 3-K also blocked growth-factor induced activation of p70 S6K.

Chung et al. (1994) have reported that mutations of the PDGF receptor which block binding of the regulatory subunit of PI 3-K also prevent activation of p70 S6K in response to PDGF. Furthermore, wortmannin and LY294002 both inhibit the stimulation of p70 S6K by PDGF and insulin (Chung et al., 1994). A study by Ming et al. (1994) demonstrated that p70 S6K/p85 S6K regulation is through a Ras-independent pathway. Despite the fact that mitogen-induced sites contain recognition determinants for proline-directed kinases, Erk did not activate p70 S6K after phosphorylation of these sites in vitro (Mukhopadhyay et al., 1992). Thus, it is well established that p70 S6K activation is distinct from the Ras/Raf/Mek/Erk pathway (Ballou et al., 1991), but involves PI 3-K and the PIK-related kinase, mTOR, as well as PKC and an unidentified proline-directed kinase (Vanhaesebroeck et al., 1997).

1.2.4 Protein Kinase C (PKC)

Protein kinase C family members are implicated in transducing lipid-dependent signals. In the central nervous system, PKC is involved in several physiological functions including desensitization of receptors, release of neurotransmitters, gating of ion channels, induction of gene expression, long-term synaptic changes and long-term potentiation in hippocampus which is a basis for learning and memory (Huang, 1990; Tanaka and Nishizuka, 1994). PKC has been shown to phosphorylate some glutamate receptors of the AMPA and NMDA type (Roche et al., 1994), thereby this enzyme can potentially regulate the EAA receptor. The regulation of the EAA receptor may be crucial in the nervous system in relationship to the possibility that excitotoxicity by PKC can be produced by activation of these receptors.

PKC has a number of distinct isoforms; all of these consist of a single polypeptide chain with one amino-terminal regulatory region and one catalytic region at the carboxy-terminal (reviewed by Newton (1995), Nishizuka (1995), and Ron and Kazanietz (1999)). Cloning studies in the mid-1980's demonstrated that PKC has four conserved domains (Coussens et al., 1986). The C1 domain is the DAG/phorbol ester binding site containing cys-rich motifs which are duplicated in most isoforms. An autoinhibitory pseudosubstrate sequence lies in front of C1. The C2 domain recognizes acidic lipids and in some isoforms this is the Ca^{2+} binding site. The C3 and C4 domains form the ATP- and substrate-binding sites of the kinase core. The regulatory and catalytic portions of PKC are separated by a hinge region that becomes proteolitically unstable when PKC is membrane-bound. Proteolysis of PKC by calpain generates PKM, which is a Ca^{2+}-independent kinase and is constitutively active (Newton, 1995). PKC isoforms have been classified into three subclasses, conventional PKCs (α, $\beta 1$, $\beta 2$ and γ), novel PKCs (δ, ε, θ, and η) and atypical PKCs (ζ, ι and λ). Another related enzyme, termed PKCμ or PKD possesses some similar features of PKC such as regulation by phorbol ester and DAG. However, it differs from the other members of PKC in terms of substrate specificity and its regulation (Johannes et al., 1994; Valverde et al., 1994; Ron and Kazanietz, 1999). Novel "nonkinase" phorbol ester receptors have been identified: the mammalian α and β chimaerins, Ras-GRP and *C. elegans* Unc-13. These receptors are highly expressed in the brain and could have a potential role in neurotransmitter release and regulation of the small GTP binding proteins (reviewed in Ron and Kazanietz, 1999).

The conventional PKC are the best characterized isoforms. The hallmark of this subclass of PKCs is that they contain a Ca^{2+}-binding site in the C2 domain and is

regulated by Ca^{2+}. The conventional types of PKC are thus regulated by DAG, phosphatidylserine and Ca^{2+}. The novel PKC isoforms are similar in structure to conventional PKCs except that their C2 domain does not mediate Ca^{2+} binding and thus this group is regulated by DAG and phosphatidylserine, but not by Ca^{2+}. The atypical PKCs lack the C2 domain and the C1 domain contains only one cys-rich motif. The regulation of this group of PKCs is less understood, although phosphatidylserine and unsaturated fatty acids were found to stimulate members of the atypical PKCs.

The regulation of PKC has been studied extensively the past few years. Beside its regulation by lipid and Ca^{2+} (Nishizuka, 1992; Newton, 1997; Bazzi et al., 1990; Keranen and Newton, 1997), phosphorylation of PKC plays an important role in the activation of this kinase. A model for the regulation of PKC by phosphorylation and localization was proposed by Newton (1997). Her hypothesis is that PKC associates with the cytoskeleton in a conformation that exposes the activation loop. Phosphorylation of PKC by a putative PKC kinase on the exposed activation loop will produce the correct alignment of residues for catalysis, and allow autophosphorylation at two carboxy-terminal positions. Autophosphorylation at the first position locks PKC in a catalytically competent conformation. Autophosphorylation at the second site releases PKC into the cytosol. In 1998, two groups have identified PDK-1 as a kinase that phosphorylates PKC (Le Good et al., 1998; Chou et al., 1998). It turns out that PDK-1 is able to phosphorylate the classical, novel and atypical PKCs (Le Good et al., 1998; Chou et al., 1998; Dutil et al., 1998). PDK-1 phosphorylates PKCα, βII, δ and ζ within the activation loop. This is a critical step in the regulation of PKC, since an inactive PDK-1 when coexpressed

with PKCβII lead to a failure in activating PKC (Dutil et al., 1998). As proposed by Newton (1997), phosphorylation of PKC by PDK-1 is then followed by autophosphorylation of two sites in the C-terminus (for example, Thr-638 and Ser-657 for PKCα) (Bornancin and Parker, 1996; 1997). The first autophosphorylation (Thr-638) step is important for the duration of phosphorylation and the rate of dephosphorylation of PKC. The second autophosphorylation (Ser-657) contributes to lock the fully phosphorylated enzyme in a close conformation to prevent the action of phosphatases (Bornancin and Parker; 1997). Thus, the phosphorylation followed by autophosphorylation of PKC is required for localization of PKC and to reach a state of catalytical competence. A study using mutants of PKCβII has revealed that autophosphorylation of PKCβII plays a role in relocating PKC from the membrane to the cytosol after its activation (Feng and Hannun, 1998).

Tyrosine phosphorylation of PKCδ has also been reported (Li et al., 1996; Song et al., 1998; Konishi et al., 1997). The importance of tyrosine phosphorylation is still unclear, however Nishizuka and colleagues have reported that this event is a response to stress stimuli such as H_2O_2 (Konishi et al., 1997). It has been shown that different PKC isoforms are localized to different cellular compartments where they control different functions (reviewed by Jaken (1996) and Battaini et al (1997)). Therefore, subcellular targeting through protein-protein interactions comes into play, where different PKC-binding proteins have recently been discovered to play a crucial role in serving as an anchor to localize PKC subcellularly. A number of PKC binding proteins have been discovered and reviewed recently by Ron and Kazanietz (1999) and Jaken and Parker (2000). Some of the PKC anchoring proteins include the perinuclear "proteins interacting with C kinase" (PICKs), "receptors for activated C-

kinase" (RACKs), substrates that interact with C kinase (STICKs)"cAMP protein kinase anchoring protein" with M_r 79000 (AKAP 79) and the "PKC substrate-binding proteins" (PSBP). It is proposed that PKC in its active state binds to RACKs or STICKs, whereas the inactive form will bind to either the AKAPs or the protein 14-3-3 (see Ron and Kazanietz (1999)). Thus, after being released into the cytosol, specific targeting of PKC isoforms is believed to be regulated by these anchoring proteins. The interaction of PKC with these anchoring proteins may position the enzyme closer to its substrate and allow for a rapid response to its second messenger, DAG. DAG binding to the C1 domain and PS binding to the C2 domain causes removal of the pseudosubstrate sequence from the active site and converts the enzyme to an active state.

It has been shown that activation of PKC can result from various signaling pathways, such as by the activation of receptor-tyrosine kinases, non-receptor-tyrosine kinases and G protein-coupled receptors which then stimulate either phospholipase C (to produce IP3 and DAG) or phospholipase D (to produce phosphatidic acid and then DAG). The group of Nakanishi (1993) first reported that one of the atypical PKC isoforms (PKC ζ) was activated in vitro by the lipid product PI (3,4,5)-P3 of PI 3-K. This finding was followed by other reports demonstrating that the novel PKC isoforms (δ and ε) were also activated in vitro by 3-phosphorylated inositol phospholipids (Singh et al., 1993;Toker et al., 1994). These results were challenged in a study conducted by Palmer et al. (1995) which demonstrated that activation of some PKC isoforms by the lipid products of PI 3-K showed no specificity in an in vitro condition.

However, some experiments demonstrate a relation between PI 3-K and the activation of PKC in vivo. A study in permeabilized platelets showed that addition of PI (3,4,5)-P3 to the platelets resulted in the phosphorylation of pleckstrin, a major PKC substrate (Zhang et al., 1995). Although the specific isoform of PKC responsible for the phosphorylation could not be determined in this experiment the pattern of pleckstrin phosphorylation was identical to that seen with phorbol ester stimulation. This result indicates that PI 3-K-mediated activation of PKC isoforms may have a parallel function with the classical phospholipase C pathway. Another in vivo experiment by Akimoto and colleagues (1996) demonstrated activation of the atypical PKCλ by EGF or PDGF receptors through PI 3-K pathway supporting the presence of a signaling pathway from receptor-tyrosine kinases to atypical PKCs through PI 3-K. Furthermore, the same group showed that PDGF caused translocation of PKC ε as well as activation of a TPA responsive element which was attributed to the ability of PDGF to activate the PI 3-K pathway. They further investigated the independent activation of PLCγ and the PI 3-K pathway using PDGF receptor add-back mutants to activate PKCε and found that either pathway results in activation of PKCε (Moriya et al., 1996). This data raises the possibility that two separate signaling pathways (Classical PI through PLCγ and PI 3-K pathways) involving different lipid metabolites could activate distinct but overlapping sets of downstream signaling kinases, which can be PKC family members. This evidence indicates that PI 3-K signaling through its lipid products could act as second messenger that may be important for the regulation of some PKC isoforms. A study by Ettinger et al. (1996) showed that PI 3-K associates with the novel PKCδ and PKCε in platelets and in a human haemopoetic cell line. Another report demonstrated an association of PI 3-K with the atypical PKCζ

during IL-2 stimulation of a mouse T cell and suggested that PI 3-K may be phosphorylated by the associated PKCζ (Gómez et al., 1996). The finding that classical PKC are also phosphorylated by PDK-1, has further established a connection between PI 3-K and PKC (Dutil et al., 1998). Thus, activation of the PI 3-K signaling pathway could stimulate different isoforms of PKC.

1.2.5 Mitogen Activated Protein Kinase (MAPK)

The MAPK cascade is a major intracellular signaling network whose family members have the characteristic of being activated by phosphorylation on both threonine and tyrosine residues, located adjacent to proline residues. The features of the MAPK cascade are highly conserved and homologues are found in yeast, *C. elegans*, Drosophila and mammalian cells.

In mammals, the first and best characterized members of the MAPK family are the 44 kDa Erk1 (extracellular-signal-regulated kinase-1) and the 42 kDa Erk2. Erk 1 and Erk 2 are activated following stimulation with mitogens such as EGF and PDGF (Cobb et al., 1991) and are currently known to be activated by over 50 extracellular stimuli (reviewed in Pelech and Charest (1995). Erk1 and Erk2 play a role in cell proliferation, long-term potentiation in neurons and the production of insulin in pancreatic β cells (Lewis et al., 1998; Cobb, 1999). The activation of Erk 1 and Erk2 by growth factor receptors involves the recruitment of Ras activators to the membrane. Once the receptor is activated it will recruit SH-2 -containing adaptor proteins such as Grb-2 (Growth receptor binding-2). Grb-2 can interact with other proteins through two of its SH3-domains (Schlessinger, 1994). One of the proteins containing proline-rich regions that is known to bind Grb-2 is the guanine nucleotide

exchange protein SOS (Son of sevenless). Binding of Grb-2 to SOS results in recruitment of SOS to the membrane. SOS can then interact and activate Ras by catalyizing the release of GDP and the subsequent binding of GTP (Aronheim et al., 1994). An important effector of Ras signaling is the protein-serine/threonine kinase, Raf-1. Binding of Ras to Raf is believed to localize Raf to the plasma membrane, where Raf becomes activated. The precise mechanism of Raf activation is still unclear, however, several lines of evidence have demonstrated that phosphorylation of Raf and interaction of Raf with membrane lipids and the 14-3-3 phosphoserine-binding protein could activate Raf (Marais et al., 1995; Morrison and Cutler, 1997). Src, PKC and a ceramide-activated protein kinase (CAPK) have been shown to activate Raf.

The activation of Raf leads to the phosphorylation and activation of its downstream kinase MAPK/Erk kinase (MEK1/2, also known as MKK1), which in turn activates Erk1 and Erk2 through phosphorylation of tyrosine and threonine residues in a conserved TEY motif (Ahn et al., 1992). Erk1 and Erk2 have potential substrates which include p90 Rsk and MAPKAP kinases (Blenis, 1993; Stokoe et al., 1992). The activated forms of Erk1 and Erk2 translocate to the nucleus where they phosphorylate transcription factors such as Elk-1, leading to the transcription of mitogen-inducible genes, such as c-fos (Cahill et al., 1996; Coffer et al., 1994). The best candidates for a physiological substrate for Erk1 and Erk2 are the various Rsk isoenzymes, a family of ribosomal S6 kinases. The protein-serine/threonine kinases, Rsk1 and Rsk2, are known to be activated during meiotic maturation of oocytes. In somatic cells they show an early response to treatment with growth factors (Sturgill and Wu, 1991; Blenis, 1991). The actual signaling events leading to Ras activation

and stimulation of its downstream targets is more complex than described, as other molecules (such as Shc, SHP2, PI 3-K) may also be involved.

A second subfamily of MAPKs was identified as a kinase that was activated in response to stress factors, such as UV irradiation, osmotic or heat shock and DNA damage. Studies of the regulation of the transcription factor c-Jun led to the discovery of protein kinases that respond to stress stimuli and phosphorylate c-Jun at two serine residues in the amino-terminal region. These kinases are known as c-Jun amino-terminal kinases (JNKs) or stress-activated protein kinases (SAPKs). This pathway is distinct from that of Erk1 and Erk2, since SAPKs are activated by different stimuli than Erk1/2. SAPKs are also activated by cytokines such as tumor necrosis factor-alpha (TNFα) and interleukin-1 (IL-1) (Minden et al., 1994; Sluss et al., 1994). JNK/SAPKs are implicated in cytokine biosynthesis, cell transformation and apoptosis (Kyriakis and Avruch, 1996; English et al., 1999). An analysis of the JNK knockout mice revealed that JNK1 and JNK2 are required for apoptosis in certain areas of the brain during early brain development (Kuan et al., 1999). The upstream kinase that phosphorylates SAPK in a conserved TPY motif is SEK1/MEK4, which in turn is phosphorylated by MEKK1 (Yan et al., 1994; Lin et al., 1995). Ras and Raf do not activate the JNK pathway, whereas the Ras-related GTP-binding protein, Cdc42 and Rac1, have been shown to activate JNK family members (Minden et al., 1995). Recent reports show that a growing number of protein kinases are capable of activating the JNK pathway. These new kinases act upstream of MEK4, including the TGF-β activated kinase (TAK), germinal center kinase (GCK), tumor progression locus-2 (Tpl-2), specific mixed lineage kinase (MLKs) and the p21-activated kinases (PAKs) (Fanger et al., 1997). However, the mechanisms that regulate these kinases

are still poorly understood. The activated form of JNKs translocate to the nucleus where they phosphorylate transcription factors such as c-Jun and ATF2, resulting in gene transcription that mediates growth arrest or apoptosis.

A third member of the MAPK family was cloned from peptide sequences and is a mammalian homologue of a MAPK in yeast which responses to high osmolarity (HOG1). The mammalian counterpart of HOG1, p38, is defined by the sequence TGY for its phosphorylation by its upstream dual specificity kinase (Han et al., 1995). Four p38-like MAPKs have been discovered (Rouse et al., 1994). This family of MAPKs plays a role in cytokine biosynthesis, muscle differentiation and B cell proliferation (Lee et al., 1994; Zhao et al., 1999; Craxton et al., 1999). The p38 family members are activated by a cytokine inducer, lipopolysaccharide (LPS), and by high osmolarity produced by NaCl or sorbitol. There is some overlap in the stimuli that activate the p38 and JNK family members as JNK1 is also activated by hyperosmolarity (Gargova et al., 1994). The p38 MAPKs are activated by MEK 3/6, which may be a downstream target of TAK1 and thousand and one amino acid kinase (TAO) (Robinson and Cobb, 1997) or an indirect target of PAK1 (Fanger et al., 1997). It appears that the regulation of p38 and its upstream kinases is still poorly understood. However, many of the downstream events mediated by the p38 pathway seem to occur via phosphorylation and activation of MAPKAP kinase-2, a direct target of p38 (Pulverer et al., 1991). The MAPKAP kinase-2 is shown to phosphorylate the small heat shock protein (hsp25/hsp27) as well as ATF-2 in the nucleus. Less is known about other isoform of MAPKs such as Erk3, Erk5 and Erk7.

The three major pathways within the MAPK family members reflects a diversity of stimuli that converge on these complex regulatory pathways to selectively mediate physiological events.

1.3 Glutamate receptors

Glutamate is the main excitatory neurotransmitter in the mammalian central nervous system. It plays a role in the formation of neural networks during development and modulates changes in synaptic transmission efficacy which are involved in LTP (Long term potentiation) and LTD (Long term depression) (Bliss and Collingridge, 1993; Dingledine et al., 1999). The glutamate receptors can be classified into ionotropic and metabotropic receptors (Seeburg, 1993; Hollman and Heinemann, 1994). The ionotropic receptors are categorized into three groups based on their agonist specificities: (1) N-methyl-D-aspartate (NMDA) receptors (2) α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors and (3) kainate receptors. The metabotropic glutamate receptors (mGluRs) are coupled to the G proteins and consist of three gene families, mGluR1, mGluR2 and mGluR3.

The common structure for the ionotropic glutamate receptors includes a large extracellular N-terminus domain, four hydrophobic membrane segments (M1-M4) in between which are three transmembrane domains (M2 does not span the membrane, instead it does a hairpin turn within the membrane), and an intracellular C-terminus domain (Hollman et al., 1994). Based on molecular cloning and expression studies, 14 cDNAs were discovered for the iGluRs. There are four cDNAs for AMPA receptor subunits (GluR1-4), 5 for Kainate receptors (GluR5-7, KA1 and KA2) and 5 for NMDA receptor subunits (NR1, NR2A, NR2B, NR2C and NR2D). The NR1 subunit appears

to be essential to form a functional receptor, whereas NR2 subunit plays a regulatory role when co-assembled with the NR1 subunit (Meguro et al., 1992). NMDA receptors are permeable to Na^+, K^+ and Ca^{2+}, whereas AMPA and kainate receptors are mainly permeable to Na^+ and K^+ (reviewed in Hollman and Heinemann, 1994).

1.4 Animal models of neurodegenerative diseases

The rapid advances in molecular biology have provided useful tools such as the ability to produce animal models that reproduce clinical and neuropathological characteristics of neurodegenerative disorders in humans. Animal models may also permit preclinical therapeutic trials which can be helpful to assess drug efficacy and toxicity. In addition, animal models provide opportunities to examine the basis of selective vulnerability, the spatial and temporal evolution of the disease and the mechanism of nerve cell degeneration.

Several animal models have been developed to address aspects of the pathophysiology of ALS. Some models arise from spontaneous mutations such as the progressive motor neuronopathy (pmn) and Wobbler mice, as well as muscle deficient mouse (mdf) (Schmalbruch et al., 1991; Mitsumoto and Pioro, 1995; Blot et al., 1995). Other models have been developed in transgenic mice. These models exhibit diverse clinical symptoms (eg, age of onset, life span) and different responses to pharmacological agents. For example, CNTF administration was effective in slowing down but not arresting disease progression in pmn and wobbler mice. However, a comparable effect has not been observed in transgenic mice overexpressing human mutant SOD-1 (Sagot et al., 1997). Synergistic effects may be observed when applying more than one trophic factor (eg. CNTF and NT-3, BDNF

and CNTF) into Wobbler mice (Mitsumoto and Pioro, 1995). A possible explanation for the differing response of murine models to growth factors is that motoneuron subpopulations may be heterogeneous. The actions and clinical status of neurotrophic factors tried in various animal models of motoneuron disease has been reviewed by Sagot et al., (1997).

Some animal models are transgenic animals. Transgenic animals express a foreign gene, the transgene, that is introduced into the germline of the animal usually by microinjection of DNA into the fertilized eggs of the animal (Rudolph and Mohler, 1999). Transgenic animals could be useful to study the role of a particular gene in the pathogenesis of a disease. Overexpression or deletion of a specific gene can be manipulated in these transgenic animals by using various techniques (reviewed in Shuldiner, 1996). In some patients with FALS, mutations of the SOD1 gene are observed. Thus, transgenic mice can be generated with an identical mutation or with altered expression of this gene to address the role of mutated SOD1 gene in the pathogenesis of ALS (Gurney et al., 1994; Wong et al., 1995). Since abnormal neurofilament accumulation was also a feature found in some ALS patients, transgenic mice which overexpress NF-H or NF-L were developed to investigate the role of these proteins in murine motoneuron disease (Collard et al., 1995; Julien, 1997). The transgenic mice with overexpressed NF-H or NF-L demonstrated abnormal neurofilament accumulation similar to that observed in ALS patients. Thus, animal models can provide useful information to further understand the role of a specific gene in the pathogenesis of a disease.

1.5 Rationale and research objective

1.5.1. Rationale

Autoradiographic studies have indicated that abnormal regulation of NMDA receptors is present in ALS. For instance, NMDA receptor binding is reduced in both dorsal and ventral horns of spinal cords from patients who died with ALS, compared to control subjects, whereas AMPA binding is unchanged in both groups (Allaoua et al., 1992; Krieger et al., 1993; Shaw et al., 1994b). The changes in NMDA binding appear to result from a reduction in receptor number, since no change in receptor affinity was observed (Krieger et al., 1994). PKC could be involved in this process, as it has been shown that NMDA receptor function can be modulated through the activation of PKC (Ben Ari et al., 1992; Markram and Segal, 1992; Chen and Huang, 1991; Raymond et al., 1994; Logan et al., 1999; Zheng et al., 1999). Furthermore, PKC activation also alters neurotransmitter receptor binding to the NMDA receptor channel (Kitamura et al., 1993). The role of PKC in altering NMDA receptor function was studied by exposing autopsied spinal cord sections from control subjects and ALS patients to phorbol ester, a PKC activator, before incubation with [^{3}H] MK-801 (a non-competitive NMDA channel antagonist). Treatment with phorbol ester resulted in increased [^{3}H] MK-801 binding in both ALS and control groups to a similar level and concurrent exposure of spinal cord sections to H7 (an inhibitor that has some specificity towards PKC but could also inhibit PKA and PKG), completely blocked the increase in NMDA binding (Krieger et al., 1993b).

This data indicated that the reduction in the number of neurons in ALS tissue is not the only factor contributing to the loss of NMDA receptor binding observed in ALS spinal cord sections. Phorbol ester application was able to normalize NMDA

receptor density in ALS spinal cord sections indicating the abnormal regulation of NMDA receptor by PKC (Krieger et al., 1993b). These data were interpreted as providing evidence for dysregulation of EAA receptors in ALS which could have been related to aberrant phosphorylation. Protein phosphorylation is one of the most widely used mechanisms for signal transduction in eukaryotic cells (Krebs, 1985). Protein phosphorylation is a reversible process and is achieved by the coordinated actions of protein kinases and protein phosphatases. An imbalance between the actions of kinases and phosphatases could cause impairment in the biological and physiological function of a cell. There has been evidence that links altered protein phosphorylation with diseases including neurodegenerative diseases (Saitoh et al., 1991). An impairment in certain signaling pathways could cause cell death. Thus, it is important to study the role and the regulation of PKC and other protein kinases in the signaling pathway that may be associated with cell survival or cell death in ALS.

1.5.2 Objectives

The objective of this study was to evaluate whether aberrant regulation of certain protein kinases is present in motoneuron dysfunction. Specifically, analysis of PKC, PI 3-K, PKB, p70S6K and MAPK was performed in human postmortem tissue from ALS patients and control subjects. To further study the role of PKC in excitotoxic cell death, a human cell line (HEK 293) that mimics some neuronal properties was transfected with specific subunits of the NMDA receptor. This transfected cell line was further used to evaluate cell death in the presence of specific inhibitors or agonists of PKC. The role of PI 3-K and MAPK in NMDA-mediated cell death was also analysed by applying the inhibitors of these kinases to the transfected cell line.

An animal model of motoneuron disease, the pmn/pmn mouse, was used to establish whether the activity and protein level of PI 3-K, PKB, p70 S6K, Erk1 and Erk2 were altered in neuronal tissues of pmn/pmn mice, compared to controls. Furthermore, the role of PI 3-K and MAPK in the uptake and/or axonal retrograde transport of fluorescent tracers were studied in the affected mice compared to normals. The effect of exogenous BDNF on the uptake or retrograde transport of fluorochromes was also analysed in the pmn/pmn and control mice. The information obtained from these three different approaches can be used to establish the role of some signaling molecules in motoneuron dysfunction. The ultimate goal of this work is to provide information on the pathogenesis of ALS.

The work discussed in this thesis has been published in:

Krieger C., Wagey R., Lanius R., Shaw C.A. (1993) Activation of PKC reverses apparent NMDA receptor reduction in ALS. *Neuroreport* 4: 931-934

Krieger C., Wagey R., Shaw C. (1993) Amyotrophic lateral sclerosis: quantitative autoradiography of [^{3}H]MK-801/NMDA binding sites in spinal cord. *Neurosci Lett* 159: 191 -194.

Lanius R.A., Paddon H.B., Mezei M., Wagey R., Krieger C., Pelech S.L. and Shaw C.A. (1995) A role for amplified protein kinase C activity in the pathogenesis of ALS. *J. Neurochem.* 63: 927-930.

Wagey R., Krieger C., and Shaw C.A. (1997). Abnormal dephosphorylation effect on NMDA receptor regulation in ALS spinal cord. *Neurobiol. Disease* 4: 350-355.

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Wagey R., Pelech S.L., Krieger C. (1999) The validity of protein and lipid kinase measurement in ALS. Abstract, 10th International Symposium on ALS, November, 1999, Vancouver, Canada. p.148.

CHAPTER 2. MATERIALS AND METHODS

2.1 General Materials

2.1.1. Chemical reagents

Acetic acid	Fisher
Acrylamide	ICN/Fisher
Adenosine 5'-triphosphate disodium salt (ATP)	Sigma
Agar	Difco-Fisher/VWR
Ampicillin (D[-]-α-aminobenzylpenicillin)	Sigma
[γ-^{32}P]ATP	Amersham/Mandel/ICN
Bactotryptone	Fisher/VWR
Bactoyeast extract	Fisher/VWR
Bis-acrylamide	ICN/Fisher
Bovine serum albumin (BSA)	Sigma
5-Bromo-4-chloro-3-indoyl phosphate (BCIP)	Sigma
Bromophenol blue	ICN
Chloroform	Fisher
Coomassie Brilliant Blue G	Aldrich
Coomassie Brilliant Blue R-250	Fisher
5,7-dichlorokynurenic acid	Sigma
Dithiothreitol (DTT)	BDH
Enhanced chemiluminescence kit (ECL)	Amersham
Ethanol	Fisher
Ethylene bis (oxyethylenenitrilo) tetraacetic acid (EGTA)	Fisher/ICN
Ethylene diamine tetraacetate disodium salt (EDTA)	Fisher/ICN
Fetal Bovine Serum	Gibco BRL
Formaldehyde	Gibco BRL
Formamide	Gibco BRL
Glutamine	Gibco BRL
Glutaraldehyde	BDH

Glycerol	Anachemia
β-Glycerophosphate	ICN/Fisher
Glycine	ICN/Fisher
Histones H1	Sigma
Hydrochloric acid (HCl)	Fisher
Lipofectamine	Gibco
LY 294002	Calbiochem
Lysozyme	Sigma
Magnesium chloride ($MgCl_2 \cdot 6H_2O$)	Fisher
Manganous chloride ($MnCl_2 \cdot 4H_2O$)	Fisher
β-Mercaptoethanol	Fisher
Methanol	Fisher
Minimum Essential Medium (MEM)	Gibco BRL
MonoQ	Pharmacia
MOPS 3-[N-Morpholino]ethanesulfonic acid	Sigma/ICN
Myelin basic protein	Kinetek/Sigma
Nitric acid	Fisher
Nonidet P-40 (NP-40)	BDH
N-methyl-D-aspartate	Sigma
Opti-MEM	Gibco/BRL
PD 98059	Gibco/BRL
Penicillin-streptomycin stock	Gibco/BRL
Phenol	ICN
Phenyl methylsulphonyl fluoride (PMSF)	Sigma
Phorbol 12-myristate 13-acetate	Sigma
Phosphate buffered saline (PBS)	Gibco/BRL
Phosphoric acid (H_3PO_4)	Fisher
PKI - cAMP-dependent protein kinase inhibitor peptide	Sigma
Poly-D-lysine	Sigma
Ponceau S concentrate	Sigma
Potassium dichromate ($K_2Cr_2O_7$)	BDH/VWR
Potassium dihydrogen orthophosphate (KH_2PO_4)	BDH/VWR

Potassium ferricyanide	Sigma
Potassium ferrocyanide	Sigma
di-Potassium-hydrogen-orthophosphate3-hydrate (K_2HPO_4)	BDH/VWR
Prestained SDS-PAGE standards	BioRad/Kinetek
Protamine chloride	Sigma
Protein A Sepharose CL4B	Pharmacia
Pesudo A, Pseudo Z	Quality control Biochem.
Qiagen kits	Qiagen Inc.
RO 32-0432	Calbiochem
Silver nitrate ($AgNO_3$)	Fisher
Skim milk	Safeway
Sodium acetate (NaOAc)	BDH/VWR/Fisher
Sodium azide	Fisher
Sodium carbonate (Na_2CO_3)	BDH/VWR
Sodium chloride (NaCl)	Fisher
Sodium dodecylsulphate (SDS)	Fisher
Sodium fluoride (NaF)	BDH/VWR/Fisher
Sodium hydroxide	Fisher
Sodium orthovanadate (Na_3VO_4)	Fisher
Sodium pyruvate	Gibco
Tris (hydroxylmethyl) methylamine (Tris)	Fisher
Tris hydroxylmethyl aminomethane hydrochloride (Tris-HCl)	Fisher
Tween-20 (polyoxyethylene-20-sorbitan monolaurate)	Fisher
Trypsin	Fisher
Wortmannin	Gibco
X-gal	Calbiochem
Xylene cyanol	Gibco BRL

2.1.2 Laboratory supplies

Nitrocellulose	Gelman Sciences/VWR
P81 filter paper	VWR
3MM filter paper	VWR

2.1.3 Photography supplies

Developer and fixer	Medtec
Reflection autoradiography film	Mandel

2.1.4 Plasmids and bacterial strains

cDNA NR1A, NR1C	Gift from S. Nakanishi
cDNA NR2A	Gift from M. Mishina
pCMVβ	Clontech lab
pRK5 plasmid	Stratagene
DH5α bacteria	Pharmacia

2.1.5 General Antibody reagents.

4G10 antiphosphotyrosine antibody	UBI
Goat anti-rabbit IgG - horse radish peroxidase conjugate	Calbiochem
Goat anti-mouse IgG - horse radish peroxidase conjugate	BioRad

2.1.6. Human postmortem tissue

For experiments in chapter 3.1, human spinal cords (cervical segments) were obtained at autopsy from 14 patients diagnosed with ALS (mean ± SE, age 69.8 ± 2.8 years; mean ± SE, death to freezing interval 11.4 ± 3.1 h) and 14 subjects who did not have neurological disease (mean ± SE, age 69.9 ± 3.8 years; mean ± SE, death to freezing interval 13.8 ± 3.2 h). Human motor and visual cortex was obtained at autopsy from 4 patients who had been diagnosed with ALS (motor and visual cortex:

mean ± SE, age 71.7 ± 4.3 years; mean ± SE, death to freezing interval, 8.2 ± 0.9 h); and 4 subjects who did not have neurological disease (motor cortex: mean ± SE, age 70 ± 6.8 years; mean ± SE, death to freezing interval 7.9 ± 3.4 h; visual cortex: mean ± SE, age 49 ± 4.9 years; mean ± SE, death to freezing interval 19.6 ± 7.7 h). The causes of death for control subjects were myocardial infarction, cancer, pancreatitis, diabetes and bronchopneumonia. Spinal cord and brain tissue was stored at -70°C until analysis. Platelets and leukocytes were taken from 3 patients with ALS (mean ± SE, age 65 ± 4.9 years) and 3 healthy controls (mean ± SE, age 79 ± 5 years) and extracted as described by Baenzinger and Majerus (1974).

To obtain protein, platelets, leukocytes, spinal cord or brain tissue were placed in 1.5 mL homogenization buffer (75 mM sodium-β-D-glycerophosphate, 20 mM MOPS, 15 mM EGTA, 2 mM EDTA, 1 mM Na_3VO_4), homogenized by three 30-s strokes with a sonicator, and centrifuged at 70 000 rpm in a Beckman TL 100 centrifuge for 15 min at 4°C to obtain the cytosolic fraction. The supernatant was quickly frozen and stored at -70°C. One ml of homogenization buffer containing 1% Nonidet P-40 was added to each pellet which was resuspended at 4°C after three 30-s treatments with an ultrasonic probe. The resuspended material was centrifuged at 70,000 rpm for 15 min and the supernatant (solubilized particulate fraction) was quickly frozen at -70°C.

For experiments in chapter 3.2, human postmortem spinal cord tissues (restricted to cervical levels including dorsal and ventral regions) were obtained from 8 patients with sporadic ALS (mean age ± SE, 69.5 ±1.8 years; mean death to freezing interval ± SE, 10.5± 2.4 h) and 8 control subjects (mean age ±SE, 73.1 ± 5.1 years; mean death to freezing interval ± SE, 10.1± 2.0 h). None of the patients had

evidence of a familial form of ALS. Human visual cortex was obtained from 6 ALS patients (mean age ± SE, 73.0 ± 2.0 years; mean death to freezing interval ± SE, 9.8± 1.4 h) and 6 control subjects (mean age ± SE, 64.3 ± 3.9 years; mean death to freezing interval ± SE, 11.7 ± 2.1 h). Motor cortex tissue was obtained from 5 ALS patients (mean age ± SE, 72.6 ± 2.4 years; mean death to freezing interval ± SE, 8.8± 0.9 h) and 5 control subjects (mean age ± SE, 65.8 ± 5.1 years; mean death to freezing interval ± SE, 7.4 ± 2.7 h), respectively. Motor cortex tissue was also obtained from 3 patients with Alzheimer's disease (AD) (mean age ± SE, 77.3 ± 1.4 years; mean death to freezing interval ± SE, 5.3 ± 0.3 h) and 3 control subjects (mean age ± SE, 68.3 ± 8.4 years; mean death to freezing interval ± SE, 5.7 ± 0.4 h). Brain and spinal cord tissues were stored at -80°C until analysis.

2.2. General Methods

2.2.1. Preparation of human postmortem tissues

Brain and spinal cord sections were weighed, placed in 2.4 ml of homogenization buffer (75 mM sodium-β-D-glycerophosphate, 20 mM MOPS, 15 mM EGTA, 2 mM EDTA and 1 mM sodium orthovanadate, pH 7.2), sonicated, and centrifuged (100,000 x g, 15 min, 4°C) in a Beckman TL-100 ultracentrifuge. The supernatant (cytosolic fraction) was stored at -80°C. Homogenization buffer containing 1 % Triton X was added to the pellet. The pellet was resuspended by sonication and the resuspended material was centrifuged under the same conditions mentioned above. The supernatant, which is the solubilized particulate fraction, was stored at -80°C. Protein was assessed using a Bradford assay and approximately

500 μg of protein was used per sample for immunoprecipitations and in vitro kinase assays.

2.2.2. Assessment of protein concentration

The Bradford method (1976) was used to assess the protein concentration. A series of protein standards ranging from 0-30 μg BSA were prepared. The Bradford reagent (100 mg Coomassie Blue G, 50 ml ethanol, 100 ml H_3PO_4, 850 ml dH_2O) was added to each standard. The samples to be quantitated were diluted with dH_2O to within 5-20 μg/10 μl, 2.5 ml of the Bradford reagent was added to each sample and mixed by gentle vortexing. After 5-10 min incubation, the absorbances of the solutions were measured at 595 nm in a spectrophotometer and the concentrations of the samples calculated through linear regression plotting of the standards.

2.2.3. Mono Q fractionation

Cytosolic or solubilized particulate extracts from postmortem brain and spinal cord tissue, platelets or leukocytes were fractionated by fast protein liquid chromatography (FPLC; Pharmacia) on a Mono Q column using standard procedures (Pelech et al., 1991). Approximately 2 mg of protein for each sample was loaded onto the Mono Q column (Pharmacia; 1 ml) equilibrated in column buffer (25 mM sodium-β-D-glycerophosphate, 10 mM MOPS, 5 mM EGTA, 2 mM $MgCl_2$ 1 mM DTT and 2 mM Na_3VO_4). A standard elution program using a 10 ml 0-0.8 M NaCl linear gradient was used. The eluate was collected in either 0.25 ml or 0.50 ml fractions. These samples could then be further analyzed by SDS-PAGE or by using a protein kinase assay.

2.2.4. Electrophoresis and immunoblotting

2.2.4.1 SDS-polyacrylamide gel electrophoresis.

Samples containing proteins were separated using sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Proteins were diluted with 30 μl of 5x concentrated SDS-sample buffer (125 mM Tris-HCl (pH 6.8), 4% SDS (w/v), 20% glycerol (v/v), 10% β-mercaptoethanol, 0.01% bromophenol blue (w/v)), boiled for 5 min then loaded onto an SDS-PAGE gel. Proteins were subjected to electrophoresis on 1.5 mm thick polyacrylamide gels with 4% stacking gels and 11% separating gels. The gels were electrophoresed for 15 h at 10 mA in running buffer (25 mM Tris, 192 mM glycine, 3.5 mM SDS)

2.2.4.2. Western blot analysis

After electrophoresis, the separating gel was equilibrated in transfer buffer (20 mM Tris, 120 mM glycine, 20% methanol (v/v), pH 8.6) for 5-15 min to remove SDS. Nitrocellulose membrane was hydrated in transfer buffer for about 5 min and assembled with the gel into a sandwich between pieces of 3 MM filter paper. The protein on the gel was electrophoretically transferred to the nitrocellulose membrane for 3 h at 300 mA in a Hoeffer transfer cell system.

Transferred proteins were stained using Ponceau S dye by incubating the membrane in the stain for 1 to 5 min in order to visualize the proteins on the nitrocellulose. The excess stain on the nitrocellulose membrane was destained with water. The nitrocellulose membranes were blocked in 5% skim milk (w/v) in TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) for 1 h. Membranes were rinsed briefly with

TTBS (0.05% Tween-20 (v/v) in TBS) to remove excess blocking solution. The membrane was incubated with the optimized concentration of primary antibody diluted in TTBS containing 0.05% sodium azide (w/v) for several hours or overnight with agitation at room temperature. After incubation with the primary antibody, the membrane was rinsed in TBST before incubation with the appropriate secondary antibody (horseradish peroxidase goat anti- mouse or anti-rabbit antibodies, BioRad) for 45 min. The membranes were then washed with TBST again to remove the excess secondary antibody and a final rinse with TBS to rinse away the detergent Tween-20. The Western blots were incubated with enhanced chemiluminescence (ECL) detecting reagents for 1 min and exposed to film to visualize the immunoreactive protein.

If a phosphotyrosine antibody was used, the membranes were blocked overnight at room temperature using low-salt TBS (20 mM Tris, pH 7.5, and 50 mM NaCl) containing 3% BSA (w/v). Primary antibody was incubated for 4 h, and alkaline phosphatase conjugated secondary antibody incubated for 2 h. All washes were performed with low-salt TBS containing 0.05% Nonidet P-40 (NP-40). Blots were developed as described above.

2.2.5. Stripping and reprobing of Western Blots

To reuse a nitrocellulose membrane for another antibody detection, the nitrocellulose membranes were stripped by incubation in a stripping solution (100mM β-mercaptoethanol, 2% SDS (w/v), 62.5 mM Tris-HCl (pH 6.7)) for 30 min at 50°C with occasional agitation. The membranes were rinsed with TTBS several times to

wash out the β-mercaptoethanol. The membranes were reblocked in 5% skim milk in TBS for 1 h and the Western blot procedure was used as described.

2.2.6 Immunoprecipitations and in vitro kinase assay

2.2.6.1 Immunoprecipitation

Protein A-Sepharose CL4B beads were incubated for 15 min in 3% NETF (3% NP-40 (v/v) in 100 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl (pH 7.4), 50 mM NaF). The beads were washed twice in 3% NETF and resuspended in an equal volume of 3% NETF.

The extracts (cytosol, particulate or total lysates), containing 500 μg total protein were diluted with an equal volume of 3% NETF (3% NP-40 in NETF buffer). Extracts were precleared with 10 μl of a Protein A-Sepharose slurry, incubated at 4°C for 15 min with agitation. The beads were removed by centrifugation for 1 min at 10,000 rpm. Ten μg of antibody were incubated with the supernatants for 1 h at 4°C with agitation. To the mixture were added 20 μl of Protein A-Sepharose slurry and the antibody was allowed to complex for 45 min at 4°C with agitation. The beads were washed 2 x with 3% NETF and once with KII buffer (12.5 mM sodium-β-D-glycerophosphate, 12.5 mM MOPS, pH 7.2, 5 mM EGTA, 20 mM $MgCl_2$, 50 mM NaF and 0.25 mM DTT, pH 7.2), to rinse out the detergent in the NETF buffer. Immunoprecipitates were either used for the in vitro kinase assay or boiled for 5 min in 4x SDS sample buffer and subjected to SDS-PAGE and Western blotting as described.

For experiments in chapter 3.2, immunoprecipitation of PI 3-K was done using a mouse monoclonal antibody against the p85 subunit and an anti-phosphotyrosine

antibody (4G10). Briefly, 4 μl of antibody and 40 μl of protein A-Sepharose (PAS) beads (50% slurry) were added to the samples and incubated overnight. The beads were washed with lysis buffer (20 mM Tris, 137 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Triton-X-100, pH 8.0) followed by a wash in 10 mM Tris, pH 7.4. The enzyme assay was performed by adding 10 μg of phosphatidylinositol (Avanti polar lipids, Alabaster, AL) as a substrate to each sample and mixing with 40 μl of the lipid kinase buffer (30 mM HEPES pH 7.4, 30 mM $MgCl_2$, 50 μM ATP, 200 μM adenosine and 10 μCi [γ^{32} P] ATP. Some samples were incubated in kinase buffer with 100 nM wortmannin, a fungal metabolite which irreversibly modifies the catalytic domain of PI 3-K rendering the enzyme inactive. Samples were incubated at room temperature for 15 min. The reaction was terminated by adding 1 N HCl and lipids were extracted by adding 200 μl of chloroform/methanol (1:1, v:v). Each sample (25 μl) was spotted and then separated on an oxalate-treated thin layer chromatography (TLC) plate using a solvent mixture of chloroform/methanol/water/ammonium hydroxide (18:14:3:1, v:v). TLC plates were dried and exposed to autoradiography film (Dupont, NEN). Quantitation was done by excising the part of the TLC plate that corresponds to the lipid product (PI 3-phosphate), followed by quantitation of the radioactivity using a liquid scintillation counter.

PKC was immunoprecipitated with anti-PKC αβ antibody. NETF buffer 3% was added to 500 μg of the tissue sample to equalize the volume of all samples, precleared with 10 μl PAS for 30 min followed by centrifugation at 10,000 x g to remove insoluble material. Antibody (5 μl of 0.5 mg/ml) was added to the supernatant for 1.5 h, followed by the addition of PAS for another 1.5 h at 4°C. Immunoprecipitated PKC was then used for an in vitro kinase assay by adding KII

Buffer, 1 mg/ml HH1 (Histone H1) as substrate, and 10 μCi [γ^{32}P] ATP (specific activity 2000 cpm/pmol). To assess the lipid-dependent activity, additions of Mg^{2+}, Ca^{2+} and lipid (6 μg/ml of diacylglycerol and 60 μg/ml of phosphatidylserine) were made; Mg^{2+} alone was added for the lipid-independent assay. The immunoprecipitated PKC bound to PAS beads was washed with 3% NETF and then KII buffer. The assay was conducted for 20 min at 30°C, then 20 μl aliquots were spotted onto Whatman P81 phosphocellulose paper and washed 10 times in 1% phosphoric acid.

Immunoprecipitation of PKB and Erk1 was performed with 10 μl (PKB:0.5 mg/ml; Erk: 0.1 mg/ml) of the appropriate antibody for 1.5 h using 500 μg protein per sample diluted in 3% NETF. PAS was then added for another 1.5 h at 4°C. After washing the pellet with 3% NETF and KII Buffer, the pellets were used in a kinase assay by addition of 10 μl of 200 mM $MgCl_2$ and 25 μl of KII buffer. The assay for MBP phosphotransferase activity (10 μl of 5 mg/ml MBP and 15 μl of KII buffer) was commenced by adding 10 μCi [γ^{32}P] ATP and incubating the mixture for 20 min at 30°C. The reactions were stopped by adding 5x SDS-PAGE sample buffer. The samples were boiled and used for SDS-PAGE. The immunoprecipitation of S6K was also performed by a 3 h incubation with 5 μl (0.1mg/ml) of the antibody and PAS. After washing with the buffer as for PKB, the assay was performed by adding "S6 cocktail" (10 μg S6-10 peptide as substrate, assay dilution buffer (vol: 50 μl), a PKC inhibitor (50 μM RO 38220, Roche compound 3), a peptide inhibitor of cAMP-dependent protein kinase (0.5 μM PKI) and 10 μl [γ^{32}P] ATP for 20 min at 30°C. The 15 μl aliquots were spotted onto Whatman P81 phosphocellulose paper and counted

in the scintillation counter. The reactions were stopped and the samples were used for further immunoblotting analysis.

2.2.6.2. In vitro kinase assay

Kinase assays were performed on immunoprecipitated samples or Mono Q column fractions. Phosphotransferase reactions were carried out in a specific buffer (depending on the kinase) in a total volume of 30 µl containing, 0.5 µM PKI, 50 µM [γ-^{32}P] ATP (~2000 cpm/pmol), 5 µg of protein substrate or 2 µg of peptide substrate. Reaction mixtures were incubated at 30°C for several minutes (depending on the protein kinase) and stopped by spotting 20 µl of the mixtures onto P81 filter paper squares (Whatman). Filter papers were washed with 1% H_3PO_4 (v/v) to remove free ATP, placed into scintillation vials containing 200 µl scintillant and counted in a scintillation counter. An alternative way to measure the phosphorylation reactions was to stop the kinase reaction with 5x sample buffer, boil for 5 min and analyze by SDS-PAGE. Proteins were transferred to nitrocellulose and Ponceau S stained to visualize the substrate bands. Substrates were excised from the membrane and counted as described for the filter papers.

For experiments in chapter 3.1, PKC assays were carried out as described previously (Pelech et al., 1991). Assays contained "Buffer A" (25 mM sodium-β-D-glycerophosphate, 10 mM MOPS, 2 mM EGTA, 2 mM EDTA, 0.25 mM DTT), 10 mM $MgCl_2$, 500 nM Protein kinase A inhibiting peptide (PKI), 50 µM [γ-^{32}P]-ATP (-2000 cpm/pmol (ICN)), 1 mg/ml histone H1 or 1 mg/ml protamine chloride (Sigma), and 5 µl of the cytosolic or particulate column fraction in a volume of 25 µl. Histone H1 phosphorylation was measured in the absence or presence of 4.5 mM Ca^{2+}, 6 µg/ml

diacylglycerol, and 60 μg/ml phosphatidylserine. The kinase assays were initiated by addition of [γ-^{32}P]- ATP and incubated for 5 min at 30°C. At the conclusion of the reaction period, 20-μl aliquots were spotted onto Whatman P81 phosphocellulose paper, washed 10 times for at least 5 min in a solution of 1% phosphoric acid, transferred into scintillation vials with 0.5 ml of Aquasol scintillation fluid and analyzed in a Beckman model 6000IC scintillation counter.

SDS-PAGE was performed as described in Section 2.2.4.1. For experiments in Chapter 3.1, the membrane was incubated with affinity purified rabbit polyclonal PKC α antibodies (Santa Cruz Biotechnology) or whole serum rabbit polyclonal PKC α antibodies (Sphinx Pharmaceuticals) (in TBST containing 0.5% milk and 0.05% sodium azide; diluted to 2 mg/ml) raised against isozyme-specific peptides.

For experiments in Chapter 3.2, the membranes were each incubated with the following antibodies: monoclonal antibody against the p85 subunit of PI 3-K (Upstate Biotechnology), anti-PKB (Rac pH, Kinetek Pharmaceuticals), anti-PKC (PKC αβ monoclonal, gift of Dr. Susan Jaken), anti-Erk1/Erk2 or anti-S6K (Kinetek Pharmaceuticals).

2.2.7. Cell culture and transfection

HEK-293 cells from the American Type Culture Collection (Rockville, MD; CRL 1573) were cultured in Minimum Essential Medium (MEM) containing 10% fetal bovine serum, penicillin/ streptomycin (100 U/ml), L-glutamine (2 mM) and sodium pyruvate (1 mM) as described previously by Chen et al. (1997). Cells were plated at a density of 1 x 10^6 cells /1.5 ml MEM medium onto poly D-lysine pre-coated tissue culture plates and incubated in a 5 % CO_2 incubator at 37° C for 24 h.

cDNAs for NR1A, NR1C and NR2A were subcloned from pBluescript KS^+ into pRK5, a mammalian expression vector containing the cytomegalovirus (CMV) promoter. Plasmid purification was done using the Qiagen plasmid mega kit (Qiagen Inc.) according to the manufacturer's instructions. The DNA yield was determined by spectrophotometry.

Cells were transiently transfected using lipofectamine. A total of 1.5 μg of cDNA was added to each 35 mm plate in 150 μl of Opti-MEM. In experiments that used two different plasmids, equal amounts of the plasmids were added to give a total of 1.5 μg. With co-transfection of lacZ, lacZ cDNA comprised 20% of total cDNA. Transfection employed 7.5 μl lipofectamine/ plate (5 μl lipofectamine for 1 μg cDNA). Cells were left for 5 h at 37^0 C in a 5% CO_2 incubator. The transfection was terminated by adding serum-containing MEM and the NMDAR antagonist, 5-7 dichlorokynurenic acid (dichloro; 250 μM).

2.2.8. Plasmid transformation and purification

Competent cells (DH5α) were thawed on ice, mixed, and aliquoted into microcentrifuge tubes. As a control for transformation efficiency, 0.5 ng of pUC 19 was added to one vial of competent cells. The DNA (1-10 ng) of interested was added to one of the microtubes containing competent cells and incubated on ice for 30 min. Cells were then heat shocked at 37°C for 20 seconds then placed on ice. SOC (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$ 10 mM $MgSO_4$ and 20 mM glucose) medium was added to the cell-DNA mixture and shaken for 1 h at 37°C for expression. After expression, the mixture containing the control pUC19 was diluted into medium (1:10). Then 100 μl of the diluted and the

undiluted mixture were spread onto LB plates with 100 μg/ml ampicilin and 50 μg/ml X-gal. For the experimental mixture, after expression the mixture was diluted and spread (150 μl) onto LB plates and incubated overnight at 37°C.

After transformation of the required plasmid, an *E coli* culture containing the plasmid was grown overnight at 37° C. A toothpick was immersed in the culture and streaked onto an agar plate (LB, agar and ampicilin) which was incubated at 37°C overnight. The next morning a colony was picked from the agar plate and a starter culture of 3 ml LB medium containing ampicillin was inoculated and left in the shaker for about 8 h. The starter culture was diluted 1 ml into 400 ml of LB medium (+Ampicillin). The culture was grown at 37°C for 16 h, shaking at 225 rpm for a preparation of high copy plasmids.

Plasmid purification was done using Qiagen plasmid mega kit which is a purification procedure based on a modified alkaline lysis followed by binding of plasmid DNA to the anion- exchange resin. Other impurities such as RNA and proteins are removed through washes with a medium salt buffers. The DNA is eluted in a high-salt buffer and concentrated further through isopropanol precipitation

2.2.9. DNA yield

The yield of DNA concentration obtained from plasmid DNA purification was determined by UV spectrophotometry. The DNA plasmid was diluted (1: 5) with ddH_20 and measured at OD (Optical Density) 260 nm to determine the DNA concentration. The ratio of OD 260 nm over 280 nm was used to determine the purity of the obtained plasmid DNA

2.2.10. Treatment protocols

Cells were exposed to various treatments 22 h after the start of the transfection. Two plates (35 mm) were prepared for each treatment. Control cells were incubated in a physiological salt solution (PSS; 140 mM, NaCl; 1.4 mM $CaCl_2$; 5.4 mM KCl; 1.2 mM NaH_2PO_4; 21 mM glucose; 26 mM $NaHCO_3$; pH 7.4) containing 250 μM dichlorokynurenic acid for 10 min in a 5 % CO_2 incubator at 37^{0}C. Activation of NMDARs was performed by incubating the cells in PSS containing NMDA (1 mM) and glycine (50 μM) for 10 min. Experiments using the PKC inhibitor, RO 32-0432 (1 μM), were performed in the presence and absence of NMDA and glycine. In the presence of the NMDAR agonists, RO treatment was begun 10 min prior to incubation with NMDA and glycine. In the absence of NMDAR agonists, RO was applied in PSS with dichlorokynurenic acid for 10 min. After the 10 min treatments at 5% CO_2, and 37^{0}C, cells were placed in fresh media containing 250 μM dichlorokynurenic acid for 6 h before assessment of cell death.

Cells were exposed to NMDAR agonists in the presence and absence of phorbol 12-myristate-13-acetate (PMA, 85 nM), a PKC agonist. As a control, transfected cells were incubated in PSS containing 250 μM dichlorokynurenic acid for 10 min. Treatment with NMDAR agonists was performed as described above. Treatment with PMA was performed by adding the compound directly to the media 10 min before replacing the media with PSS and 250 μM dichlorokynurenic acid. In the presence of the NMDAR agonist, cells were exposed to PMA 10 min prior to incubation in PSS containing NMDA and glycine. Following treatment with the NMDAR agonists, cells were incubated in fresh media containing dichlorokynurenic acid for 6 h prior to cell death assesment.

To determine the effect of the PKC inhibitor RO-32-0432 on PMA and NMDA-mediated cytotoxicity, this compound was added 10 min prior to adding PMA, as described above. Cells were then incubated for 10 min in PSS containing NMDA, glycine and RO before replacing PSS with fresh media containing 250 μM dichlorokynurenic acid and RO for 6 h. These same procedures were used for cells transfected with NR1C and NR2A subunits.

To evaluate the role of different PKC isoforms in cytotoxicity, isoform-specific PKC inhbitors were used such as pseudo A (40 μM), which inhibits the action of Ca^{2+}-dependent PKC isoforms, and pseudo Z (40 μM) which inhibits activation of PKCζ, an atypical PKC isoform (Liu et al., 2000). These two inhibitors were added separately 30 min prior to exposure of cultures to PMA. Cells were further incubated in PSS in the presence of NMDAR agonists and pseudo A, or pseudo Z for 10 min before replacing PSS with media containing dichlorokynurenic acid in the presence of pseudo A or pseudo Z.

To evaluate the role of PI 3-K and MEK in NMDA-mediated cell death, inhibitors of PI3-K and MEK, LY 294002 (25 μM) and PD 98059 (30 μM), respectively, were applied separately to HEK 293 cells transfected with NR1A and NR2A subunits. The inhibitors were applied 20 min before treatment of cells with glutamate agonists. Cell death counts were performed as described.

2.2.11. Trypan Blue assay

Cell death was determined by trypan blue exclusion by live cells (see Cik et al., 1993). Two plates of HEK-293 cells were prepared for each treatment. Six hours following treatment, cell death was assessed both in attached and floating cells by

trypan blue exclusion. To determine the percentage of dead cells in the medium, medium from each plate (1.5 ml) was removed and trypan blue added. Cell counts were performed on a hemacytometer in 10 counting areas and the average number of live and dead cells was calculated as a percent cell death. Death of attached cells was determined by trypsinizing cells and assessing the percent cell death in a similar manner as for floating cells. All data points correspond to the mean of values performed in duplicate from at least 3 separate experiments. Statistical comparisons of cell death were performed using Student's t-test.

2.2.12. β-Gal staining

To determine transfection efficiency, β-Gal staining was performed on the plates transfected with the NMDA receptor subunits. Briefly, in addition to the NMDA subunit/s, the cells were co-transfected with a plasmid containing *E. coli* β-Gal gene. After transfection and treatments, cells were washed once with PBS (37°C), fixed at room temperature for 5 min with a fixative solution (37% formaldehyde, 25% glutaraldehyde and PBS, pH 7.3) and washed again with PBS (3 times). Cells were then stained with a freshly made solution of 1.2 mM 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal), 5 mM potassium ferrocyanide, 5 mM potasium ferricyanide and PBS, pH 7.3, then incubated at 37°C for about 16 h. β-Gal expression was assessed under the microscope by counting the ratio of cells that incorporated β-Gal (blue) versus cells that did not incorporate β-Gal (white). β-Gal expression was calculated as the percentage of cells that incorporated β-Gal. Plates with transfection efficiencies ranging from 60-80% were used for further experiments.

2.2.13. Production of homozygous pmn/pmn mice

The studies of pmn/pmn mice (Chapter 5) were conducted in collaboration with Dr. Yves Sagot (Dept. of Pharmacology, Centre Medical Universitaire, 12 11 Geneva, Switzerland). Heterozygous pmn breeder mice (SV129 strain) were obtained from the laboratory of Dr. J.L. Guenet (Institut Pasteur, Paris, France); the litters (usually 6-8 animals) had an average of 25% pmn/pmn mice. Homozygous pmn mice undergo progressive paralysis beginning in the hindlimbs during the third week of life. Mice were studied at 28 days (moderately affected) and 41 days of age (severely affected). Control mice constitute homozygous normal mice and pmn heterozygotes. Efforts were made to minimize animal suffering and the number of animals used for these experiments.

Mice were decapitated, the brains and spinal cords removed and these were immediately frozen in cooled isopentane (-70°C). Brains and spinal cords were cut into small pieces, weighed and placed in 0.4 ml of homogenization buffer. Samples were prepared as described in section 2.2.1. Immunoprecipitation, in vitro kinase assay and Western blotting for PI3-K, PKB, p70S6K and Erk1 were performed as described in Section 2.2.6.

2.2.14. Fluorogold labeling of motoneurons

The left sciatic nerve of an anesthetized mouse (28 day-old; 16-18 g for control; 7-8 g for pmn/pmn mice) was sectioned in the mid-thigh. A small polythene tube (Portex, U.K.) containing 2.5% Fluorogold (Fluorochrome Inc, Denver, CO, USA) in 75 mM PBS (Mg^{2+}/Ca^{2+}-free) mixed with inhibitors, with or without BDNF (1 μg/μl, Regeneron, Inc. Tarrytown, NY, USA), was applied to the central nerve stump. The

incision was sutured and the animals were kept on a warming plate (35°C) until they had recovered from narcosis.

2.2.15. Preparation of inhibitors

LY-294002; PD 98059 and wortmannin were purchased from Alexis Biochemicals (Loufelfingen, Switzerland). All inhibitors were initially dissolved in 100% DMSO at 5 mM, with the exception of LY-294002, which was solubilized at 5 mM in 100% ethanol. Prior to mixing with Fluorogold, LY-294002 and wortmannin were further diluted with water, while PD 98059 was diluted with 0.66% BSA in water in order to prevent precipitation at low concentrations. Appropriate controls were made with vehicle solution. Due to the instability of some substances, solutions having color changes or precipitate were discarded.

2.2.16. Histological analysis and motoneuron counting

Twenty-four hours after surgery, mice were deeply anesthetized with pentobarbital and perfused with PBS followed by 4% paraformaldehyde in PBS. In every experiment, we verified that the polyethylene tube was still in place, and that Fluorogold did not stain the adjacent muscles. Animals in which the tube had not remained in place around the nerve stump, or where the tube contained blood, were discarded. The spinal cords were removed and processed for cryosectioning. Cryostat sections (30 μm) were viewed under a Reichert-Jung fluorescence microscope using a wide-band UV filter. Fluorogold labelled cells, identifiable as motoneurons by their size, shape and location in the ventral horn, were counted in

every section. The number of labelled motoneurons after 24 h was referred to as the labelling index.

2.2.17. Intraperitoneal injection of BDNF

A saline solution of BDNF (100 μl; 5 mg BDNF/kg body weight) was injected intraperitoneally using a 21G syringe. Eighteen hours after injection, animals were decapitated and the tissues processed for immunoprecipitation and Western blot analysis.

2.3. Statistical analysis

Protein was measured by scanning the film (Abaton Scan 300, Color Adobe Photoshop) into a TIFF format file and band densities were quantified in arbitrary units using the NIH Image Program (version 1.61). Results are expressed as mean ± SEM, with $p<0.05$ being considered significant using Student's T-test (unpaired, two-tail).

CHAPTER 3. PROTEIN KINASES IN POSTMORTEM TISSUE FROM ALS PATIENTS

3.1. Amplified PKC activity in the pathogenesis of ALS

3.1.1. Introduction

Amyotrophic lateral sclerosis is likely a disease with more than one pathogenic mechanism (see Chapter 1, reviewed in Eisen and Krieger, 1998). One view of the origin of sporadic amyotrophic lateral sclerosis (ALS) holds that motoneuron death arises due to increased levels of intracellular Ca^{2+} through various processes such as abnormal regulation of EAA receptors (Plaitakis, 1990), activation and modification of voltage dependent Ca^{2+} channels by antibody in some ALS patients (Llinas et al., 1993; Delbono et al., 1991; Smith et al., 1992) and mitochondrial dysfunction (Beal, 1995). An increase in intraneuronal Ca^{2+} concentration by whatever route could lead to a sustained activation of protein kinase C (PKC).

There is evidence of an alteration in the level or activity of PKC in some neurodegenerative diseases such as Alzheimer's and Huntington's diseases (Krieger et al., 1996). PKC might be abnormally regulated in ALS. PKC activation has been associated with several aspects of ALS. Some studies have found a reduction in receptor binding of [^{3}H] MK-801, a NMDA receptor antagonist, in dorsal and/or ventral horns in ALS spinal cord compared to control (Shaw et al., 1994; Allaoua et al., 1992; Krieger et al., 1993). Treatment with phorbol ester was able to increase [^{3}H] MK-801 binding in postmortem tissue from ALS patients more extensively than in controls, indicating that [^{3}H] MK-801 binding was downregulated in ALS and this downregulation could be reversed by PKC activation (Krieger et al., 1993). Activation of PKC has been associated with neuron death in several cell types (Favaron et al.,

1988; Candeo et al., 1992; Felipo et al., 1993), and inhibitors of PKC such as calphostin C have been shown to reduce glutamate-induced neurotoxicity in cultured cerebellar neurons (Felipo et al., 1993). Furthermore, activation of PKC has been linked to loss of intracellular Ca^{2+} homeostasis and abnormal glutamate metabolism (Favaron et al., 1990). Finally, activation of PKC contributes to changes in neurofilament organization, similar to those observed in neuropathological studies of tissue from ALS patients (Doroudchi and Durham, 1996).

The present study examined the activity and amount of PKC in ALS and control central nervous system tissue for regions known to be pathologically affected (cervical spinal cord and motor cortex) as well as regions pathologically spared in the disease (visual cortex). As a control for non-neuronal PKC alterations, PKC activity in platelets and leukocytes was examined in the two groups. In addition to PKC, the activity and amount of PKM, a Ca^{2+}/ phospholipid-independent 45-50 kDa fragment of PKC (Kishimoto et al., 1989), were evaluated in ALS and control tissue.

3.1.2. Results

To measure PKC activity from spinal cord tissue of ALS and control subjects, extracts of spinal cord tissue were subjected onto Mono Q columns. Figure 2 shows two peaks of histone H1 phosphotransferase activity in cytosolic (panel A) and Nonidet P40-solubilized particulate (panel B) extracts from ALS and control cervical spinal cord samples which were resolved by FPLC on MonoQ columns. Histone H1 phosphorylation was measured in the absence and presence of Ca^{2+}, phosphatidylserine (PS), and diacylglycerol (DAG).

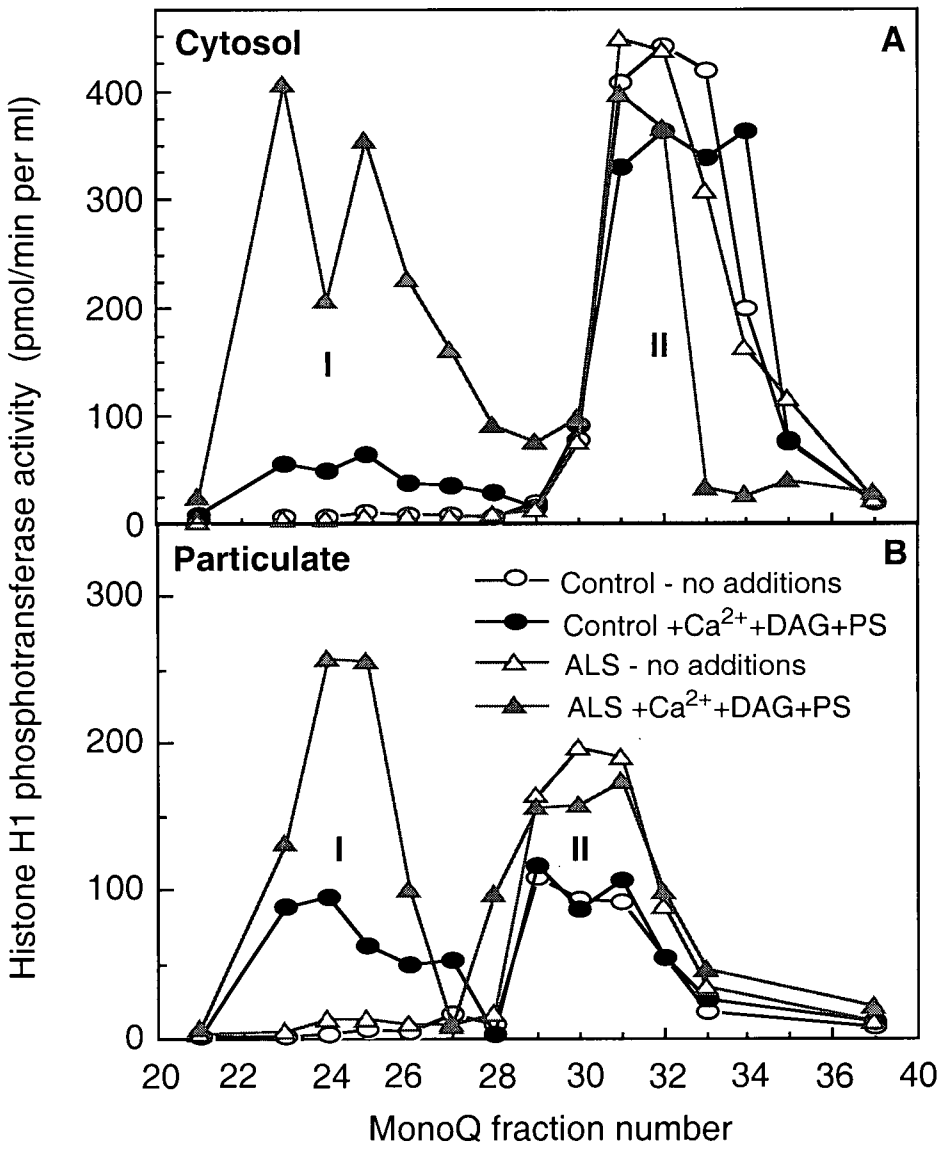

Fig.2. Mono Q chromatography of PKC HH1 phosphotransferase activity. Representative results of Histone H1 phopsphotransferase activity from Mono Q chromatography of cytosolic (A) and particulate (B) fractions of tissue from one control and one ALS spinal cord.

Peak I histone H1 phosphotransferase activity (fractions 23-27) in both cytosolic and particulate-derived extracts from ALS and control patients was highly dependent on Ca^{2+}, PS and DAG and could therefore be ascribed to PKC. To further confirm the identity of the two MonoQ peaks as PKC and PKM, column chromatography fractions from ALS and control patients were subjected to SDS-polyacrylamide gel electrophoresis. The proteins were transferred to nitrocellulose and immunoblotted with affinity-purified polyclonal antibodies specific for the α isoform of PKC. The PKC α antibody immunoreacted with an 80 kDa protein which eluted in fractions 23-27 as well as with a 50 kDa protein which eluted in fractions 28-32 for both the cytosolic (Fig.3A and B) and particulate-derived extracts (Fig. 3C and D). Western blotting of total lysates of spinal cord tissue from 6 control and 6 ALS subjects using a PKC $\alpha\beta$ monoclonal antibody revealed a significant increase in PKC protein level in ALS patients compared to control subjects (Fig. 3E, $p< 0.01$). Positive controls for PKC α, β, and γ showed that PKC α antibodies were not specific for PKC α. The antibodies used in this study reacted equally well to PKC α , β, and γ (Fig. 3F).

Significantly higher PKC activity was observed in ALS spinal cord tissue compared to control: cytosolic and particulate-derived extracts from ALS patients showed 330% and 118% increases in PKC activity compared to control, respectively (Fig. 4A and C, $p<0.05$, Students t-test). Peak 2 histone H1 phosphotransferase activity (fractions 28-31) was Ca^{2+}-, PS- and DAG-independent and had a molecular mass of approximately 45 kDa which are the characteristics of PKM (Nishizuka, 1984; Kishimoto et al., 1989).

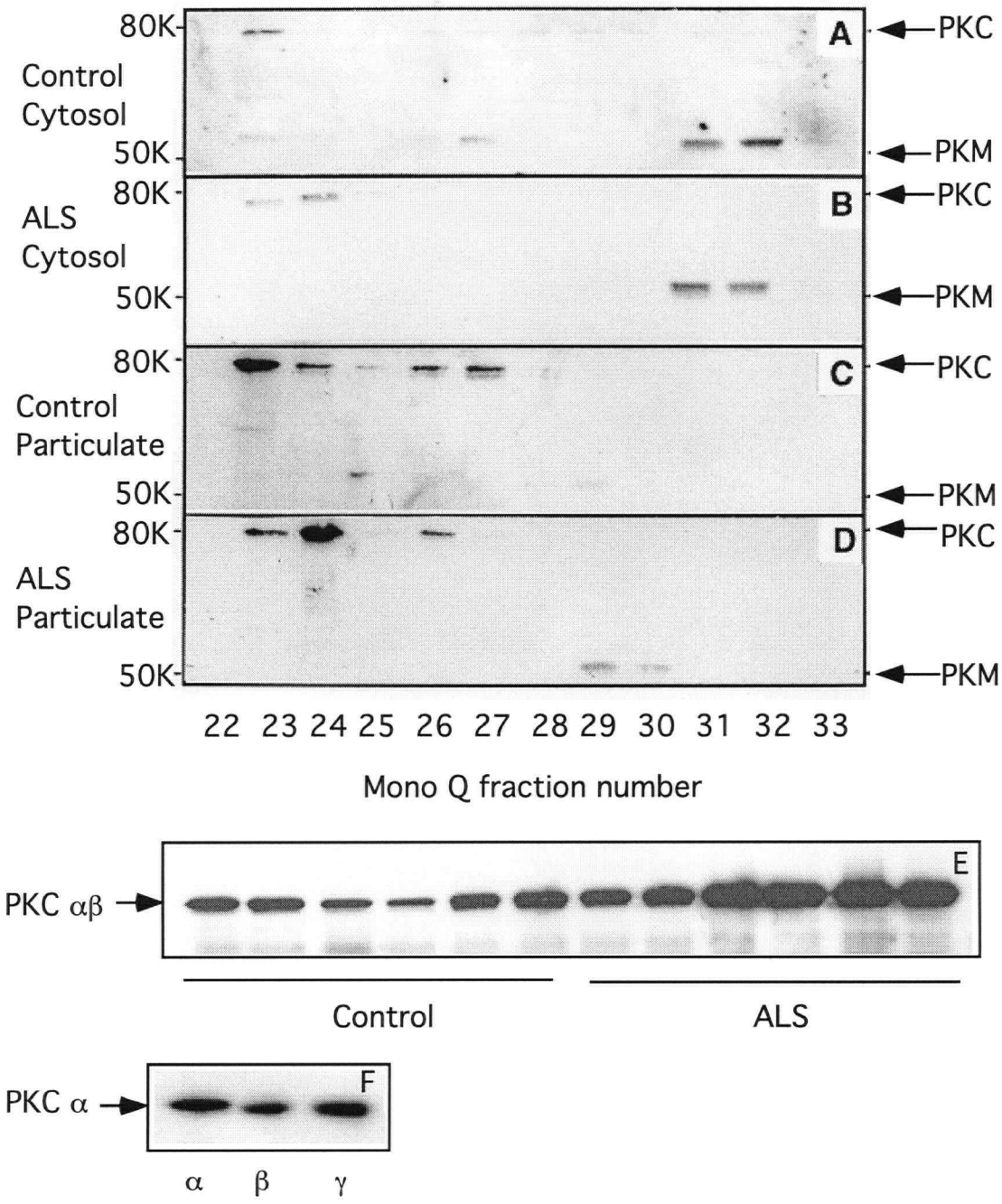

Fig. 3. Western blot analysis of PKC from Mono Q and total lysates. Western blotting of Mono Q chromatography fractions of cytosolic (A and B) or particulate (C and D) PKC and PKM pooled from tissue obtained from spinal cords of 5 control and 5 ALS patients probed against anti PKC α polyclonal antibodies. E. Western blotting of spinal cord total lysates from 6 control and 6 ALS patients probed against PKC αβ antibody. F. Positive controls of PKC α,β and γ probed against PKC α antibody.

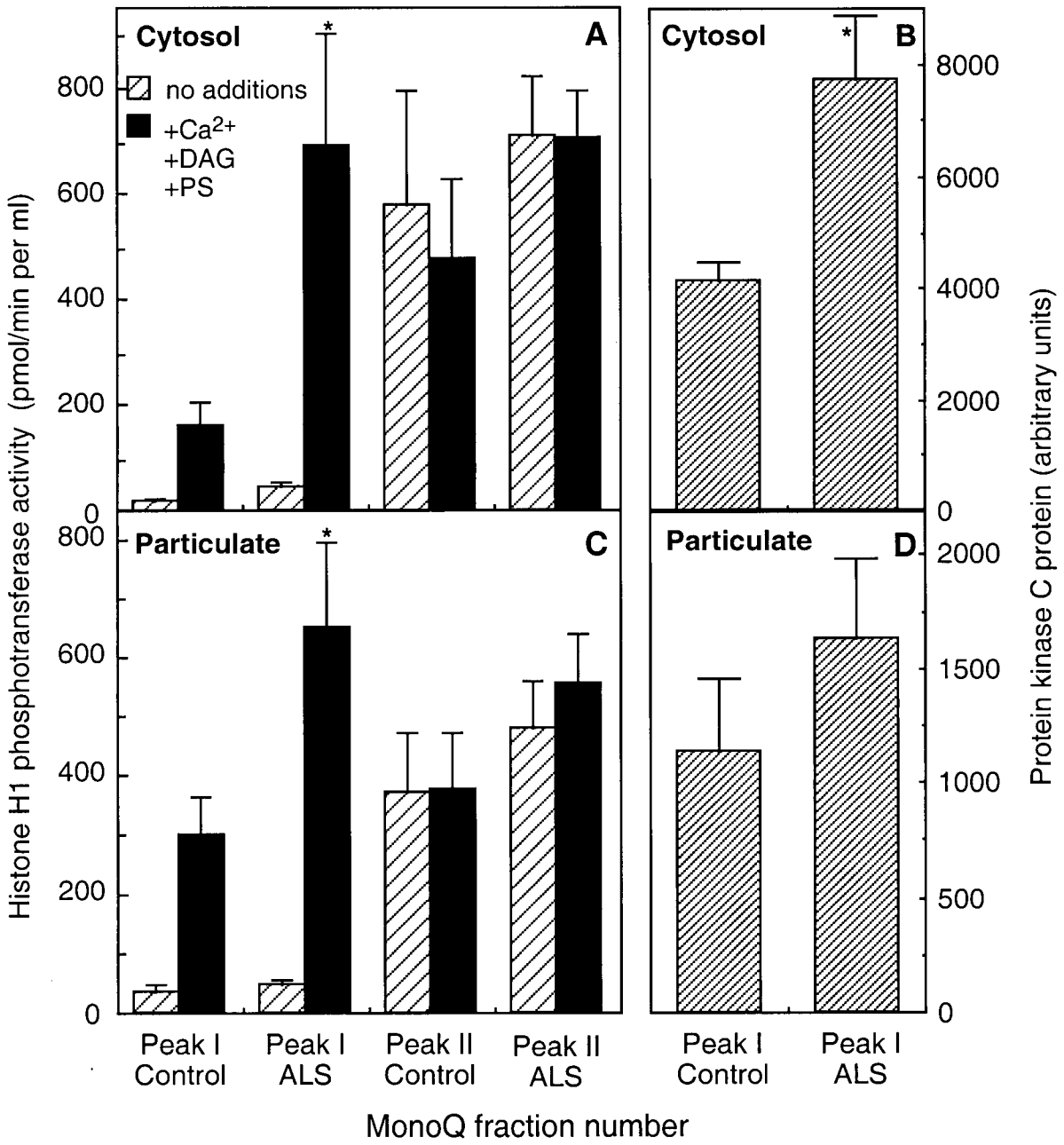

Fig. 4.PKC activity and protein level from control and ALS spinal cord tissue. A and C. Combined data for peaks I and II from spinal cord tissue obtained from 14 control and 14 ALS patients. B and D. Quantification of Peak I from spinal cord tissue from 4 control and 4 ALS patients using anti-PKC α antibody. Data are presented as mean $\pm$ SEM (bars) values. *significantly different from control ($p<0.05$)

The kinase activity of PKM was larger in ALS spinal cord tissue than control, showing 46% and 47% increases in cytosolic and particulate-derived extracts, respectively (Fig. 4A and C). However, these increases did not reach statistical significance ($p>0.05$, Students t-test). Similar increases of PKC and PKM activity in ALS patients were seen when protamine chloride was used as a substrate for PKC phosphorylation. The inclusion of a pseudosubstrate inhibitory peptide (PKC 19-36, RBI) (House and Kemp, 1987) in a parallel set of experiments abolished the MonoQ peaks 1 and 2 histone H1 phosphotransferase activity observed with extracts from ALS and control patients (data not shown). This inhibitory peptide data indicated that the Mono Q peaks were contributed by PKC activity.

Quantitation of peak I in Western blots of spinal cord tissue from 4 ALS and 4 control patients probed with a PKC α antibody showed a significant increase in the amount of PKC protein in cytosolic fractions from ALS spinal cord compared to controls (Fig. 4B, $p<0.05$). Particulate-derived fractions in the ALS patient samples were not significantly different from control (Fig. 4D). Thus, the increased PKC protein level did not entirely parallel the 330% and 118% elevations in PKC histone H1 phosphotransferase activity observed in cytosolic and particulate-derived extracts, respectively.

Autophosphorylation of PKC has been reported to increase the rate of PKC histone H1 phosphotransferase activity (Mochly-Rosen and Koshland, 1987). To determine whether autophosphorylation of PKC played a role in the mechanism underlying the increased PKC histone H 1 phosphotransferase activity observed in ALS, cytosolic and particulate-derived extracts from ALS or control patients were incubated with protein phosphatase 2A (PP2A) (0.5 units/reaction). PP2A has

previously been shown to catalyze the dephosphorylation of the autophosphorylated form of PKC. However, incubation with PP2A for up to 90 min had no effect on PKC histone H1 phosphotransferase activity in either ALS or control samples (data not shown), suggesting that autophosphorylation does not play a role in the mechanism underlying the increased PKC activity observed in ALS patients.

In patients who died with ALS, PKC and PKM histone H1 phosphotransferase activity was not found to be significantly elevated in cytosolic-and particulate-derived extracts obtained from motor and visual cortex (Fig.5), Moreover, cyotosolic-derived extracts obtained from platelets and leukocytes from patients who died from ALS showed no elevated PKC and PKM histone H1 phosphotransferase activity compared to control (Fig. 6).

3.1.3. Discussion

The present data demonstrate that the activity of the Ca^{2+}-activated phospholipid-dependent PKC is increased in cytosolic and particulate derived-extracts from ALS spinal cord tissue compared to control tissue. This increase was partially attributable to an increase in the amount of PKC protein present in ALS spinal cord tissue. Due to a lack of specificity of the PKC α antibodies it was not possible to determine whether the increase in PKC protein reflected an increase in the α, β, or γ of PKC isoform(s). PKC and PKM histone H1 phosphotransferase activity were not found to be significantly different in cytosolic- and particulate-derived extracts obtained from motor and visual cortex in ALS patients compared to control subjects.

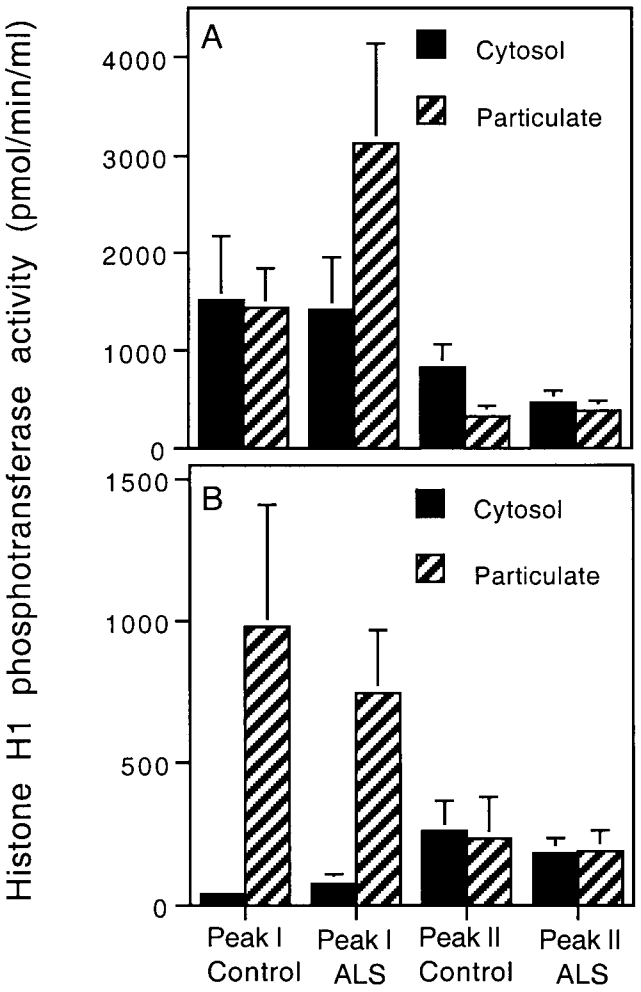

Fig.5. PKC activity from motor cortex and visual cortex. Mean HH1 phosphotransferase activity of Mono Q peaks I and II of cytosol and particulate fractions from A. motor cortex and B. visual cortex tissue obtained from 4 control and 4 ALS patients. Data are presented as mean ± SEM (bars) values.

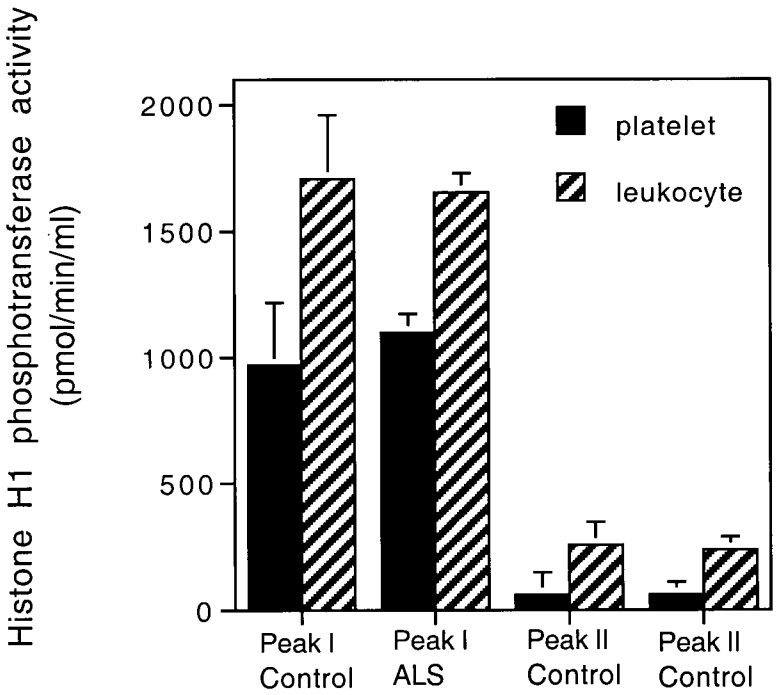

Fig.6. PKC activity from platelets and leukocytes. Mean HH1 phosphotransferase activity of Mono Q peaks I and II of total lysates from platelets and leukocytes obtained from 3 control and 3 ALS patients. Data are presented as mean ± SEM (bars) values.

Moreover, non-neuronal tissue, including platelets and leukocytes, showed no difference in PKC and PKM histone H1 phosphotransferase activity in ALS patients compared to control. These results indicate that the alterations in PKC histone H1 phosphotransferase activity are specific to the spinal cord, an area pathologically affected in ALS.

The increased PKC activity observed in ALS tissue may be a result of increased intracellular Ca^{2+} concentrations ($[Ca^{2+}]i$). Elevated $[Ca^{2+}]i$ might be generated by increased Ca^{2+} influx through activation of EAA (excitatory amino acid) receptors or by influx through voltage-gated Ca^{2+} channels (Llinas et al., 1993). Mitochondrial release of $[Ca^{2+}]i$ has also been reported to lead to elevated $[Ca^{2+}]i$ for at least several hours in a motoneuron hybrid cell line (Hasham et al., 1994) and could be a mechanism operating in ALS. Moreover, decreased buffering of $[Ca^{2+}]i$ may be present in motoneurons, as only trace amounts of immunoreactivity to the calcium buffering proteins, calbindin D28 and parvalbumin, can be observed in motoneurons (Ince et al., 1993; Alexianu et al., 1994;). A protective effect of cultured motoneurons from mutant SOD1 toxicity was observed after increasing the expression of calbindin (Roy et al., 1998). This group provided evidence that influx of Ca^{2+} during neurotransmission through AMPA/kainate receptors and voltage gated calcium channels contributes to potentiation of the toxicity of SOD1 mutants in primary culture of motoneurons. Furthermore, increased $[Ca^{2+}]i$ through stimulation of NMDA receptors could lead to activation of PKC which has been associated to alteration of neurofilaments in motoneurons (Doroudchi and Durham, 1996). This group suggested that PKC activation acts cooperatively with NMDA receptor stimulation to activate CaMK and other neurofilament-directed kinases to

phosphorylate neurofilament proteins. This mechanism could cause alteration in the cytoskeleton of motoneurons (Doroudchi and Durham, 1997).

Nagao and colleagues (1998) have reported that calcium-and phospholipid dependent PKC immunoreactivity is significantly decreased in motoneurons in sections of spinal cord from sporadic ALS patients compared to control subjects. The PKC immunoreactivity was measured in thoracic and lumbar regions by immunostaining using a commercially available mouse monoclonal antibody against PKC. They also evaluated phosphorylated neurofilaments using SMI-31; an antibody which reacts with highly phosphorylated neurofilaments. A mild to moderate loss of anterior horn cells and myelinated fibers in the pyramidal tracts were observed in spinal cord from ALS patients compared to control subjects. Despite their finding that many atrophic motoneurons did not show PKC immunoreactivity, some motoneurons with simple atrophy showed intense PKC immunoreactivity. Furthermore, in ALS spinal cord tissue, many non-neuronal cells such as endothelial cells, Schwann cells and fibroblasts were reported to show PKC immunoreactivity in the nucleus, similar to control spinal cords. Nagao and colleagues (1998) also demonstrated that SMI-31 immunoreactivity was only found in the cytoplasm of motoneurons and spheroids in ALS spinal cord but not in control spinal cord. The expression of SMI-31 was found in the atrophic motoneurons that did not show PKC immunoreactivity indicating that PKC was not associated with accumulation of phosphorylated neurofilaments in these neurons. Based on this observation, this group proposed two possibilities of why PKC immunoreactivity is decreased in ALS. First, a subset of neurons expressing PKC immunoreactivity could be selectively vulnerable in ALS tissue causing a decrease in the number of neurons staining intensely for PKC. Second, the

cell signalling involving PKC may be decreased in motoneurons of ALS patients. They speculated that the decrease of PKC signalling could be related to dysfunction of Golgi apparatus in ALS caused by abnormal SOD. It is believed that SOD toxicity in motoneurons is associated with downregulation of protein-tyrosine kinases (Beckman et al., 1993; Gurney et al., 1994). Nagao and colleagues (1998) have suggested that downregulation of PKC is secondary to down regulation of tyrosine kinase receptors by SOD abnormality. Moreover, they speculated that cytokines could also downregulate PKC. However, they did not rule out the possibility that PKC could also be activated at later stages of motoneuron degeneration in ALS.

The measurement of PKC activity and protein level in our study was from tissue sections of cervical spinal cord. Thus, we could not distinguish which types of cells in the spinal cord are responsible for the elevated PKC level. However, based on the immunocytochemical findings of Nagao and colleagues (1998) that intense PKC immunoreactivity was present in some motoneurons with simple atrophy and non-neuronal cells such as Schwann cells and fibroblasts, it is possible that the elevated PKC activity and protein levels found in spinal cord tissue from ALS patients, could be produced by these type of cells. Another possibility is that the increased PKC activity and protein levels found at a late stage of motoneuron degeneration in our study could be a compensatory response by non-neuronal cells. This compensatory response could be generated by elevations in circulating growth factors secondary to muscle denervation due to motoneuron loss in ALS patients. Non-neuronal cells such as glial and fibroblasts could respond by upregulating PKC due to presently unidentified circulating growth factors.

Increased PKC activity is believed to be an important event leading to neurotoxicity as determined from studies using cultured cells which have shown that down regulation of PKC by pre-exposure to phorbol ester (PMA) decreases the number of neurons dying as a result of exposure to EAA (Favaron et al., 1990). A role for PKC is also supported by evidence that EAA-induced cell death and apoptotic (naturally-occurring cell death) are associated with changes in transduction cascades and that inhibition of the signal transduction pathways, especially PKC-, lipid-and calcium-mediated pathways, prevents the progression of neuron death (Saitoh et al., 1991). Furthermore, another study demonstrated that application of phorbol ester to the eyes of mutant fruit flies induces degeneration of their photoreceptors (Minke et al., 1990). The increased PKC activity found in spinal cord tissue from ALS patients indicates that PKC may be involved in the pathogenesis of this neurodegenerative disease. However, this study could not determine whether the PKC alteration is a primary or secondary cause to motoneuron death. Although it has been established that PKC can modulate NMDA receptor currents and Ca^{2+} influx, further study is required to determine whether PKC contributes directly to NMDA-induced cell death. If increased PKC levels are causal to neuron death in ALS, drug therapy designed to normalize PKC levels in affected cells may provide a strategy for the slowing the progression of the disease.

3.2. Phosphatidylinositol 3-kinase: increased activity and protein level in ALS

3.2.1. Introducton

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disorder with a hereditary and nonhereditary form. Most ALS patients have a non-hereditary, sporadic form of the disease which may have a multifactorial

pathogenesis. One of the hypothesized pathogenic mechanisms for ALS is impaired action of trophic factors such as insulin-like growth factor 1 (IGF-1) (Dore' et al., 1997; Sagot et al., 1997). Attention has been focused on IGFs in ALS, as IGF-1 promotes neurite extension of cultured chick motoneurons (Caroni and Grandes, 1990), enhances nerve regeneration after sciatic nerve crush in mice (Contreras et al., 1995), induces sprouting of motor axons in innervated skeletal muscle (Caroni and Grandes, 1990) and may slow the progression of clinical deterioration in ALS patients (Lange et al., 1996). Recent studies of IGF receptor distribution in ALS patients have demonstrated elevated [^{125}I] IGF receptor binding throughout the grey matter of spinal cord, especially in the ventral horns and intermediate regions (Adem et al., 1994; Dore' et al., 1996).

The trophic actions of IGF-1 appear to be mediated largely by the activation of the phosphatidylinositol 3-kinase (PI 3-K) signalling pathway (D'Mello et al., 1997; Feldman et al., 1997). PI 3-K has also been implicated as a mediator of the actions of other growth factors affecting cells of the central nervous system. For instance in rat PC-12 cells, the anti-apoptotic actions of NGF can be prevented by specific inhibitors of PI 3-K (Yao and Cooper, 1995). Cultured rat cerebellar neurons undergo apoptosis following a reduction in extracellular potassium and this neuronal death can be attenuated by IGF-1. The survival effect of IGF-1 administration under these circumstances is mediated in part by PI 3-K (D'Mello et al., 1997).

The lipid and protein-serine kinase, PI 3-K, is a heterodimer consisting of an 85 kDa regulatory subunit and a 110 kDa catalytic subunit (Kapeller and Cantley, 1994). This enzyme phosphorylates inositides at the D-3 position of the inositol ring to generate the lipid products PI-3-phosphate, PI-3,4-bisphosphate and PI-3,4,5-trisphosphate (Whitman et al., 1988). PI 3-K is associated with and is activated by a number of growth factor receptors with intrinsic or associated protein-tyrosine kinase activity. Insulin and IGF-1 activate PI 3-K by tyrosine phosphorylation of the insulin receptor substrate (IRS-1) which acts as an adapter protein, binding to the p85

subunit of PI 3-K via its SH2 domain and thereby mediating the activation of the enzyme (Kapeller and Cantley, 1994). PI 3-K has recently been studied very extensively due to its role in various cellular processes such as cell growth and differentiation, neurite outgrowth, cell survival and membrane trafficking (Vanhaesebroeck et al., 1996). Activation of PI 3-K and phosphorylation of phosphoinositides leading to its lipid products are currently thought to stimulate directly or indirectly, several protein kinases such as protein kinase B (PKB), some isoforms of protein kinase C (PKC) and p70 ribosomal S6 kinase (S6K).

To determine whether PI 3-K is altered in ALS tissue, the activity and protein amount of PI 3-K and other kinases that may be regulated by PI 3-K was measured in postmortem tissues from ALS patients and control subjects.

3.2.2. Results

PI 3-K activity was detectable in human post-mortem tissue from patients with ALS and controls as shown in a representative thin layer chromatography autoradiogram of spinal cord tissue. PI 3-K was immunoprecipitated prior to phosphotransferase activity measurements using an antibody against the p85 subunit of PI 3-K (Fig. 7A). Particulate fractions of spinal cord from ALS patients demonstrated significantly elevated PI 3-K activities which were approximately 250% of those in control spinal cord (Fig. 7A, Fig. 8A). There was variability in the PI 3-K activity measurements of different samples, likely reflecting patient variability, slight differences in the death to freezing interval, or post-mortem tissue storage. A similar increase of PI 3-K activities in particulate fractions from spinal cord tissue of ALS patients was identified using anti-phosphotyrosine (4G10) antibodies for immunoprecipitation. The increase in PI 3-K activity appeared to reflect a commensurate rise in PI 3-K protein amounts in the particulate fractions of spinal cord from ALS patients (approximately 200% higher; Fig. 7B, Fig. 8B).

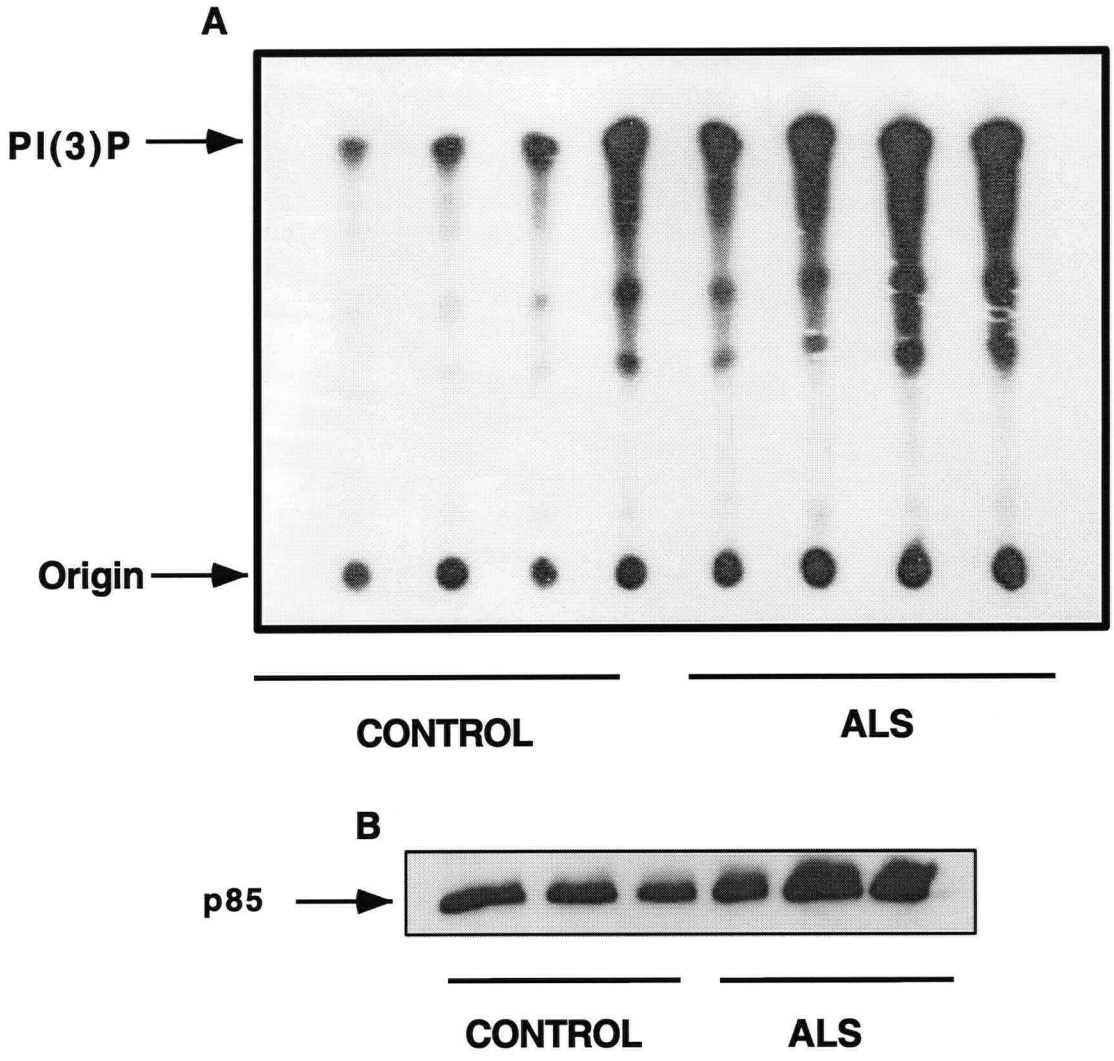

Fig.7. PI 3-K activity and protein level of spinal cord tissue from control and ALS patients. A. A representative of thin layer chromatography autoradiogram of immunoprecipitated particulate fractions of spinal cord tissue from control and ALS patients, using the antibody against p85 subunit of PI 3-K. The lipid product PI 3 (P) which reflects PI 3-K activity was significantly higher in spinal cord tissue from ALS patients compared to control subjects. B. Western blot analysis of p85 subunit of PI 3-K in spinal cord from control and ALS patients. ALS patients have a significant increase in the p85 protein level compared to control subjects ($p < 0.01$; $n=3$).

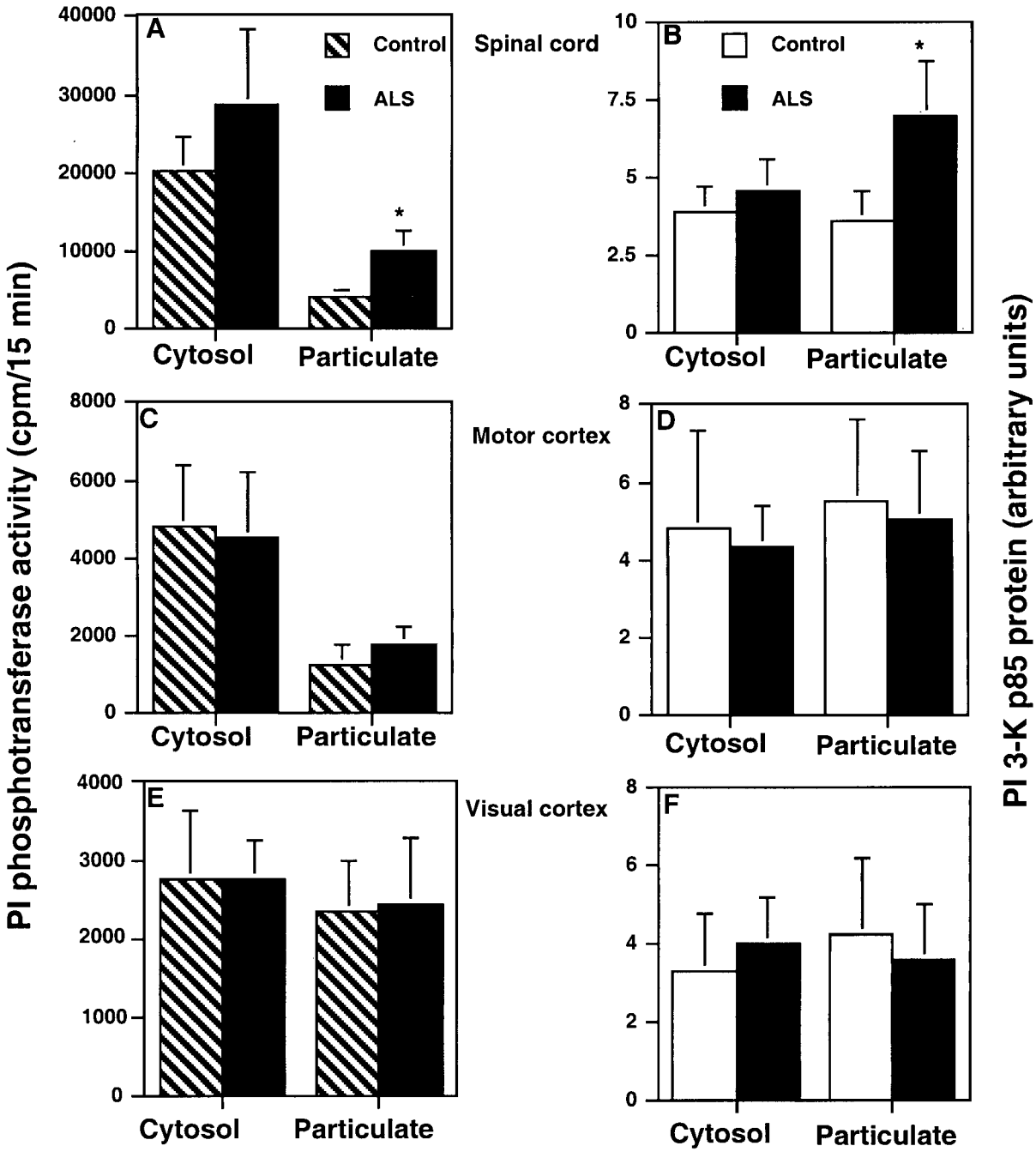

Fig. 8. PI 3-K activities (A,C,E) and protein levels (B,D,F) from three control nervous system regions of control subjects (hatched bars) and ALS patients (filled bars). Regions included spinal cord (A,B), motor cortex (C,D) and visual cortex (E,F). PI 3-K activities in the particulate fractions of spinal cord were significantly increased ($p < 0.05$) in tissues from ALS patients compared to control subjects. The protein level in the particulate fraction was also significantly elevated in ALS spinal cord tissue fractions ($p < 0.05$). Values shown indicate mean ± SEM.

In contrast, cytosolic fractions of spinal cord tissue from ALS patients had activities and protein levels that were not significantly different from controls (Fig. 8A, Fig. 8B). PI 3-K activities and protein amounts were unchanged in motor and visual cortices from ALS patients compared to controls (Fig. 8C-F). As an additional control, PI 3-K activities and protein levels were evaluated in motor cortices from patients with Alzheimer's disease (AD). No significant differences were found in AD tissue compared to controls (Fig. 9). Addition of 100 nM wortmannin resulted in a 50% inhibition of the production of PI 3 (P) by PI 3-K isolated from control, ALS and AD tissue (Fig.9). PI 3-K activities and protein levels were higher in motor cortices from the three samples used as controls for AD tissue, compared to the five samples used as controls for the ALS tissue. The reason for this difference could be that brain samples used as controls for AD tissue had shorter death to freezing intervals, as a group, than did controls used for ALS tissue (see death to freezing intervals in Chapter 2. Materials and Methods).

The protein kinases PKB and S6K are regulated by PI 3-K in some systems and here the activities and protein levels of these kinases were evaluated in tissue from ALS patients. There was a significant elevation of PKB protein amounts in particulate fractions of spinal cord in ALS patients. This was not accompanied by significant differences in the activity of PKB either in the particulate or cytosolic fractions (Fig.10A&B). Elevated levels of S6K protein were also seen in ALS spinal cord, whereas the activity of S6K showed no significant differences between ALS and control tissue (Fig.10C&D). As a control Erk1/2 was also evaluated, a protein kinase which is not directly regulated by PI 3-K. The activities and protein levels of Erk were unchanged in ALS tissue compared to controls (Fig.11A&B). Activities and protein levels of PKC were also measured using immunoprecipitation with anti-PKC $\alpha\beta$ antibodies followed by an <u>in vitro</u> kinase assay and immunoblotting.

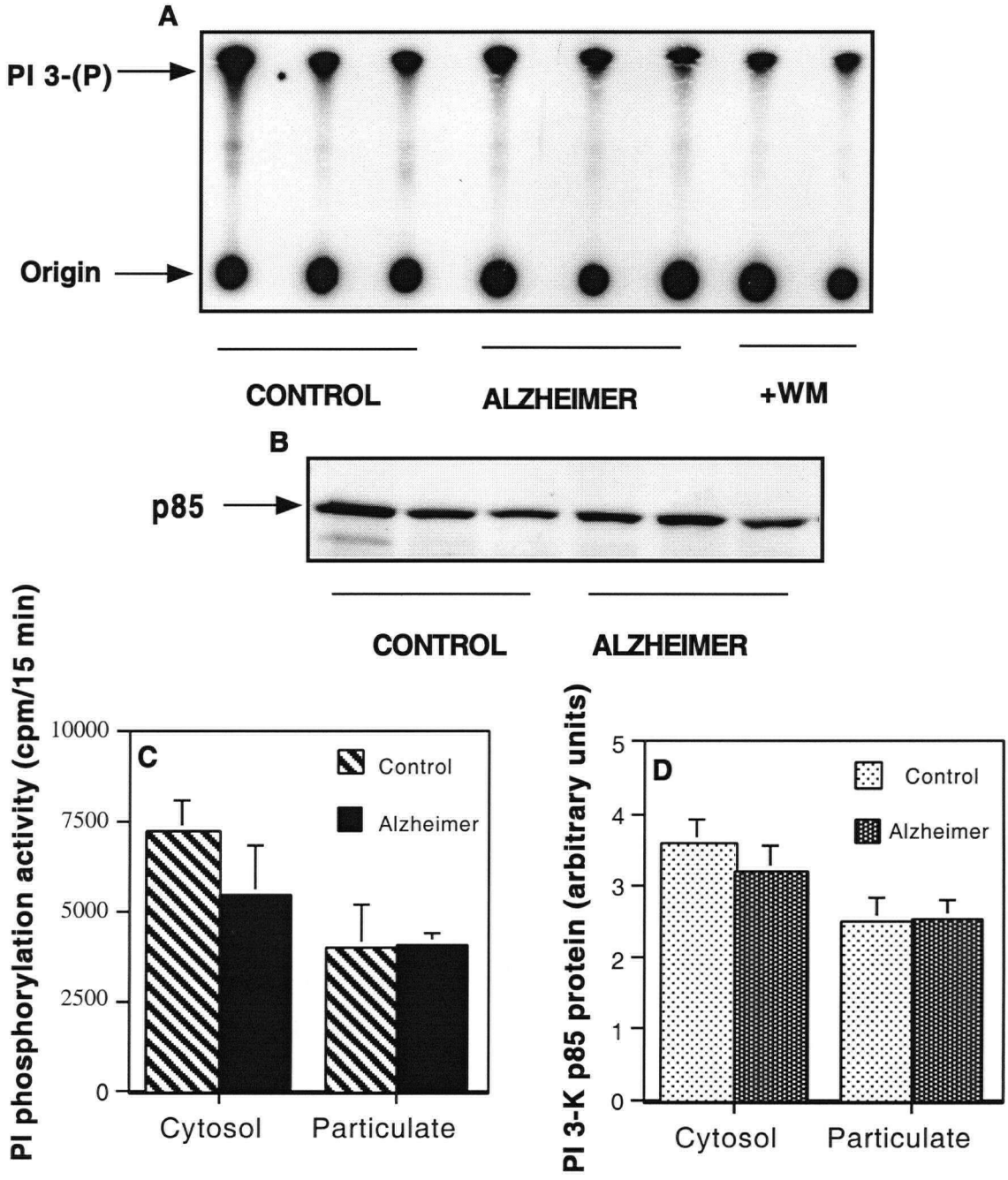

Fig.9. PI 3-K activity and protein level of motor cortex tissue from control and Alzheimer's patients. A. Autoradography of thin layer chromatography of immunoprecipitated particulate fractions of motor cortex from control and Alzheimer's patients detected using antibody against the p85 subunit of PI 3-K. The lipid products PI 3-(P) were not significantly different between the two groups. B.No differences in p85 protein level were seen in the particulate fractions of motor cortex from control and Alzheimer's patients. C&D. No differences were found in PI 3-K activity and protein level of motor cortex from patients who died with Alzheimer's disease compared to control subjects in both cytosolic and particulate fractions. Error bar indicates SEM; n=3.

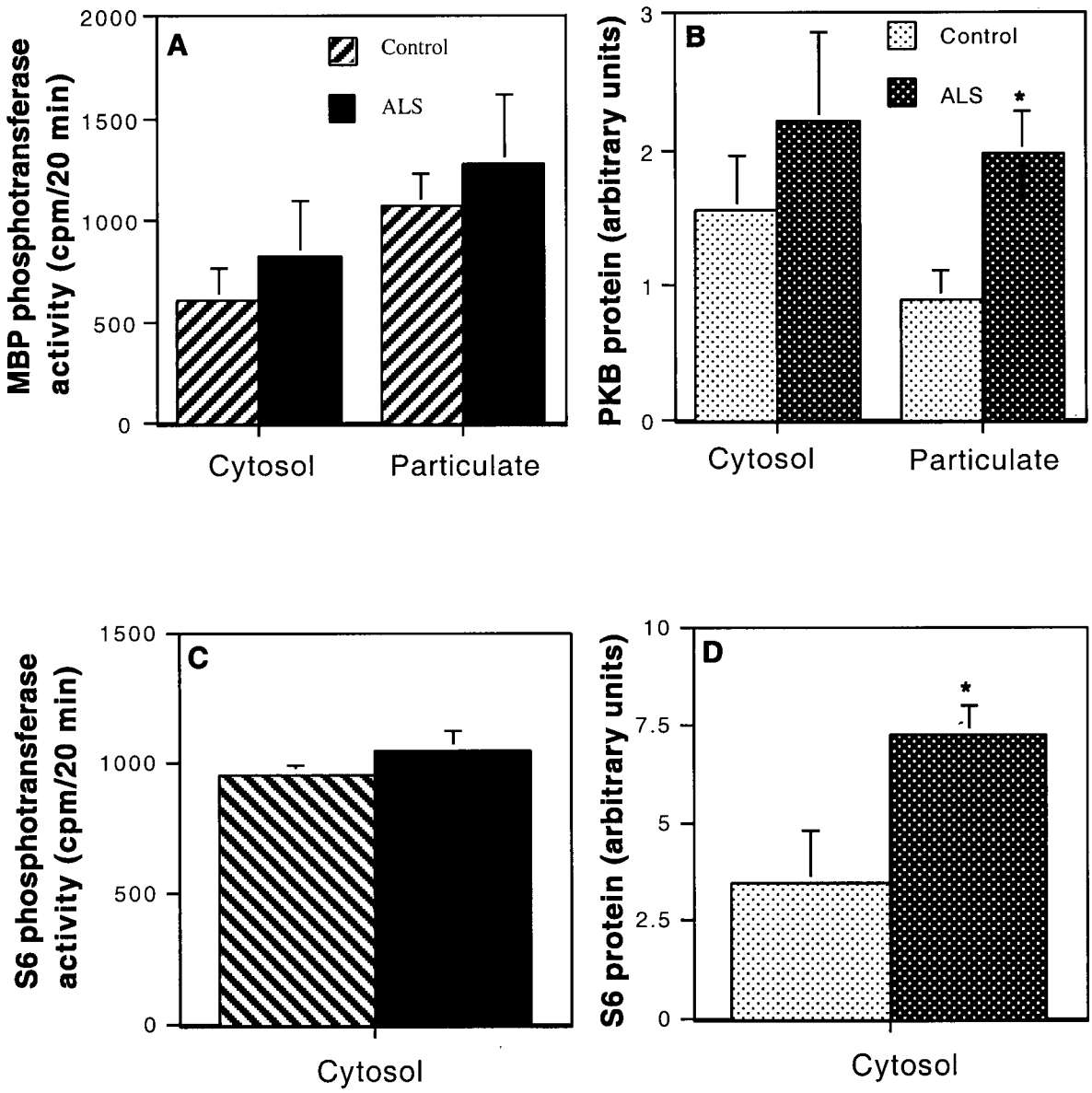

Fig.10. PKB and S6K activities and protein levels of spinal cord tissue from control and ALS patients. A.&B. PKB activity and protein measurement from ALS and control spinal cord tissue in cytosol and particulate fractions. No significant difference was seen in PKB activity between control and ALS tissue. Protein amount of PKB was significantly higher in particulate fractions from ALS patients ($p < 0.05$; n=5). C&D. In vitro kinase activity and protein amount of S6K from cytosolic fraction of spinal cord tissue from control and ALS patients. Protein amount of S6 was significantly increased in ALS samples compared to controls ($p < 0.05$;n= 5). Error bar indicates SEM.

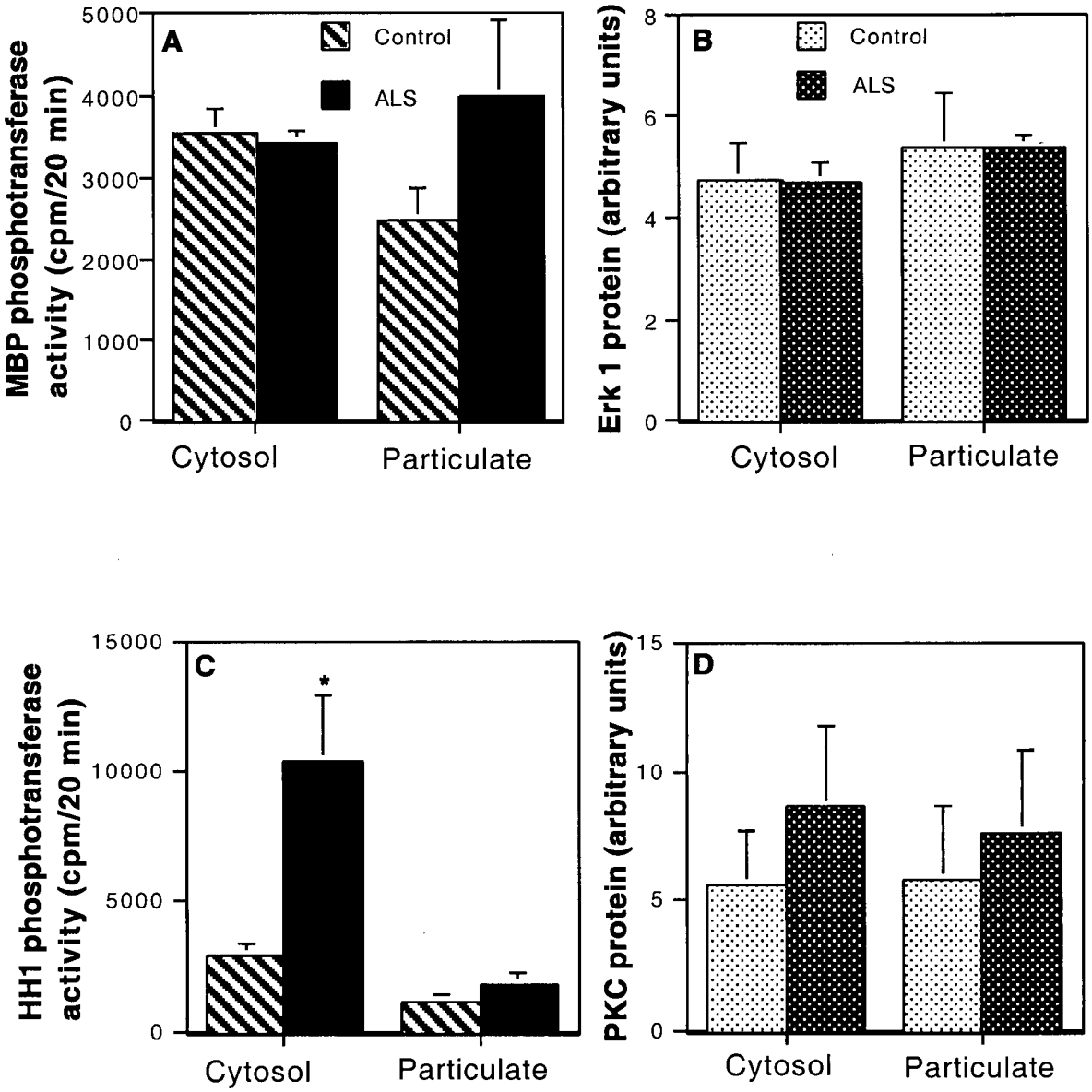

Fig.11. Erk1 and PKC activities and protein levels of spinal cord tissue from control and ALS patients. A&B. Erk1 activity and protein amount in cytosol and particulate fractions of spinal cord tissue from control and ALS patients. No significant differences were found in activity and protein amount between control and ALS tissues. C&D. Activity and protein measurement of PKC in spinal cord tissues from control and ALS patients. There is a significant increased of PKC activity in cytosolic fractions of ALS compared to control subjects ($p < 0.01$; $n= 5$). Error bars indicates SEM.

PKC activity was found to be significantly increased in cytosolic fractions from ALS samples (Fig.11C&D). No significant differences were found in the activities or protein levels of PKC in particulate fractions from ALS patients.

3.2.3. Discussion

To my knowledge, the present results demonstrate for the first time that PI 3-K activities and protein levels are detectable in human nervous system tissue. The use of monoclonal p85 antibody which does not activate the PI 3-K enzyme in these experiments, suggests that the PI 3-K activity measured here reflects the activity of the immunoprecipitated PI 3-K in the tissue lysates. In the spinal cord and motor cortex, PI 3-K activities were three to five-fold higher in the cytosolic fractions than in particulate fractions, an observation which is consistent with PI 3-K activities in resting 3T3 fibroblasts (Susa et al., 1992). Upon activation, PI 3-K is thought to translocate from the cytosol to the plasma membrane (Kapeller and Cantley, 1994; Susa et al., 1992) and for this reason PI 3-K activities were assayed in both fractions. In the cytosol, the p85 protein content is believed to reflect PI 3-K activity, whereas in the membrane, other factors such as intrinsic enzymatic activation influence PI3-K activity (Susa et al., 1992). Spinal cord tissue from patients who died with ALS has significantly higher PI 3-K activities and protein levels in particulate fractions compared to control tissue. Cytosolic fractions of spinal cord tissue from ALS patients did not show significant differences in activities and protein levels from controls. The increase in PI 3-K activities in the particulate fractions of ALS spinal cord tissue can be attributed to the elevated p85 protein levels. Protein levels of PI 3-K were elevated two-fold in ALS tissue consistent with a comparable rise in PI 3-K activities. Thus, these changes would represent an increase in total PI 3-K activity of

the particulate fraction but with unchanged specific activity. Analysis of activities and protein levels of PI 3-K in other brain regions from patients with ALS and controls did not reveal any statistically significant differences. This included both motor cortex, an area that is pathologically involved in ALS, as well as visual cortex, a region which is spared. As an additional control, PI 3-K activities and protein levels were evaluated from motor cortices of patients who died with Alzheimer's disease (AD) and unaffected controls. However, no statistically significant differences were found.

To extend these observations, signalling pathways which could be affected by PI 3-K were explored, and the activities and protein levels of PKB were evaluated, as this protein-serine/threonine kinase is regulated by PI 3-K in many cell types (Dudek et al., 1997; Franke et al., 1997). The result showed that protein levels of PKB were significantly elevated in particulate fractions of spinal cord from patients with ALS. However, no significant differences were observed in PKB activities in spinal cord tissue between ALS patients and controls in either cytosolic or particulate fractions and protein levels were unchanged in the cytosol between the two groups. A second protein kinase that is downstream of PI 3-K in some cells is S6K, which is primarily cytosolic in distribution. Analysis of this kinase revealed elevated protein levels in ALS spinal cord tissue with unchanged activity. In contrast, both the activities and protein amounts of Erk1/2, which is not directly regulated by PI 3-K, were unchanged in ALS spinal cord tissue. Activities of PKC were elevated in cytosolic fractions of spinal cord tissue from ALS patients, an observation reported previously (Lanius et al., 1995). Although protein levels were higher in ALS tissue than controls, these differences did not reach statistical significance.

The reason for elevated PI 3-K activities and protein levels in particulate fractions of ALS spinal cord tissue is unclear. This observation could be attributed to activation of PI 3-K by growth factors which are stimulated by the muscle denervation, as well as motoneuron loss. In support of this view, Ito and colleagues (1996) have demonstrated elevated PI 3-K gene expression in motoneurons whose axons have been subjected to a crush injury. IGF-1 gene expression is also found to be elevated distal to the site of crush in rat sciatic nerve (Glazner et al., 1994). In ALS, there is prominent muscle denervation which arises as a consequence of the progressive motoneuron loss. It is likely that denervated muscle tissue, Schwann cells surrounding degenerating axons, and other cells such as microglia and reactive astrocytes will respond to this insult with an increase in IGF-1 protein. [^{3}H] IGF-1 receptor density is increased in spinal cord tissue from ALS patients indicating receptor upregulation (Adem et al., 1994; Dore et al., 1996; Dore et al., 1997). However, levels of immunoreactive IGF-I and IGF-II in ALS spinal cord do not differ from controls (Kerkhoff et al., 1994). Although serum levels of IGF-1 in ALS patients are unchanged compared to controls, serum levels are not a reflection of local IGF concentrations in spinal cord tissue (Braunstein and Reviczky, 1987). Other trophic factors beside the IGFs are altered in ALS (Duberley et al., 1997). It is likely that other stimuli beside trophic factors increase PI 3-K activity in ALS tissue, for instance many cytokines activate PI 3-K (Duronio et al., 1998). It is also possible that oxidative injury in ALS (Ferrante et al., 1997) either acting directly, or indirectly through the mediation of cytokines could augment PI 3-K activity in ALS. It is unclear whether the elevated PI 3-K protein level observed in ALS is causative for the disease, a consequence of neuron loss, or a compensatory protective response of

surviving cells.

PI 3-K activation has been well established following exposure to growth factors making it likely that the observed elevation of PI 3-K activity is a compensatory response. The nature of the cell types producing PI 3-K in ALS tissue is unknown. Immunocytochemical studies will be required to identify the sources of elevated PI 3-K protein. PI 3-K protein and activity are elevated in spinal cord tissue of ALS patients, but not in motor or visual cortices. Visual cortex is preserved in ALS, but neuropathological involvement of motor cortex is frequent. Failure to detect changes in PI 3-K activity in motor cortex likely results from the absence of prominent denervation in this region. In tissue from motor cortex in patients with AD, we observed no changes in PI 3-K activities or levels. This was expected as motor cortex is typically unremarkable on neuropathological study in AD. As PI 3-K activities and protein levels are assayed from tissue, it is not possible to identify the specific cell type(s) responsible for the elevated PI 3-K activities (e.g. neurons, glia, microglia). It is also possible that the elevated PI 3-K activities and protein levels derive from surviving neurons which are responding to unknown growth factors arising from nerve or muscle denervation, or injury.

A further explanation for the altered PI 3-K protein levels in ALS could be increased activity of enzymes which degrade the 3-phosphorylated inositol phospholipids resulting in enhancement of PI 3-K activity in ALS tissue as a compensatory mechanism. Additional support for the involvement of a PI 3-K-mediated pathway in ALS is the observation that elevated protein levels of PKB and S6K are found in ALS tissue. It is tantalizing that protein levels of these two kinases which lie downstream of PI 3-K are also elevated in ALS. This observation may reflect an upregulation of protein levels of kinases that could be directly stimulated by the activation of PI 3-K. In spite of the increased protein levels of PKB and S6K there was not a commensurate increase in the activity of PKB or S6K indicating an

impairment in the connections between PI 3-K and some of its downstream effectors. The basis for an impairment in the connection between PI 3-K and its effectors is unclear. However, it could result from either a rapid degradation of the lipid products of PI 3-K in ALS tissue or dephosphorylation of enzymes such as PKB and S6K in ALS. Although there is currently no direct evidence indicating that dephosphorylation reactions are impaired in ALS tissue, there is indirect evidence indicating that protein phosphatase (PP2B) activity may be decreased in spinal cord tissue from ALS patients (Wagey et al., 1997). The lipid products of PI 3-K, PI-3,4-bisphosphate and PI-3,4,5-trisphosphate, have been reported to activate some Ca^{2+} -independent isoforms of PKC such as the novel PKC-ε, PKC-η and the atypical PKC-ζ (Duronio et al., 1998; Nakanishi et al., 1993; Toker et al., 1994). In stimulated cells, both PKC-ε and PKC-λ are activated downstream of PI 3-K (Toker and Cantley, 1997). Wortmannin was reported to inhibit the phosphorylation of pleckstrin, a substrate of PKC in stimulated platelets indicating that PI 3-K acts upstream of a physiological substrate of PKC (Toker and Cantley, 1997). There is evidence that 3-phosphorylated inositol phospholipids activate several PKC isoforms in vitro, including the conventional isoform PKC-α (Duronio et al., 1998). It is of interest that previous work has shown elevated activities and protein levels of Ca^{2+} -dependent PKC isoforms in spinal cord tissue from ALS patients (Lanius et al., 1995; Krieger et al., 1996).

These analyses were performed in human tissue obtained at post-mortem and are confounded by variables arising from the delay between death to freezing of tissue, medication effects, as well as other factors related to concurrent illness. In these experiments efforts were made to minimize variables arising from the selection of tissue from patients who died with ALS and controls. Nonetheless, it would be of interest to evaluate the activities and protein levels of PI 3-K, PKC, PKB and S6K in animal models which are believed to mimic human ALS. An important issue which remains to be addressed is the measurement of the PI 3-K lipid products in neurons

and other cells. These studies have typically been performed using radioactive tracers to label lipids in cell lines. At present there is no simple method of measuring small amounts of these lipids extracted from human tissue.

CHAPTER 4. MODULATION OF NMDA-MEDIATED EXCITOTOXICITY BY PROTEIN KINASE C

4.1. Introduction

N-methyl-D-aspartate (NMDA) receptors are heteromeric proteins composed of NR1 subunits (ζ1), along with one or more subunit such as NR2A (ε1), NR2B (ε2) NR2C (ε3), or NR2D (ε4) (Sucher et al., 1996). The regulation of NMDA receptor (NMDAR) function has been extensively studied using recombinant NMDARs expressed heterologously in human embryonic kidney (HEK)-293 cells, or other cells. Studies using these cell lines have established that HEK cells transfected with NMDAR subunits die following exposure to NMDA, depending on the NMDAR subunit composition (Cik et al., 1994; Anegawa et al., 1995; Raymond et al., 1996). For instance, transfection of HEK-293 cells with cDNAs for the NR1 subunit of the NMDAR, produces much less cell death than when cells are co-transfected with cDNAs for NR1 and NR2A subunits (Raymond et al., 1996). Analysis of whole-cell currents from NR1/ NR2A transfected cells have demonstrated that NMDA-evoked currents were much larger than from NR1 transfected cells, indicating that the increased cell death observed with the NR1/ NR2A co-transfected cells is dependent on the expression of functional NMDAR channels having appreciable ionic flux. Both Na^+ and Ca^{2+} influx appear to contribute to the cytotoxicity following NMDAR activation. However, experiments in HEK cells using mutant NMDARs with decreased Ca^{2+} permeability have shown that cell death is more frequent in cells with NMDARs having high Ca^{2+} permeability (Raymond et al., 1996).

Protein kinase C (PKC) activation modulates NMDAR-mediated currents (Ben-Ari et al., 1992; Markram and Segal, 1992; Urushihara et al., 1992; Raymond et al.,

1994; Zukin et al., 1995; Xiong et al., 1998; Logan et al., 1999; Zheng et al., 1999; see: MacDonald et al., 1998 for review) and Ca^{2+} influx through NMDAR channels (Murphy et al., 1994; Grant et al., 1998). Although PKC activation has been thought to be responsible for modulating excitotoxic neuronal death, the evidence is contradictory (e.g. Favaron et al., 1988; Durkin et al., 1997). For example, Felipo and colleagues (1993) claim that inhibitors of PKC are able to protect cultured cerebellar neurons from excitotoxic death. In contrast, Durkin and coworkers (1997) report that cerebral cortical neurons which have been in culture for 8 days become highly vulnerable to neuron death when exposed to excitatory amino acids (EAAs), including NMDA, in the presence of PKC inhibitors. In the absence of PKC inhibitors the neurons are relatively resistant to neurotoxicity.

To establish if the activation of PKC is involved in NMDA-induced cell death, the extent of cell death in HEK cells transfected with NMDAR subunits was evaluated.

4.2. Results

4.2.1. PKC protein and activity in transfected HEK 293 cells

To demonstrate the presence of PKC protein in HEK-293 cells transfected with NR1A or NR1A/NR2A subunits, Western blotting was performed using a monoclonal antibody against PKC $\alpha\beta$. As shown in Fig.12A, an 80 kDa protein band was present in HEK cells transfected with either NR1A alone, or NR1A/NR2A subunits.

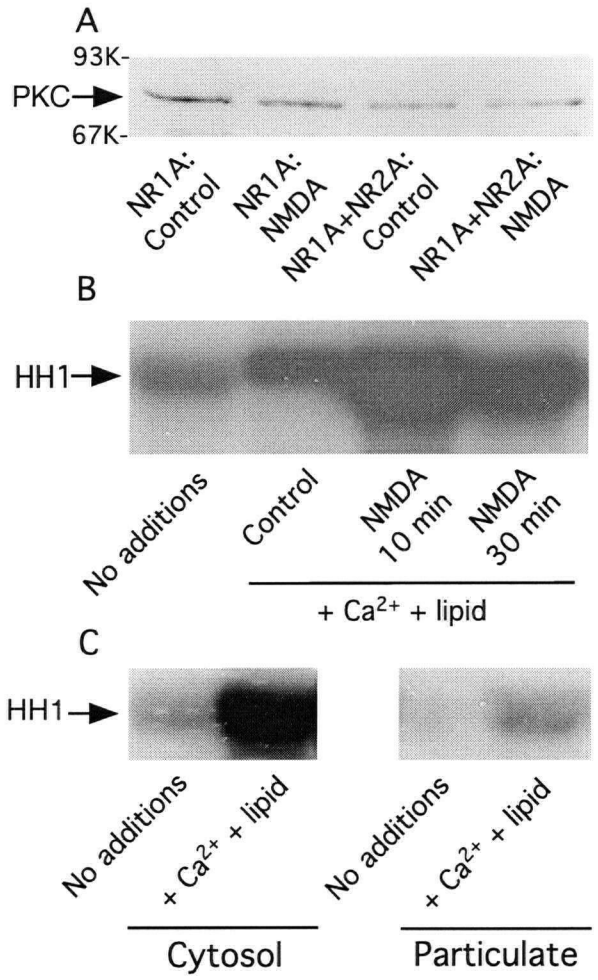

Fig.12. PKC activity and protein level in transfected HEK-293 cells. A. Western blot analysis of PKC αβ. HEK-293 cells transfected in parallel with NR1A, or NR1A +NR2A subunits in the presence of NMDA and glycine (NMDA), or unstimulated (control) were immunoblotted with a monoclonal antibody against PKC αβ. The 80 kDa immunoreactive band corresponds to PKC (arrow). B. Autoradiography of HH1 phosphotransferase activity of PKC in transfected cells. Total lysates of NR1A/NR2A transfected HEK cells were used for an *in vitro* PKC assay using HH1 as a substrate. Samples were assayed for Ca^{2+} and lipid-dependent (control) and independent (No additions: Ca^{2+} & lipid) activity. Parallel cultures were unstimulated and stimulated with NMDA and glycine (NMDA) and assayed at 10 min and 30 min following NMDA. C. Autoradiography of HH1 phosphorylation from an *in vitro* assay using immunoprecipitated PKC from cytosolic and particulate fractions of NR1A/ NR2A transfected cells under basal conditions (unstimulated).

PKC activities were assessed in total lysates from HEK cells transfected with NR1A/NR2A subunits prior to and following exposure to NMDA using histone H1 as a substrate for PKC. Ca^{2+}- and lipid-dependent PKC activity was evident in unstimulated cells (Fig. 12B). PKC activity increased 10 min following exposure to NMDA and glycine (NMDA) and declined by 30 min. Analysis of cellular fractions demonstrated that most of the PKC activity was cytosolic (Fig. 12C).

PKC activity towards HH1 and protamine was increased by two-fold (105 ± 28%; n=3) compared to control cells following PMA (85 nM) stimulation of HEK cells transfected with NR1A/ NR2A subunits. As an indirect measure of PKC activation, the expression of Erk1 was evaluated, as Erk1 is activated by PKC, or PMA stimulation in many cell types (Fiore et al., 1993; Schwenger et al., 1996). The increased tyrosine phosphorylation of Erk1 and Erk2 reduces their mobility in SDS-PAGE (Fiore et al., 1993). This bandshift phenomena is particularly evident with Erk1. Western blot analysis using an anti-Erk1 antibody demonstrated that PMA exposure led to time-dependent Erk1 activation (Fig. 13A). Ten min following PMA (85 nM), a reduction in mobility (bandshift) was evident compared to unstimulated cells (pp44). The bandshift associated with PMA stimulation was maximal at 10 to 20 min and gradually decreased over 60 min. Further confirmation of Erk1 activation was obtained using a mouse monoclonal anti-phospho-Erk antibody which reacts specifically with Thr-202 and Tyr-204 of phosphorylated Erk1 and Erk2 (Crews et al., 1992). Western blotting with the anti-phospho-Erk antibody demonstrated the presence of phosphorylated Erk1 and Erk2 following PMA stimulation, with the peak phosphorylation occurring at 10 to 20 min (Fig 13B, pp44).

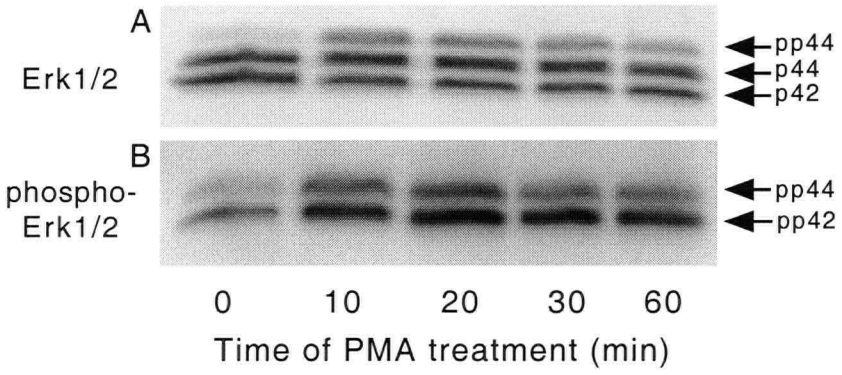

Fig. 13. Western blot analysis of Erk following PMA stimulation in HEK-293 cells. A. HEK cells were treated with PMA for different time periods (10 -60 min) and probed with an anti-Erk1-CT antibody. The Western blot of Erk exhibited a reduced mobility band (pp44) which is attributed to the activation of Erk in cells pre-treated with PMA. Erk activation is time-dependent and is maximal between 10 - 20 min. B. Western blot of anti-phospho-Erk in transfected HEK cells following PMA treatment (10-60 min). Each lane in A and B represents the same amount of protein loaded from cell lysates of sister cultures.

4.2.2. PKC activation potentiates NMDA-induced cell death

To assess cell death in HEK-293 cells, a trypan blue exclusion assay was performed 6 h following various treatments. Cultures transfected with NR1A/NR2A and maintained in PSS (control) had 13.8 $\pm$ 1.4 % cell death (Fig.14A). Exposure to NMDA and glycine (NMDA) significantly increased cell death ($p < 0.005$, Fig. 14A) and pre-incubation with a selective PKC inhibitor, RO 320432 (RO), prior to NMDA exposure led to a reduction in cell death which was comparable to control cultures. RO is a selective PKC inhibitor that interacts with the ATP binding site in the catalytic region of PKC (Birchall et al., 1994) and has the highest selectivity towards PKCα, followed by PKC β (Wilkinson et al., 1993). Application of RO (1 μM) alone to NR1A/NR2A transfected HEK cells did not change cell viability significantly (17.1 $\pm$ 3.1% dead cells), compared to unexposed cultures (13.8 $\pm$ 1.4 %), indicating that RO has no cytotoxicity at this concentration (Fig. 14A). Measurement of cell death at 12 h following treatments resulted in an increase of cell death in control cultures to a similar level as cells exposed to NMDA. Treatment with RO alone or RO treatment prior NMDA exposure also increased cell death at 12 h (Fig. 14A). In cultures which were transfected with NR1A subunits alone and maintained in PSS, cell death at 6 h was 3.3 $\pm$ 0.1 % (significantly lower than cultures transfected with both NR1A/NR2A subunits, $p< 0.005$, unpaired t-test; Fig. 14B). Exposure to NMDA did not enhance cell death and incubation with RO alone or prior NMDA exposure also did not alter percent of dead cells compared to control cultures in NR1A transfected cells (Fig. 14B).

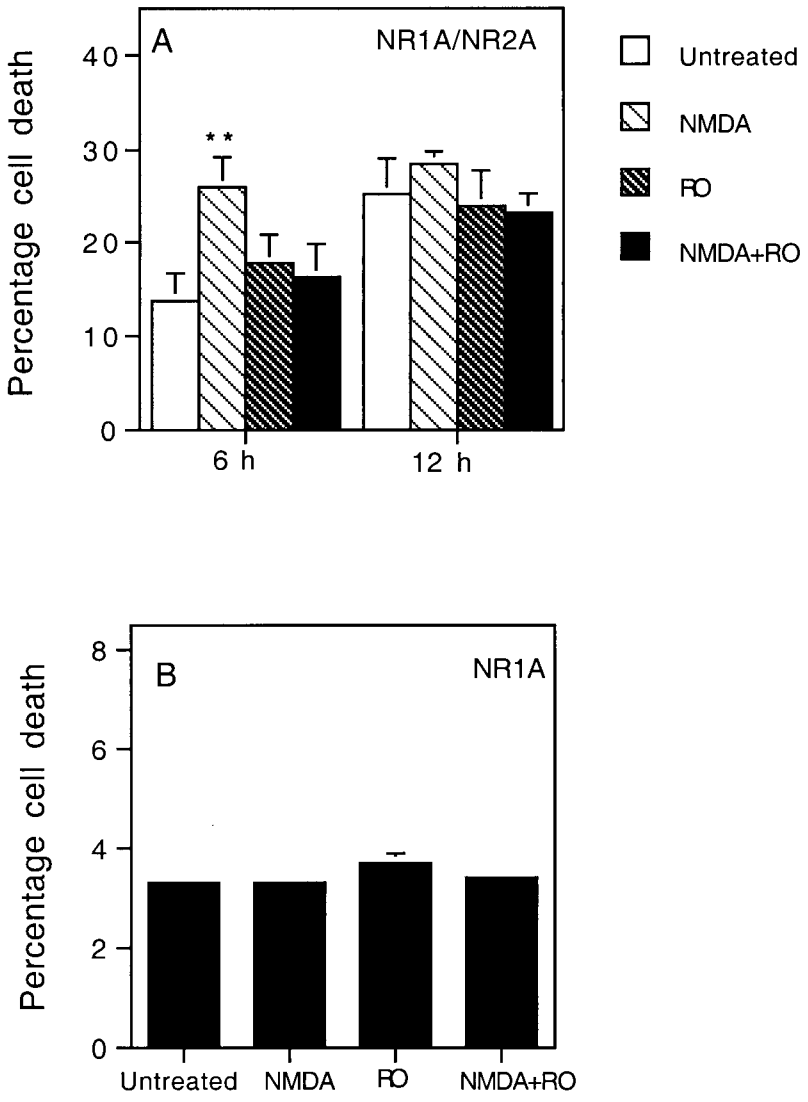

Fig. 14. Cell death in NR1A and NR1A/NR2A transfected HEK-293 cells . A. NMDA significantly increased cell death in NR1A/NR2A transfected cells at 6 h but not at 12 h post-treatment compared to control. RO alone or pre-exposure to RO prior NMDA treament was not significantly different from untreated culture. **significantly different from control ($p < 0.005$). NMDA and RO did not change cell death in NR1A transfected cells compared to control. No significant differences in cell death was observed between control and treatment with NMDA alone, RO alone and RO plus NMDA.

To determine if stimulation of PKC has a cytotoxic effect on transfected HEK cells, PMA (85 nM) was applied for 10 min, either alone (PMA), or with NMDA and glycine (NMDA) and cell death was assessed 6 h following the treatments. Under these experimental conditions, control cultures transfected with NR1A/NR2A subunits and maintained in a physiological saline solution (PSS) had a mean of 13.8 $\pm$ 1.4 % cell death at 6 h (Fig. 15). As shown in Fig.15, PMA alone did not produce a statistically significant change in the percent of dead cells compared to control cultures. Treatment with NMDA and glycine (NMDA) significantly enhanced cell death to 27.4 $\pm$ 1.3 % ($p < 0.005$). Exposure of NR1A/NR2A transfected cultures to PMA 10 min prior to NMDA stimulation significantly potentiated the cytotoxic effect of NMDA and resulted in 37.6 $\pm$ 1.4 % cell death (Fig. 15, PMA + NMDA). This result was statistically greater than the percentage of cells which died following exposure to NMDA alone ($p < 0.01$), PMA alone ($p < 0.05$) or under control conditions ($p < 0.0005$). These data indicate that pre-exposure to PMA significantly enhanced the toxicity associated with NMDA receptor stimulation.

To confirm that the cytotoxic effect was mediated by PKC, the PKC inhibitor, RO 320432 (RO), was applied prior to treatment with PMA and NMDA. When cultures were pre-treated with RO for 10 min prior to exposure to PMA and NMDA, the percentage of cell death was significantly reduced ($p < 0.01$) compared to PMA and NMDA exposure without RO pre-treatment. The RO pre-treatment appeared to block the augmentation of cell death produced by PMA exposure and produced a similar percentage of cell death as with NMDA treatment alone (Fig.15).

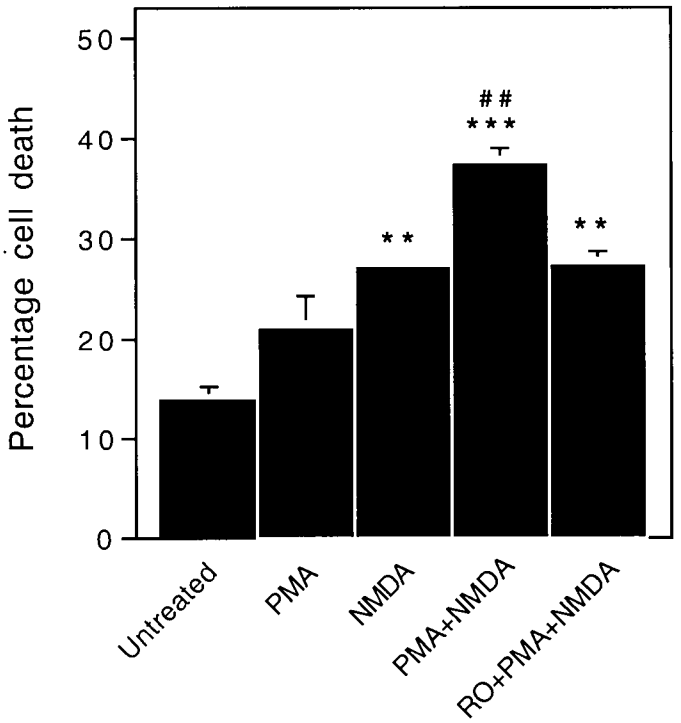

Fig. 15. Cell death following treatment with NMDA, PMA and RO 32-0432 (RO). PMA potentiates NMDA-mediated cell death in NR1A/NR2A transfected cells. Exposure to PMA alone was not significantly different from control, whereas NMDA-treated cells had a significantly higher percentage cell death compared to control ($p < 0.005$). A 10 min exposure to PMA prior NMDA-treatment potentiated the cytotoxic effect of NMDA and glycine (NMDA) and caused a significant increased in death compared to NMDA and glycine (NMDA) alone ($p < 0.01$). Pre-incubation with RO prior to exposure to PMA and NMDA led to a reduction in cell death compared to exposure to PMA and NMDA. Data derived from duplicate samples performed in at least 3 separate experiments. *** Significantly different from control ($p < 0.0005$); ** significantly different from control ($p < 0.005$); ## significantly different from NMDA and glycine alone (NMDA), and to pre-exposure to RO with PMA and NMDA ($p < 0.01$).

To further examine the potentiation effect of PMA on NMDA-mediated cell death in HEK cells transfected with NR1A/NR2A subunits, Western blotting was performed using antibodies against phosphorylated forms of PKC$\alpha\beta$ (phospho-PKC) and Erk1 (phospho-Erk). The expression of phosphorylated PKC protein did not change between control and treated cultures (Fig. 16A). In contrast, levels of phospho-Erk protein were greater when cultures were exposed to PMA and had the highest expression in cultures exposed to both PMA and NMDA (Fig. 16B). From this data it appeared that exposure to PMA and NMDA did not lead to changes in the phosphorylation of PKC itself. However we detected an increase in the phosphorylation of Erk 1 or Erk2, after exposure to cells to PMA, or to PMA with NMDA.

4.2.3. Calcium-dependent isoforms of PKC are involved in potentiating NMDA-mediated cell death

To examine which isoforms of PKC are responsible for the NMDA-mediated cell death, NR1A/NR2A transfected HEK cells were pre-treated with specific PKC inhibitors. Pseudo A is a myristoylated peptide containing amino acids 19-27 of PKC which is cell permeable and binds to the pseudosubstrate sequence of PKC specifically inhibiting Ca^{2+} and lipid-dependent PKC isoforms (α,β and γ). Pseudo Z is a myristoylated cell permeable compound which specifically inhibits the action of PKC ζ by interfering with the substrate binding site in the catalytic domain (Shen and Buck, 1990; Liu et al., 2000). Fig.17 shows the effects of these inhibitors and RO on NMDA-mediated cell death in transfected HEK cells.

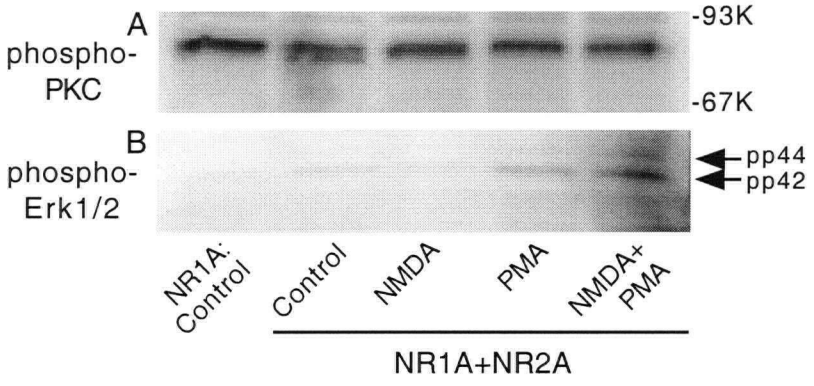

Fig. 16. Western blot analysis of phospho-PKC and phospho-Erk in NR1A and NR1A/NR2A transfected HEK-293 cells treated with PSS alone (control), NMDA, PMA or PMA and NMDA.A. Western blotting of PKC in NR1A and NR1A/NR2A transfected cells treated with PSS alone (control), NMDA, PMA or PMA and NMDA. The blot was probed with a phospho-PKC $\alpha\beta$ antibody. No differences were apparent in the band density between the different treatments. B. Western blotting analysis of phospho-Erk in NR1A and NR1A/NR2A transfected cells. Cells were treated with PSS alone (control), NMDA and glycine (NMDA), PMA, or PMA and NMDA. The blot was probed with a phospho-Erk antibody. The highest density bands of the phosphorylated Erk1 and Erk2 were present in NR1A/NR2A transfected cells which were exposed to PMA and NMDA. Each lane in A and B represents the same amount of protein loaded from cell lysates of cultures transfected in parallel.

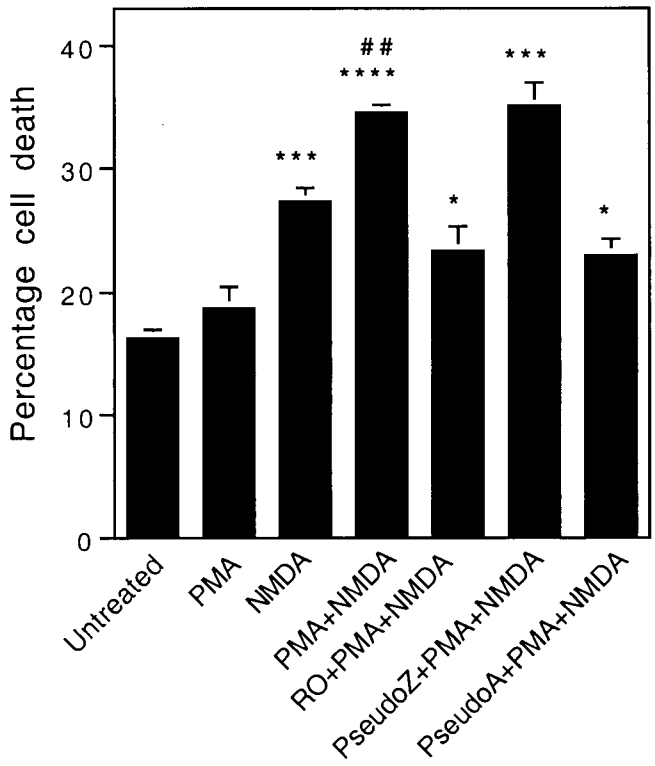

Fig .17. Cell death in NR1A/NR2A-transfected HEK-293 cells pre-exposed to different PKC inhibitors. NMDA-treated, or PMA and NMDA-treated cells had a significantly higher cell death compared to control cells. Pre-treatment with RO 32-0432 (RO), or pre-treatment with pseudo A (calcium-dependent PKC inhibitor) prior to exposure to PMA and NMDA significantly reduced cell death compared to treatment with PMA and NMDA alone ($p < 0.01$ for RO and $p < 0.005$ for pseudo A). Pre-treatment with pseudo Z (PKC ζ inhibitor) prior to PMA and NMDA treatment did not affect cell death. **** Significantly different from control ($p< 0.00005$); *** significantly different from control ($p< 0.0005$), * significantly different from control ($p< 0.05$), ## significantly different from NMDA alone, pre-exposure to RO, and pre-exposure to Pseudo A ($p< 0.01$). Data derived from duplicate batches of transfected cells for each condition performed in at least 3 separate experiments.

As shown previously, cells exposed to NMDA alone, or PMA and NMDA significantly enhanced cell death compared to control cultures. Pre-exposure to pseudo Z prior to treatment with PMA and NMDA had no effect on cell death. Preincubation with pseudo A prior to treatment with PMA and NMDA significantly reduced cell death compared to PMA and NMDA-treatment alone (Fig. 17; $p < 0.005$). The reduction in cell death seen with exposure to the classical PKC inhibitor, Pseudo A, was similar to that achieved with the pan-specific PKC inhibitor RO and was also similar to the cell death found with NMDA stimulation in the absence of PMA.

4.2.4. PKC downregulation reduces cell death in NR1A/NR2A transfected cells

PKC activity in many cell types is reduced by longer term exposure to PMA ('downregulation'). To determine whether downregulation of PKC could alter cell death in transfected HEK cells, cells were preincubated with PMA using different exposure times (from 10 min to 3 h) prior to NMDA treatment. As shown previously, exposure to PMA 10 min prior to NMDA, increased cell death compared to NMDA alone (Fig. 18). Increasing the duration of PMA pre-exposure led to a reduction in the percent of dying cells. At 20 and 30 min following PMA treatment prior to NMDA exposure, cell death was significantly lower than when NMDA was applied 10 min following PMA treatment ($p< 0.05$). A 3 h pre-treatment with PMA prior to NMDA stimulation produced a similar amount of cell death as NMDA alone and was significantly different from 10 min PMA pre-treatment ($P < 0.05$). These results indicate that downregulation of PKC leads to similar percentages of cell death as occur with exposure to NMDA alone.

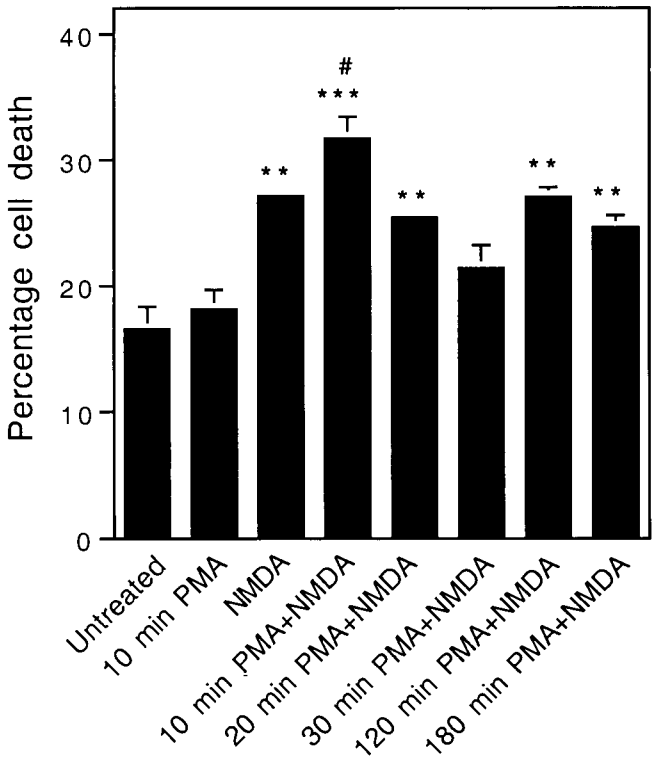

Fig.18. Cell death in NR1A/NR2A-transfected HEK-293 cells pre-exposed to prolonged PMA treatment. Pre-treatment with PMA for different time periods prior exposure to PMA and NMDA reflects a 'downregulation' of PKC. A 10-min preincubation led to increased cell death compared to NMDA alone. A longer preincubation time than 10 min resulted in a reduction in cell death to a similar level as NMDA exposed cells without PMA pre-exposure, or 20 or 30 min pre-exposure to PMA ($p < 0.01$). Data derived from duplicate batches of transfected cells for each condition performed in at least 3 separate experiments. *** Significantly different from control ($P < 0.0005$), ** significantly different from control ($p < 0.005$), # significantly different from NMDA alone, or 20 and 30 min pre-exposure to PMA ($p < 0.05$).

4.2.5. The C1 region of NR1 does not contribute to cell death

Previous work has indicated that the C1 region of NR1 is heavily phosphorylated by PKC and regulates the phorbol ester-sensitivity of the NR1 subunit (Logan et al., 1999). To evaluate the role for the C1 domain in the modulatory effect of PMA in NMDA-toxicity we compared cells transfected with a splice variant of the NR1 which did not contain the C1 domain (NR1C) with cells transfected with NR1A, which contains the C1 region. As shown in Fig. 19, there were no significant differences between the cytotoxic effect of cells transfected with NR1C/NR2A and NR1A/ NR2A subunits of the NMDAR. The percentage cell death was very similar under control conditions, following NMDA-stimulation, PMA and NMDA-treatment, or with pre-treatment with RO (Fig. 19). Thus, deletion of the C1 region did not alter the cytotoxic effect of NMDA, or change the modulatory effect of PMA in this system.

4.2.6. Inhibitors of PI3-K and MEK did not reduce NMDA-mediated cell death

To explore the role of other signaling molecules in NMDA-mediated cell death, the inhibitors of PI 3-K and MEK, LY 294002 and PD 98059, respectively, were applied separately in NR1A/NR2A transfected HEK 293 cells, either alone or prior to NMDA exposure. The PI3-K inhibitor, LY 294002, was applied at 25 μM for 20 min. Treatment with LY 294002 by itself had no cytotoxic effect on the transfected cells, as the percentage cell death was similar to control cultures. Furthermore, pre-treating cells with LY 294002 before exposure to NMDA had no significant effect on cell death compared to treatment with NMDA alone. The MEK inhibitor PD 98059 (30 μM) was applied 20 min prior to NMDA-treatment. No difference in cell death was observed between control and PD 98059-treated cells.

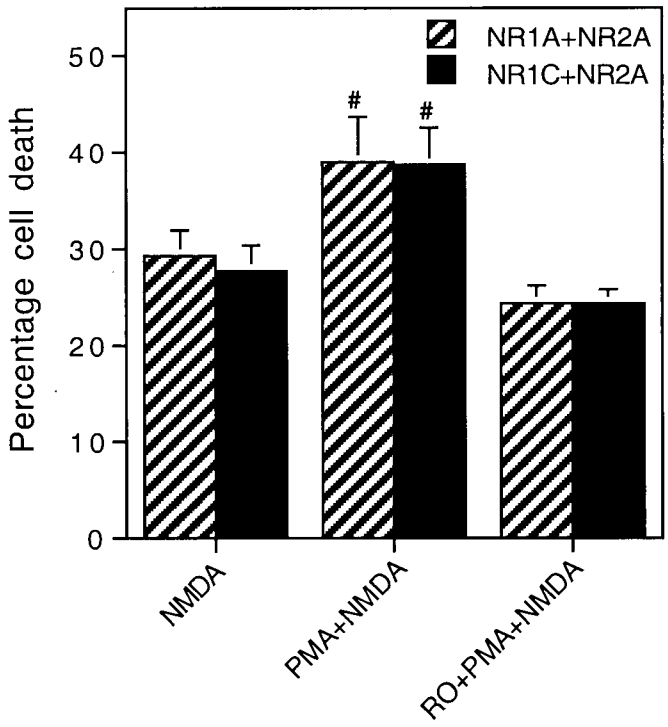

Fig.19. Cell death in NR1A/NR2A and NR1C/NR2A-transfected HEK-293 cells. Cell death of NR1C/NR2A-transfected cells treated with PSS (control), NMDA, PMA and NMDA or RO 32-0432 prior to PMA and NMDA was not significantly different from cell death of NR1A/NR2A-transfected cells treated with the same treatments. # Significantly different from NMDA alone, or RO+NMDA+PMA ($p<0.05$). Data derived from duplicate batches of transfected cells for each condition performed in at least 3 separate experiments.

Applying the MEK inhibitor prior to NMDA-treatment, also did not show any significant differences in cell death compared to cells that were exposed to NMDA alone (Fig. 20).

4.3. Discussion

HEK-293 cells transfected with NMDA receptor subunits have been used extensively as an *in vitro* model to study the mechanism of the modulation of NMDA receptor currents (Raymond et al., 1994; Grant et al., 1998) and NMDA-mediated cytotoxicity (Cik et al., 1994; Anegawa et al., 1995; Raymond et al., 1996). These cells were used for the present studies as the homogeneous cell population facilitates biochemical measurements and assessment of cell death and also permits transfection with known NMDA receptor subunits. One limitation of these cells is that since HEK cells are not neurons, it is possible that they might not contain the complement of protein kinases and other cellular constituents necessary for NMDA-mediated toxicity. However, as previous work has shown that transfected HEK cells demonstrate NMDA-mediated currents (Raymond et al., 1994), changes in free cytoplasmic Ca^{2+} concentrations (Grant et al., 1998) and cell death in response to NMDA stimulation (Raymond et al., 1996), these cells appear to mimic the responses observed in neurons. Furthermore, as these cells express only NMDARs, they provide an opportunity to study the mechanism of cell death following NMDAR activation in the absence of confounding effects from other EAA receptors.

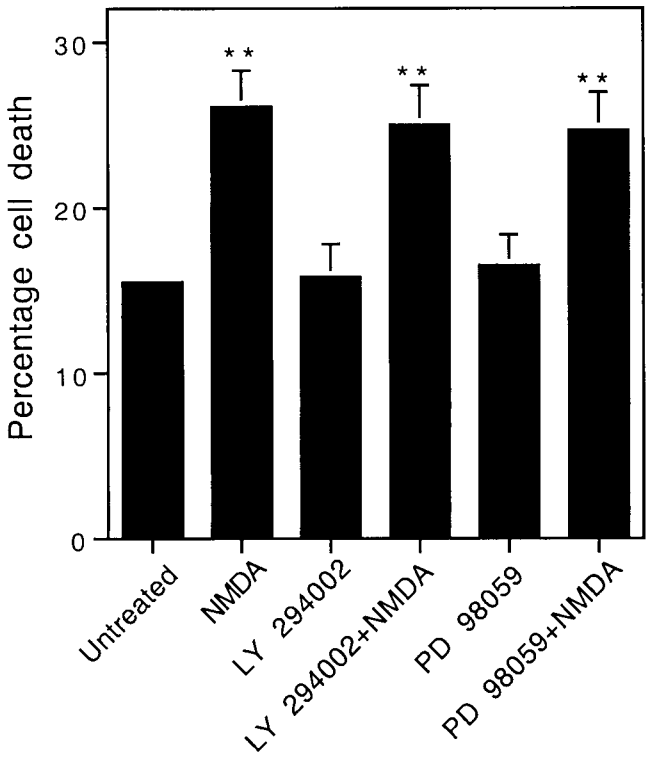

Fig. 20. Cell death in NR1A/NR2A-transfected HEK-293 cells following treatments with PI 3-K and MEK inhibitors. Inhibitors of PI 3-K and MEK did not reduce NMDA-mediated cell death. Treatment using PI 3-K inhibitor, LY 294002 (25 μM) and MEK inhibitor, PD 98059 (30 μM) 20 min before NMDA exposure did not reduce NMDA cytotoxicity in cells transfected with NR1A/NR2A subunits. These two compounds by itself had no effect since cell death produced by LY 294002 or PD 98059 was not significantly different from untreated cultures. ** significantly different from control ($p < 0.005$).

Previous experiments by Raymond et al. (1994) have demonstrated that HEK cells transfected with NR1/NR2A subunits identical to those used in the present experiments show more than 300% potentiation of NMDA receptor-mediated currents for at least 20 min, following dialysis of purified PKC into the cytosol.

This study evaluated whether PKC activation potentiates NMDA-induced cell death using HEK cells transfected with NMDA receptor subunits. Autoradiography and Western blot analysis showed that PKC activities and PKC α and/or β protein are present in unstimulated, transfected HEK cells and that NMDA augments PKC activity as determined by increased histone H1 phosphotransferase activity. These results are similar to those found in neurons and neuronal cell lines to activation by glutamate (Fukunaga et al., 1992; Hasham et al., 1997). Indirect evidence for PKC activation by NMDA and phorbol esters in HEK cells was seen, as an increase in the bandshift of Erk1. This bandshift has been attributed to the phosphorylation of Erk1 and Erk2, an event associated with the activation of Erk1 by PKC (Fiore et al., 1992). Furthermore, an increase in Erk1 phosphorylation by PMA and NMDA was also observed using phospho-Erk1/Erk2 antibodies.

Death of HEK cells was determined using trypan blue exclusion. This method suffers from the limitation that likely not all of the HEK cells in the cultures were transfected by NR1/NR2A subunits. Transfection efficiencies were generally in the range of 60-80 %, which is similar to those reported in other studies using these cells (Raymond et al., 1996). This level of transfection efficiency would have underestimated the effects observed had all HEK cells been transfected. As observed in other studies, HEK cells transfected with NR1/NR2A subunits die even in the presence of NMDA antagonists, an effect which has been attributed to incomplete

NMDA receptor antagonism. With NMDA and glycine stimulation, cell death increases substantially, as has been observed previously (Raymond et al., 1996). The present data also confirms the observations that cell death is greater in NMDA-treated HEK cells that are co-transfected with cDNAs for NR1 and NR2A, compared to cells transfected with NR1A alone (Raymond et al., 1996). In this study no attempt was made to determine if the cell death was due to necrosis or apoptosis.

Under the experimental conditions of this study, inhibitors of PI 3-K and MEK did not alter NMDA-mediated cytotoxicity. Several possible explanations could be that the concentration of the inhibitors were not high enough to exert any inhibitory effect, or the time of incubation was not sufficient for this cell type. Other investigators have used higher concentrations and longer incubation periods of these inhibitors in a chick spinal cord motoneuron culture (Dolcet et al., 1999). Another explanation could be related to the absence of growth factors in the culture medium which might have activated the PI 3-K and MAPK pathways. Since the HEK cell line used here is of neoplastic origin, it may be less sensitive to drugs or inhibitors compared to neurons in vivo (see Tiffany-Castiglioni et al., 1999). Furthermore, transfection of different receptor subunits could also alter the susceptibility of the HEK cells towards certain agonists or antagonists. Therefore, more complete dose and time response experiments need to be conducted to confirm this result.

A novel finding in this study is that pre-exposure to PMA significantly augments death of transfected HEK cells. The increased cell death results from PKC activation as it was blocked by the specific PKC inhibitor, RO. Furthermore, the potentiation of NMDA-mediated cell death by PMA was inhibited by PseudoA, a specific inhibitor of Ca^{2+} and lipid-dependent PKC isoforms, although it was not possible to determine the

specific isoform(s) involved in this effect. Additionally, the potentiation of NMDA-mediated cell death could be inhibited by PKC downregulation.

These data confirm and extend the results of several previous studies. Favaron and colleagues (1990) found that pre-treatment of cultured cerebellar granule cells with gangliosides, which inhibit PKC translocation, decreased glutamate and kainate-mediated neurotoxicity. Furthermore, primary cultures of rat cerebellar neurons can be protected from glutamate neurotoxicity by PKC inhibitors, whereas an inhibitor of cyclic nucleotide-dependent protein kinases had no effect (Felipo et al., 1993). Our data are also consistent with the observations of Manev et al. (1990) who found that downregulation of PKC in cerebellar granule cells protects neurons from glutamate-induced neurotoxicity. As these studies used primary cultures containing neurons having a number of excitatory amino acid (EAA) receptor channel types, it was not possible to draw specific conclusions about the contributions of given receptor subunits. The present data deviate from results obtained using cultured primary rat cortical neurons after 8 days in vitro. Durkin et al. (1997) reported that NMDA-mediated cell death increased in neurons which were pre-treated with the PKC inhibitor, staurosporine. As in the present experiments, they did not observe any significant effect of PMA or PKC inhibitors on cell death in the absence of NMDA stimulation. Following NMDA and staurosporine neural death increased. Durkin and coworkers (1997) attributed the increased excitatory amino acid-mediated cell death to a reduction in membrane-associated PKC. However, although pre-incubation with PMA resulted in a reduction in membrane-associated PKC, it was not associated with cytotoxicity. In contrast to the transfected HEK cells in our study, NMDA application to cortical neurons in culture for 8 days, showed little toxicity in the absence of PKC

inhibitors (Durkin et al., 1997). These observation indicate that differences in neurotoxic pathways may exist between transfected HEK cells and some classes of embryonic neurons during development. Alternatively, since cultured embryonic cortical neurons express mainly the NR1/NR2B subtype of NMDA receptor during early days in vitro (before 8 days in vitro) (Zhong et al., 1994), specific effects of PKC activation on this NMDA receptor subtype after NMDA stimulation may contribute to the apparently contradictory results. A recent study has revealed significant differences in electrophysiological properties between NR1A/ NR2A and NR1A/ NR2B subunits such as peak channel open probability (Chen et al., 1999). The results from this study may correlate best with neurons highly expressing NR1/ NR2A subtype NMDA receptors.

The mechanism by which PKC activation potentiates the toxicity of NMDA was not determined in these experiments. Consensus sequences for PKC phosphorylation are found in several distinct sites on the NMDA receptor, including several serine residues located at the alternatively spliced C1 region (Tingley et al., 1993). Specifically, phosphorylation of Ser-890 in the C1 region produces a reduction of PKC potentiation, whereas mutation of other PKC phosphorylation sites (Ser-889, Ser-896 and Ser-897) have no significant effect on phorbol ester stimulation (Zheng et al., 1999). To explore the relevance of the C1 exon in PKC potentiation of NMDA-mediated cell death in our system, we compared cell death in NR1A/ NR2A transfected cells with those in NR1C/ NR2A transfected cells (NR1C splice variants do not contain the C1 region). Results from this study indicate that PMA potentiates NMDA-mediated cell death in both splice variants and that the absence of the C1 region had no effect on cell death in transfected HEK cells. Recent data indicate that

some of the potentiation of NMDAR activity is likely not mediated by phosphorylation of NMDAR subunits themselves, but by an NMDA-associated targeting, anchoring, or signalling protein (Suen et al., 1998; Zheng et al., 1999; Lu et al., 2000).

The physiological consequences of PKC activation are highly variable and cell type dependent. For instance, in neurons possessing EAA receptors, PKC activation is often associated with cell death (Favaron et al., 1990; Felipo et al., 1993). In other cell types that do not express EAA receptors, such as mammalian oligodendrocytes, PKC activation is associated with process outgrowth and does not lead to cell death (e.g., Yoo et al., 1999).

These present observations indicate that PKC activation potentiates NMDA-mediated toxicity in a cell line expressing NMDAR. It is plausible that augmented PKC activity from whatever cause could lead to a potentiation of NMDA-mediated toxicity in neurons in vivo. These data have implications for neurodegenerative disorders in humans. We have previously reported that spinal cord tissue from patients who died with amyotrophic lateral sclerosis (ALS) have elevated PKC activities, compared to tissue from patients who died without neurological disease (Lanius et al., 1995; Wagey et al., 1998). Although the cell type associated with the increased PKC activity in ALS spinal cord tissue is unknown, it is possible that augmented neuronal PKC activity could contribute to the pathogenesis of ALS by enhancing EAA-mediated neurotoxicity, possibly at the NMDA receptor (Krieger et al., 1996). These results indicate that elevated PKC activity of postsynaptic neurons increase their vulnerability to NMDA-mediated death.

CHAPTER 5. PHOSPHATIDYLINOSITOL 3-KINASE ACTIVITY IN MURINE MOTONEURON DISEASE: THE PROGRESSIVE MOTOR NEURONOPATHY MOUSE

5.1 Introduction

Studies of post-mortem tissue from patients with the sporadic (non-inherited) form of ALS have demonstrated increased activities and protein levels of phosphatidylinositol 3-kinase (PI 3-K) in particulate fractions of spinal cord tissue, compared to control subjects (Wagey et al., 1998 and chapter 3). The enzyme PI 3-K mediates the trophic actions of various growth factors including brain-derived neurotrophic factor (BDNF), insulin-growth factor I (IGF-I) and nerve growth factor (NGF) (Duan et al., 1999; Kaplan and Miller, 1997) indicating that changes in the activity or expression of this enzyme may be important in the cellular responses to growth factors. Although BDNF administration has not clearly been of clinical benefit in patients with ALS (BDNF study group, 1999), it does significantly increase the life span of murine models of ALS, such as the Wobbler mouse (Ikeda et al., 1995). BDNF was also able to act as a survival factor in chick embryo cultured motoneurons (Becker, 1998). In the progressive motor neuronopathy mouse (pmn/pmn; pmn), BDNF, ciliary neurotrophic factor (CNTF) and neurotrophin-3 are capable of increasing the number of retrograde labelled motoneurons, 24 h following the intramuscular injection of a fluorescent tracer (Sagot et al., 1998). These data indicate that some neurotrophic factors, including BDNF, improve the function of motoneurons in pmn mice, in so far as they produce greater uptake and/ or retrograde transport of a tracer substance (Sagot et al., 1998).

The interaction between BDNF and its receptor (TrkB) has been reported to activate several signal transduction pathways including Erk1/Erk2 and PI 3-K which could modulate other downstream kinases (Segal and Greenberg, 1996; Kaplan and Miller, 1997; Kaplan and Miller, 2000). However, most reports evaluating the role and mechanism of BDNF are based on in vitro studies and very limited information exists on in vivo effect in both normal and pathological conditions. The roles of Erk1/Erk2 in trophic factor-mediated cell survival are still controversial. Some reports indicated these MAPK are required to inhibit apoptosis (eg. Parrizas et al., 1997), while others have reported that they are not critical for the survival of peripheral and sympathetic neurons (Creedon et al., 1996; Virdee and Tolkovsky, 1996). The PI 3-K pathway appears to be responsible for cell survival (Yao and Cooper, 1996; D'Mello et al, 1997). However, PI 3-K is not involved in survival of sensory neurons (Bartlett et al., 1997), or in membrane depolarization-mediated motoneuron survival (Soler et al., 1998).

Mice homozygous for the pmn/pmn trait undergo progressive paralysis beginning in the hindlimbs during the third week of life leading to an ataxic gait and eventually to impaired locomotion. Forelimb involvement follows and the mice die at six to seven weeks of age. Motoneurons become small and chromatolytic. Axonal degeneration is prominent distally and there is muscle atrophy without re-innervation (Schmalbruch et al., 1991).

The intracellular pathways involved in motoneuron survival in control and pmn/pmn mice are unknown. This study evaluated the activities and protein levels of PI 3-K and several related protein kinases in the nervous system of the control and pmn/pmn mouse. Analysis of PI 3-K activity and protein content in the presence and

absence of BDNF was performed in control and pmn/pmn mice. Furthermore, the effect of PI 3-K and MEK inhibitors on the uptake and/or retrograde transport of fluorogold in motoneurons of pmn and control mice was also examined, both in the presence and absence of BDNF.

5.2 Results

5.2.1 PI 3-K activity is reduced in spinal cord of pmn/pmn mice

PI 3-K activity in spinal cord tissue from pmn/pmn and control mice was determined by immunoprecipitation with an antibody against the p85 subunit of PI 3-K followed by phosphotransferase activity assays. PI 3-K activities in lysates of spinal cord were significantly reduced in 28-day-old pmn/pmn mice, compared to similarly aged controls (pmn/pmn, 999 $\pm$ 71 cpm/15 min, n=4; control, 1515 $\pm$ 17 cpm/15 min, n=3; $p<0.05$; Fig. 21). There was no change in total PI 3-K protein level (pmn/pmn, 72 $\pm$ 3 units, n=4; control, 74 $\pm$ 0.6 units, n=3, Fig. 21) indicating that the specific PI 3-K enzyme activity was reduced in spinal cord tissue from pmn/pmn mice.

To characterize the distribution of PI 3-K activities and protein levels in pmn/pmn and control mice, particulate and cytosolic fractions of spinal cord tissue were analyzed. In the particulate fractions, PI 3-K activities and protein amounts were significantly reduced in 28-day-old pmn/pmn mice, compared to control mice of the same age (Table 1). Cytosolic fractions from 28 day old pmn/pmn mice did not reveal any significant differences in PI 3-K activities and protein levels compared to control mice (Table 1) indicating that the reduction in specific PI 3-K activity in spinal cord occurs in the particulate fraction of pmn/pmn mice.

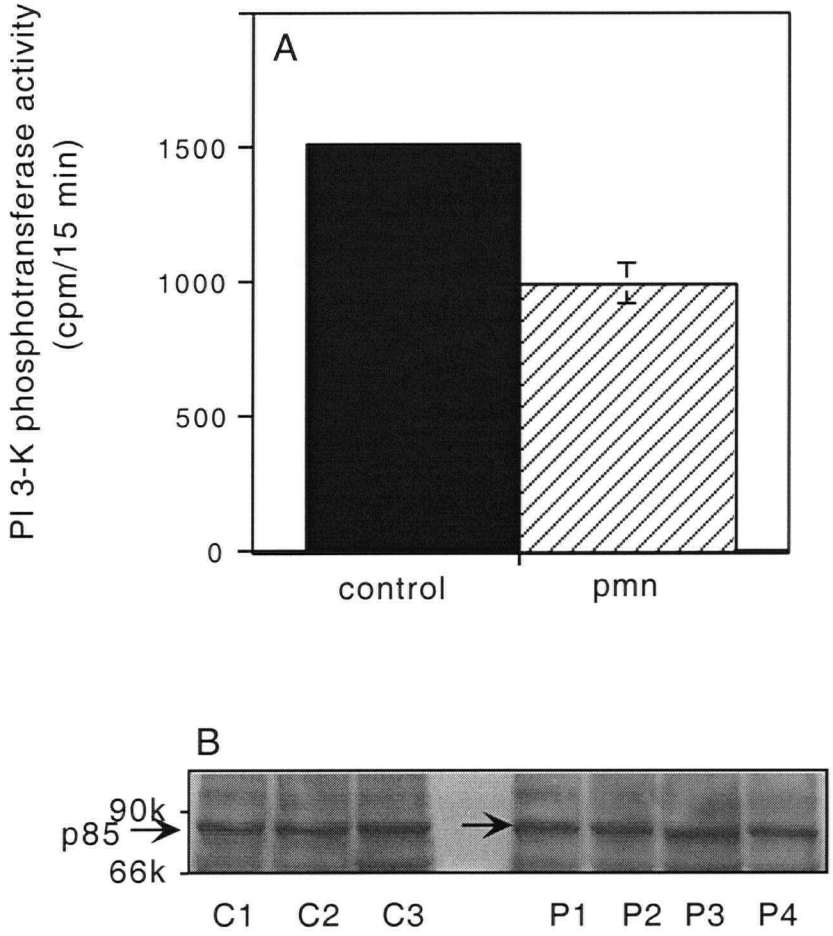

Fig. 21. PI 3-K activity and protein level of spinal cord tissue from control and pmn/pmn mice. A. PI 3-K activities of total lysates from spinal cord tissue from control (filled column) and *pmn/pmn* mice (hatched column). Data shown indicate mean ± SEM. Standard error bars on data from control animals are too small to be resolved. PI 3-K activities in *pmn/pmn* mice are significantly less than controls ($p < 0.05$). B. Western blot of the regulatory subunit of the PI 3-K protein (p85 subunit) in spinal cord tissue from each of 3 control animals (C1-3; left lanes) and 4 *pmn/pmn* mice (P1-4; right lanes). There was no significant difference in the protein level between control and *pmn/pmn* mice.

Table 1. PI 3-K activity and expression in spinal cord of control and pmn mice

	control		pmn	
	28 days old	41 days old	28 days old	41 days old
Particulate				
PI 3-K activity (cpm/15 min)	150 ± 3 (n = 4)	130 ± 19 (n = 4)	94 ± 9*** (n = 5)	93 ± 13 (n = 5)
p85 content (arbitrary units)	1.77 ± 0.14 (n = 4)	0.71 ± 0.04### (n = 4)	0.67 ± 0.05*** (n = 5)	0.62 ± 0.03 (n = 5)
Cytosolic				
PI 3-K activity (cpm/15 min)	230 ± 30 (n = 4)	229 ± 23 (n = 3)	163 ± 27 (n = 4)	134 ± 8*** (n = 5)
p85 content (arbitrary units)	10.7 ± 3.4 (n = 4)	13.0 ± 2.2 (n = 3)	6.9 ± 2.1 (n = 4)	6.7 ± 1.6*** (n = 5)

*** significantly different from control of same age ($p < 0.005$)
significantly different from 28-day old control ($p < 0.005$)

To determine if more advanced disease was associated with greater reductions in PI 3-K activity or protein levels, PI 3-K activity and protein level were examined in spinal cord tissue from pmn/pmn and control animals at 41 days of age. Pmn/pmn mice of this age are severely debilitated reflecting extensive motoneuron loss. The result showed that PI 3-K protein expression of control mice in the particulate fraction was significantly reduced at 41 days of age compared to 28 days (Table 1). However, PI 3-K activities in the particulate fractions of control mice were not significantly different at 41 days compared to 28 days (Table 1). There were no significant differences in PI 3-K activities or protein levels in 28-day-old pmn/pmn mice compared to 41-day pmn/pmn mice. As shown in Table 1, 41 day old pmn/pmn mice had PI 3-K activities and protein expression in the cytosolic fractions which were significantly less than in 41 day old controls (Table 1).

As pmn mice have a loss of spinal and facial nucleus motoneurons at 28 days of age (Schmalbruch et al., 1991), brain regions such as the brainstem and cerebellum were also evaluated to examined whether the changes in PI 3-K were also present in these two regions. In the brainstem, PI 3-K activities were not significantly different between pmn/pmn and control mice, either in the cytosolic (pmn/pmn, 310 $\pm$ 39 cpm/15 min, n=5; control, 382 $\pm$ 54 cpm/15 min, n=4) or particulate (pmn/pmn, 904 $\pm$ 37 cpm/15 min, n= 4; control, 1174 $\pm$ 221 cpm/15 min, n=3) fractions (Fig. 22). PI 3-K protein levels in the brainstem were not significantly different from control animals (cytosolic; pmn/pmn, 14.6 $\pm$ 1.1 units, n= 5; control, 12.7 $\pm$ 0.6 units, n=4; particulate: pmn/pmn, 3.5 $\pm$ 0.4 units, n= 6; control, 4.4 $\pm$ 0.4 units, n=5, Fig. 22).

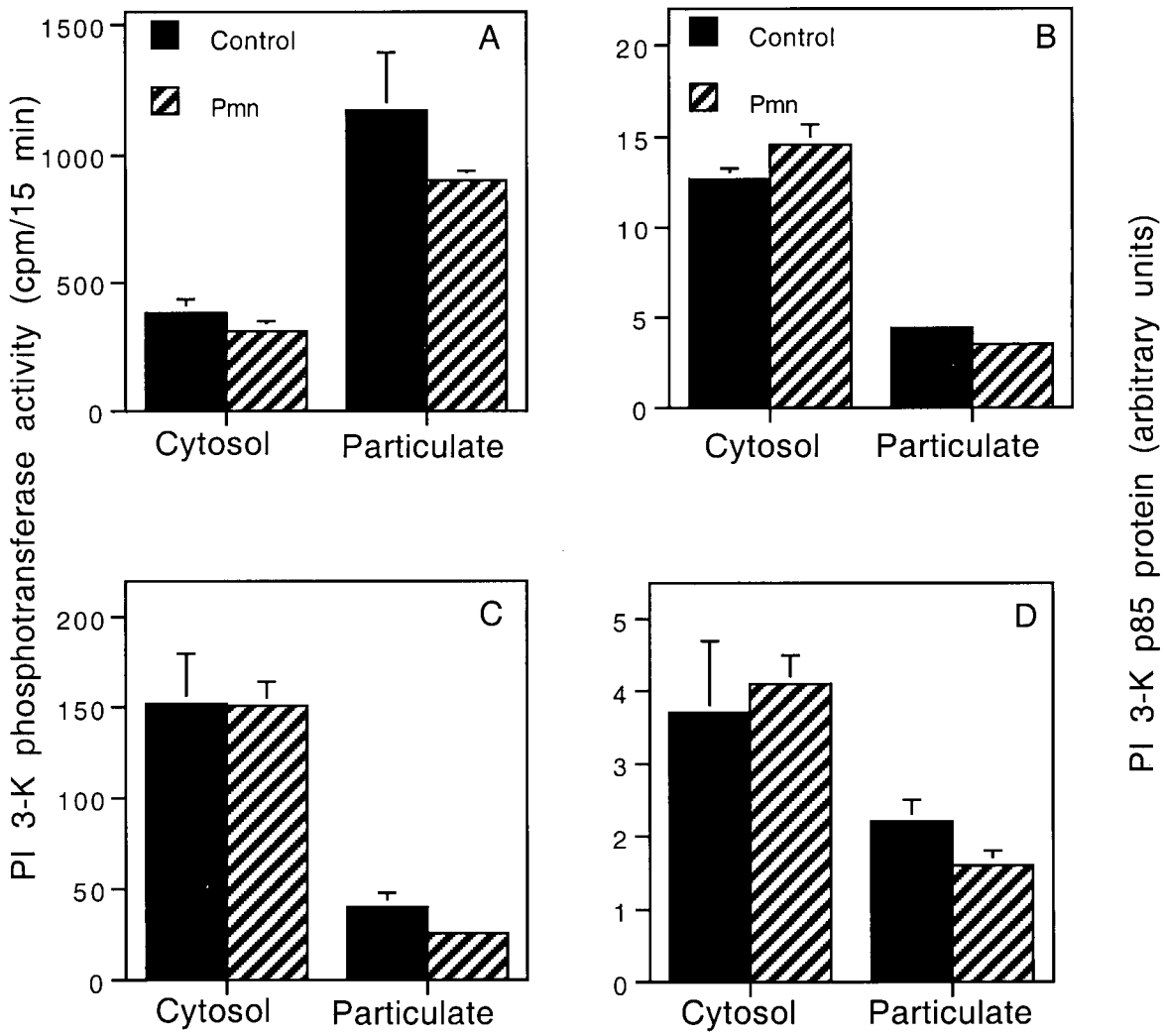

Fig.22. PI 3-K activities (A,C)and protein levels (B,D) from cytosol and particulate fractions of brainstem and cerebellum from control and pmn/pmn mice. No significant differences in activity and protein level was observed between control and pmn mice in brainstem and cerebellum. Error bar indicates SEM.

In the cerebellum also no significant differences were found in PI 3-K activities or protein levels in either the cytosol (activities, pmn/pmn, 151± 13 cpm/15 min, n= 5; control, 152 ± 28 cpm/15 min, n= 4; protein, pmn/pmn, 4.1 ± 0.4 units, n= 6; control, 3.7 ± 1.0, n=4), or particulate fractions (activities, pmn/pmn 26 ± 4 cpm/15 min, n=5; control, 40 ± 8 cpm/15 min, n= 4; protein, pmn/pmn, 1.6 ± 0.2 units, n=5; control, 2.2 ± 0.3 units, n=4, Fig. 22).

5.2.2. Activities and expression of PKB, p70 S6K and Erk1 in pmn/pmn and control mice

As these data demonstrated a reduction in PI 3-K activity in spinal cord tissue from pmn/pmn mice, several protein kinases which are regulated by PI 3-K were assayed. Table 2 shows the phosphotransferase activities and protein levels of PKB, p70 S6K and Erk1 in total lysates of spinal cord tissue from 28-day-old pmn/pmn and control mice. No statistically significant differences in the activities of any of these three kinases were observed. The protein level of p70 S6K was reduced in pmn/pmn mice, compared to controls. No significant differences in PKB or Erk1 protein levels were seen.

5.2.3. BDNF elevates specific PI 3-K activity

Previous studies have demonstrated that local injections of BDNF and Fluorogold onto the cut central stump of sciatic nerve in pmn/pmn mice results in increased numbers of retrograde labelled motoneurons after 24 h (Sagot et al., 1998).

Table 2. Phosphotransferase activities and protein expression of PKB, p70 S6K and Erk1 in total lysates of spinal cord from control and pmn mice

Kinase		**control (mean ± SEM)** n = 3	**pmn (mean ± SEM)** n = 4
PKB	Activity	358 ± 45	352 ± 38
	Protein	12.5 ± 3.2	11.7 ± 1.3
p70S6K	Activity	760 ± 40	744 ± 18
	Protein	45 ± 1.3	35.6 ± 0.1**
Erk1	Activity	2566 ± 518	2523 ± 635
	Protein	32.1 ± 0.9	29.5 ± 2.2

** Significantly different from control ($p < 0.001$)
activity expressed as cpm/15 min;
protein expressed as arbitrary units

As BDNF stimulates PI 3-K, PI 3-K activity was measured in spinal cord tissue from pmn and control mice 18 h following treatment with BDNF. After systemic BDNF treatment, PI 3-K activities were increased in both cytosolic and particulate fractions of pmn/pmn and control mice. Interestingly, in the particulate fractions, BDNF stimulated PI 3-K activity of pmn/pmn mice to a greater degree than control animals, resulting in comparable PI 3-K activities in both groups (Table 3). In cytosolic fractions, BDNF produced increased PI 3-K activities that were significantly greater in control animals than in the mutants. The increase in cytosolic PI 3-K activity appeared to reflect an augmented PI 3-K protein content, as the specific activities of cytosolic PI 3-K were similar in pmn/pmn and control animals. The specific activity of PI 3-K was significantly increased in particulate fractions from pmn/pmn mice as compared to controls (Table 3). These results indicate that BDNF enhances the PI 3-K activities of pmn/pmn and control mice, with pmn/pmn mice exhibiting a more pronounced effect, especially in the particulate fraction.

5.2.4. Inhibitors of PI 3-K alter motoneuron retrograde labelling in control mice but not in pmn/pmn

Previous studies of motoneuron retrograde labelling in pmn mice have shown that these mice have lower amounts of retrograde labelling compared to control animals (Sagot et al., 1998). To evaluate the involvement of the PI 3-K pathway in the retrograde labelling of motoneurons LY-294002, a specific, reversible inhibitor of PI 3-K, was applied together with fluorogold, to the proximal cut surface of sciatic nerve in control and pmn/pmn mice.

Table 3. PI 3-K activity and protein expression in BDNF treated and untreated control and pmn mice

	control (mean ± SEM)		pmn (mean ± SEM)	
	+ BDNF (n = 4)	-BDNF (n = 3)	+ BDNF (n = 5)	-BDNF (n = 3)
Particulate				
PI 3-K activity (cpm/15 min)	710 ± 59***	250 ± 9(#)	722 ± 50****	190 ± 17
p85 content (arbitrary units)	27.5 ± 0.9****(####)	14.8 ± 0.3	17.1 ± 0.9*	13.0 ± 1.1
PI 3-K specific activity	25.8 ± 3.0*(#)	16.9 ± 0.5(#)	42.2 ± 3.7***	14.6 ± 0.4
Cytosolic				
PI 3-K activity (cpm/15 min)	1404 ± 110***(#)	572 ± 30	1044 ± 81***	550 ± 30
p85 content (arbitrary units)	33.5 ± 1.4***(#)	23.8 ± 0.9	25.8 ± 1.9	22.2 ± 1.9
PI 3-K specific activity	41.9 ± 1.6****	24.0 ± 0.9	40.5 ± 3.5**	24.8 ± 0.9

Statistical comparison between BDNF treated and untreated animals of the same type (control or pmn): $p <$ *0.05; **0.01; ***0.005; ****0.0005

Statistical comparison between control and pmn mice which have received the same treatment (± BDNF): $p <$ (#)0.05; (####)0.0005

PI 3-K specific activity refers to PI 3-K activity/p85 content

The result indicated that concentrations of greater than 0.1 μM LY-294002 produced a significant reduction in the number of labelled motoneurons in control mice at 24 hours (approx. 20% reduction, $p<0.005$; Fig. 23). This inhibition was similar with higher concentrations of LY-294002 up to 100 μM. The decrease in retrograde labelling was not due to the presence of ethanol, as even at high concentrations of ethanol (20%), the number of labelled motoneurons remained unchanged, compared to vehicle alone (1185 ± 90, n=4 for 20% ethanol; 1236 ± 27, n=18 for vehicle alone). The inhibition of retrograde labelling could be increased to around 30% when the inhibitor (100 μM) was begun 10 min before the Fluorogold was applied to the cut end of the central nerve stump (842 ± 66, n=5, LY-294,002 pre-exposure; 1214 ± 91, n=6, $p<0.001$, control). In contrast, LY-294002 had no effect on retrograde labeling in pmn/pmn mice even at 100 μM (Fig. 23).

To extend these results the effect of wortmannin, an irreversible but slightly less specific PI 3-K inhibitor (Ui et al., 1995; Duronio et al., 1998) was evaluated on the retrograde labelling of motoneurons. As was observed with LY-294002, wortmannin significantly diminished the number of labelled motoneurons in control mice without affecting the number of labelled motoneurons in pmn/pmn mice (Fig. 24). To address the possibility that the reduction in the number of labeled motoneurons was due to motoneuron cell death, some animals were studied at six days following Fluorogold application, a time at which all motoneurons are labelled (Sagot et al., 1998). Under these conditions, the total number of labelled motoneurons was the same in control mice which received either Fluorogold alone (1811 ± 165, n=4), or those which received Fluorogold and LY-294002 (100 μM) (1929 ± 156, n=3),

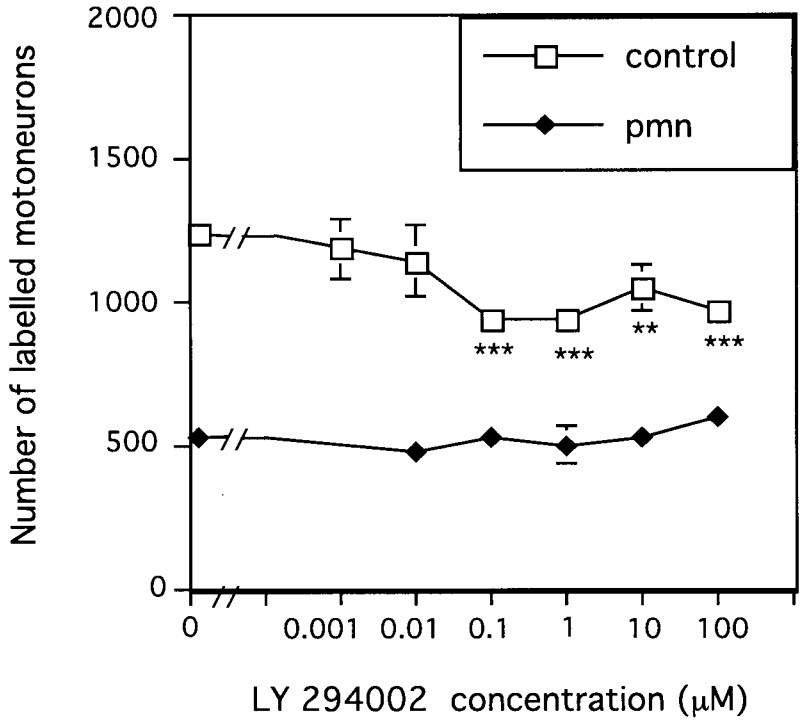

Fig. 23. Effect of LY 294002 on motoneuron retrograde labeling in control and pmn/pmn mice. LY 294002, in concentrations between 0.1 to 100 μM, produced a significant decrease in the number of Fluorogold-labeled motoneurons in control mice without affecting the labelling in pmn/pmn mice (** $p<0.01$; *** $p<0.001$). Each group represents at least 3 animals, typically 5 and error bar indicate SEM.

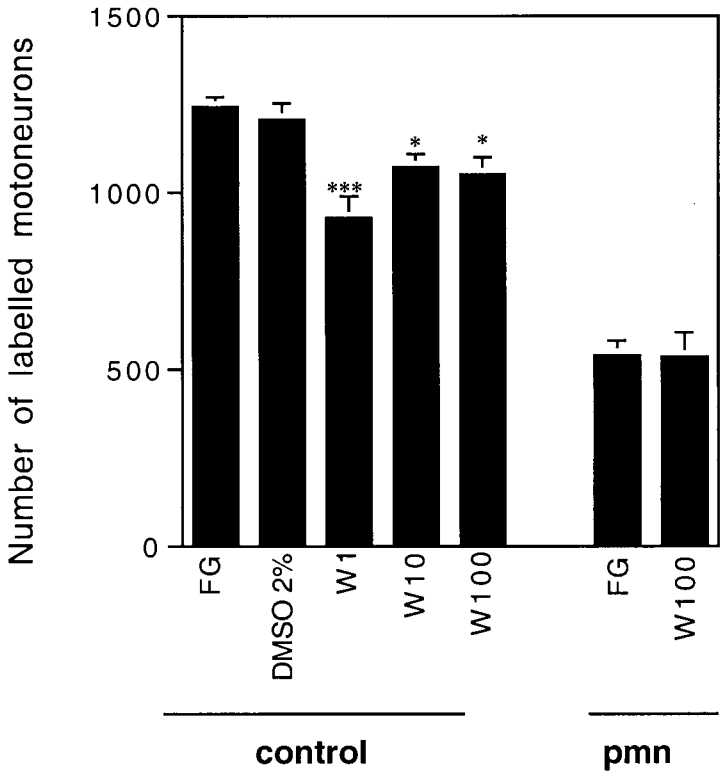

Fig. 24. Effect of PI 3-K inhibition by wortmannin (W) on motoneuron retrograde labeling in control and pmn/pmn mice. At the tested concentrations, indicated in μM inside each column, the PI 3-K inhibitor induced a decrease in the number of labelled cells in control mice but not in pmn homozygotes (* $p<0.05$; *** $p<0.005$). Each group represents at least 4 animals. Error bar indicates SEM. Control animals studied with Fluorogold alone (FG), or Fluorogold and 2% DMSO (DMSO).

or those receiving Fluorogold + LY294002 and BDNF (1707 ± 93, n=3). These results indicate that the reduction in motoneuron labelling observed 24 h after the application of PI 3-K inhibitors was not due to an effect on motoneuron survival but rather to a reduction in the uptake and/or retrograde transport of Fluorogold in the treated motoneurons.

As a control, PD 98059, a selective antagonist of MAP kinase kinase (MEK1,2) was applied. None of the concentrations of PD 98059 tested, ranging from 0.1 to 1000 μM, had any effect on the number of retrograde labelled motoneurons (control, 1241 ± 29, n=15; 0.1 μM PD, 1210 ± 103, n=4; 1 μ M PD, 1187 ± 85, n=5; 10 μM PD, 1237 ± 77, n=4; 100 μM PD, 1276 ± 73, n=7; 1000 μM PD, 1025 ± 84, n=4, 20% DMSO control, 960 ± 58, n=4). Similarly, no effect of PD 98059 was seen on the retrograde labelling index of pmn/pmn mice, even at concentrations of 100 μM (pmn/pmn: control, 539 ± 20, n=8; 100 μM PD, 509 ± 56, n=6).

5.2.5. BDNF-treated animals

BDNF treatment has been found to augment the retrograde labelling of motoneurons by fluorescent tracers (Sagot et al., 1998). As BDNF increases PI 3-K activity, it was important to evaluate whether inhibiting PI 3-K activity by the intraneural application of antagonists could modify the retograde labelling of motoneurons by Fluorogold in animals treated with BDNF. To determine whether the effect of BDNF on motoneuron retrograde labelling was affected by PI 3-K inhibitors, either LY-294002 (100 μM) or wortmannin (100 μM) was applied alone, or with BDNF (1 μg/μl) together with Fluorogold to the transected nerve stump. In control mice, both

LY-294002 and wortmannin significantly reduced the number of labelled motoneurons. This effect was unchanged by the co-application of BDNF with either of the PI 3-K inhibitors (Fig. 25). In pmn/pmn mice, the co-application of PI 3K inhibitors with BDNF completely inhibited the effect of BDNF on retrograde labelling (Fig. 25), indicating that the action of BDNF in pmn/pmn mice is associated with the activation of PI 3-K.

5.3. Discussion

5.3.1.PI 3-K activity is reduced in spinal cord tissue from pmn/pmn mice

This study demonstrates that PI 3-K activity is reduced in spinal cord tissue from pmn/pmn mice at 28 days of age, a time when these mice have substantial impairment in locomotion and neurological function. As in a previous study of mammalian spinal cord tissue, the activities of PI 3-K are higher in the cytosolic fractions than particulate fractions (Wagey et al., 1998). However, the reduction in PI 3-K activity in pmn/pmn mice of this age is related to a decrease in PI 3-K protein in the particulate fractions of spinal cord tissue. PI 3-K activities in cerebellum, a brain area which is uninvolved in pmn/pmn mice (Schmalbruch et al., 1991), are not significantly different from unaffected control animals of the same age. Although a loss of facial nucleus motoneurons has been described in pmn, brainstem involvement is very limited (Schmalbruch et. al., 1991)

Spinal cord tissue from older pmn/pmn mice when the animals were severely debilitated (41 days old) was also examined. In these mice PI 3-K activity and p85 protein levels are reduced in the cytosolic fraction.

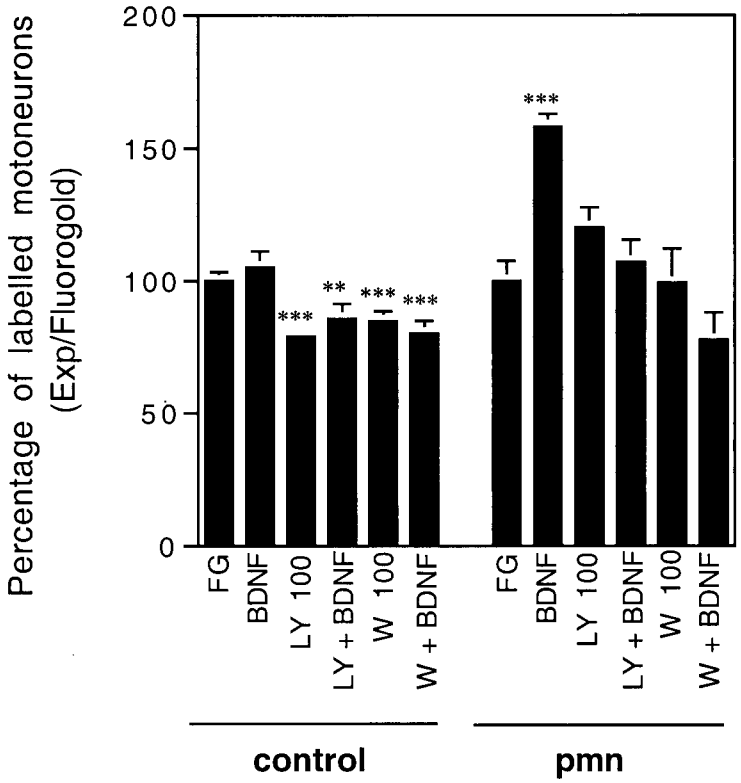

Fig. 25. PI 3-K inhibitors block BDNF effect in pmn mutant. When co-applied with BDNF, wortmannin (W) or LY 294002 (LY) (both at 100 μM) inhibited BDNF action in pmn/pmn mice. For clarity, the number of labelled motoneurons under each condition is expressed as a percentage compared to application of Fluorogold (FG) alone. Note that in control mice, despite the presence of BDNF, the labelling index was decreased by PI 3-K inhibitors. Symbol * indicates significantly different from control (** $p<0.01$; *** $p<0.001$; **** $p<0.0001$). Each group represents at least 5 animals and error bar indicates SEM.

Interestingly, control animals showed a significant reduction in p85 protein level between 28 and 41 days of age in the particulate fractions of spinal cord. This raises the possibility that a reduction in PI 3-K protein occurs normally in an age-dependent manner. This issue was not explored further but it may have implications for neurodegenerative disorders such as ALS, which typically occur in middle age or later.

5.3.2. Reduced PI 3-K activities do not alter activities of PKB, p70 S6K and Erk1

To extend these observations, signalling pathways which may be affected by PI 3-K including the activities and protein levels of PKB was evaluated, as this protein-serine/threonine kinase is regulated by PI 3-K in many cell types (Duronio et al., 1998). Measurement of activities and protein levels of p70 S6K was also examined since it has also been implicated as a downstream effector of PI 3-K (Chung et al., 1994) and may be involved in the retrograde transport of NGF in sensory neurons (Reynolds et al., 1998). As a control, the phosphotransferase activity and protein levels of Erk1 were measured, which may not directly be regulated by PI 3-K. The result was a modest reduction in p70 S6K protein level only with no change in the activities or protein levels of PKB and Erk1. This data indicates that the decrease in PI 3-K activity is not accompanied by changes in these downstream pathways.

5.3.3. Effect of BDNF on PI 3-K uptake and/or retrograde transport of fluorogold

This study showed that systemic administration of BDNF increased the activities and protein levels of PI 3-K in pmn/pmn and control mice, in both particulate

and cytosolic fractions. In the particulate fractions, specific PI 3-K activity was stimulated to a greater degree in pmn/pmn than in control mice. This result is supported by a report from Dolcet and colleagues (1999) who also found a threefold increase in PI 3-K activity following BDNF stimulation in cultured motoneurons. Potentially, the reduced particulate PI 3-K activity and protein level in pmn/pmn mice at 28 days occurs because motoneurons and other spinal cord cells have decreased exposure to presently unidentified trophic substances which stimulate PI 3-K (Sendtner et al., 1997).

Inhibitors of PI 3-K applied to transected peripheral nerve reduced the number of retrograde labelled motoneurons in control mice, but not in pmn/pmn animals. In control mice only partial inhibition of retrograde labeling was achieved, indicating either that the effect of these inhibitors was not maximal or that there co-exists a PI 3-K-independent pathway regulating axonal transport properties. The latter possibility appears more probable for two reasons. First, there was no difference in the extent of the inhibition produced by LY-294002 at concentrations ranging from 0.1 μM to 100 μM. If PI 3-K was only partially inhibited at the lowest effective concentration of LY-294002, further inhibition would be expected with increasing doses. Secondly, in the data obtained from the pmn/pmn mice, retrograde labelling that could be antagonized by inhibitors of PI 3-K occurred only in the presence of BDNF. If axonal transport and uptake properties were mediated largely by the PI 3-K pathway a decrease in the retrograde labelling rate in pmn mice when exposed to PI 3-K inhibitors would be expected, regardless of the presence of exogenous BDNF. Interestingly, the data showed that in control mice, the application of BDNF did not stimulate the retrograde labelling rate, or diminish the inhibition obtained with PI 3-K inhibitors. This indicates

that in control mice, the PI 3-K pathway was already maximally activated and that BDNF did not act on PI 3-K-independent pathways.

The data indicated that there are PI 3-K-dependent and PI 3-K-independent pathways which mediate the uptake and/or retrograde transport of fluorescent tracers from a transected peripheral nerve to the motoneuron cell body in control animals. The pmn/pmn mice likely employ a PI 3-K-independent pathway for uptake and/or transport, possibly due to the low activity of PI 3-K in motoneurons and other spinal cord cells.

When BDNF was co-applied with fluorochromes to the transected nerve stump of peripheral nerve, we found a greater number of motoneurons labelled within 24 h in pmn/pmn mice, than in untreated pmn/pmn animals. This observation indicated that augmented PI 3-K activity increases the extent of the retrograde transport and/or uptake of fluorochromes to the cell body in these animals. Furthermore, when BDNF was co-applied with Fluorogold, inhibitors of PI 3-K were able to impair the retrograde uptake and/or transport of fluorogold in the pmn/pmn mice as manifested by the reduction in retrograde labelling, compared to BDNF alone. Furthermore, the effect of BDNF on motoneuron uptake and/or retrograde labeling in pmn/pmn mice is mediated by enhancing the PI 3-K specific activity, but not by Mek1 activation. Together, these results indicate that the uptake and/or axonal transport of fluorescent tracers are closely related to the PI 3-K activity in spinal cord tissue. In untreated pmn/pmn mice, where PI 3-K activity was low, uptake and/or retrograde transport of fluorochromes did not appear to be mediated by a PI 3-K-dependent process. In BDNF-treated and control animals, a PI 3-K-dependent pathway appears to be responsible in part for the uptake and/or retrograde transport of fluorescent tracers.

Previous work has demonstrated the involvement of PI 3-K in the uptake and/or retrograde transport of substances within the mammalian peripheral nervous system. For instance, in sympathetic and sensory neurons, a wortmannin-sensitive isoform of PI 3-K is involved in the retrograde transport of ^{125}I-NGF, whereas LY-294002 inhibits transport of NGF in sensory, but not sympathetic neurons (Bartlett et al., 1998; Reynolds et al., 1998). These authors suggested that different isoforms of PI 3-K may be recruited within a given neuronal population for the retrograde transport of growth factors or other substances (Reynolds et al., 1998). The present data extend these observations to indicate that the uptake and/or retrograde transport of fluorescent dyes by motoneurons also employ PI 3-K-dependent and independent pathways. It can be further indicated that whether a PI 3-K-dependent mechanism of uptake and/or retrograde transport is used is also dependent on the specific activity of PI 3-K in the motoneurons themselves.

5.3.4. BDNF and inhibitors act on motoneurons

One assumption in this study is that the protein and lipid kinase inhibitors used, as well as BDNF act directly on motoneurons, rather than through other cell types which could secondarily influence the retrograde labeling of motoneurons. Although the sciatic nerve contains sensory as well as motor fibers, only the medium- to large-sized sensory neurons are responsive to BDNF (Lewin and Barde, 1996). In contrast, all lumbar motoneurons express TrkB, the high affinity receptor for BDNF (Merlio et al., 1992; Piehl et al., 1994). Non-neuronal cells such as Schwann cells and possibly endothelial cells may also release substances which could secondarily increase the retrograde labeling of motoneurons. However, Schwann cells express

only a non-signaling truncated form of TrkB (Funakoshi et al., 1993; Offenhauser et al., 1995), while motoneurons express the full length TrkB (Kobayashi et al., 1996; Koliatsos et al., 1993). It therefore seems likely that by applying BDNF to the cut ends of motoneuron axons, an intracellular pathways is activated in motoneurons rather than in Schwann cells or other adjacent cells.

5.3.5. Local action of BDNF and inhibitors

A previous paper has indicated that the action of neurotrophic factors on retrograde labeling was mediated by local intra-axonal events, such as dye uptake and transport rather than by somatic response such as transcription events (Sagot et al., 1998). By allowing only a short survival time after the application of protein and lipid kinase inhibitors it is likely that the inhibitors did not reach the cell body within 24 h and only affected local intracellular signaling events. This may explain the lack of effect of the MEK inhibitor PD 98059 on retrograde labelling at 24 h, since the Erk1/Erk2 pathway is mainly responsible for activating gene transcription and may have had inadequate time for modification (Segal and Greenberg, 1996).

The kinase inhibitors, including PD 98059, as well as BDNF and fluorogold were applied to the transected central stump of sciatic nerve. To avoid diffusion of the inhibitors into the surrounding muscles, a procedure was developed which allowed most of the administered solution to remain localized to the nerve stump. The position of the nerve within the tube and the absence of fluorogold staining in the muscles after perfusion were also confirmed. Therefore, the lack of effect of PD 98059, was not due to inappropriate concentrations of the inhibitor, but to an absence of an effect of this substance. In contrast, the PI 3-K inhibitor LY-294002 had clear

effects at concentrations more than 1,000 times lower than those used for PD 98059. A requirement for activation of the PI 3-K pathway but not the Erk pathway in BDNF-mediated motoneuron survival was also reported by Dolcet and colleagues (1999). Athough both pathways were activated in cultured chicken motoneurons following BDNF stimulation, only the activation of PI 3-K was contributing to motoneuron survival (Dolcet et al., 1999).

The enzymatic product of PI 3-K, PIP_3, is known to modulate the activity of several cytoplasmic proteins either directly or indirectly. These proteins include PKB (Akt), the p70 S6K kinase and the small GTP-binding proteins Rac-1/Rho. These proteins in turn regulate cytoplasmic events: Rac-1 mediates membrane ruffling (Clark et al., 1998; Cox et al., 1997) and Rho is implicated in actin cytoskeletal modification (Ridley, 1997). It has also been shown that PKB activates Rab-5, a small GTP-binding protein, which is involved in liquid phase endocytosis (Barbieri et al., 1998). Since intracellular Fluorogold is found in vesicular structures (Schmued et al., 1989; Wessendorf, 1991) the present data indicate that BDNF enhances the retrograde labeling of motoneurons by fluorescent probes through modification of membrane structures and pinocytosis following PI 3-K activation. However, the possibility that BDNF also stimulates retrograde transport cannot be excluded and previous reports have shown that NGF stimulates both pinocytosis and transport activity in primary cultures of sensory neurons (Yuki et al., 1996).

5.3.6. PI 3-K activity in murine and human motoneuron disease

Although these observations indicate that abnormalities in the PI 3-K pathway are associated with the impairment of uptake and/or axonal transport in pmn/pmn

mice, it is unclear if the abnormalities in PI 3-K precede the appearance of clinical symptoms of motoneuron dysfunction. Recent studies have shown that slowing of axonal transport is an early event in the toxicity associated with mutations of superoxide dismutase in transgenic murine models of ALS (Williamson and Cleveland, 1999). Furthermore, PI 3-K activation likely has other effects in addition to regulating uptake or retrograde transport in motoneurons, such as altering sensitivity to excitotoxins (Fryer et al., 2000).

Postmortem analysis of spinal cord tissue from ALS patients revealed an increased expression of the PI 3-K regulatory subunit, p85 which was associated with a corresponding increase in PI 3-K activity compared to controls (Wagey et al., 1998). However, this elevated PI 3-K activity is accompanied by increases in PKB and p70 S6K protein levels without a concomitant up-regulation of their activities indicating impairment in signal transduction cascades mediated of compensatory regulatory mechanisms by PI 3-K. Although the cause of motoneuron death in humans and mice is unknown the present data indicate that impairment of the PI 3-K signalling cascade may be a common feature of motoneuron diseases.

CHAPTER 6. GENERAL DISCUSSION / FUTURE DIRECTIONS

6.1. Discussion

In this study the role of PKC, PI 3-K and some of their downstream targets have been evaluated in relation to motoneuron dysfunction using several different approaches. Direct measurement of the activities and protein levels of PKC, PI 3-K, PKB, p70 S6K, Erk1and Erk2 were performed in human postmortem tissue from ALS patients and control subjects. Measurement of the activities and protein levels of protein kinases from postmortem tissues in areas that are affected in ALS patients is important, since local changes in these kinases could provide a clue to the pathogenesis of the disease. Although such studies are a relatively direct means to evaluate the disease state, they have several limitations. Postmortem tissue may represent the endstage of the disease, and studies based on this tissue might make it very difficult to determine the primary cause of the disease. One other limitation is that the measurement of the enzyme activities from postmortem tissue was an in vitro assay which does not necessarily reflect the prior activity of these enzyme in the tissue. These studies are also confounded by variables arising from the delay between the time of death and the freezing of the tissue (DF interval), which is difficult to control. Other issues such as improper tissue handling (from autopsy to freezing) and the effect of ante-mortem patient treatment or medication are parameters that could affect the results of postmortem studies. An ideal control to minimize possible postmortem artifact would be to obtain biopsy tissues. However, spinal cord or brain tissue is not biopsied in such cases.

A preliminary study of protein kinase activities and protein levels of normal rat brain and spinal cord tissue has indicated that the activities and protein levels of

some kinases are sensitive to the delay in the death to freezing interval, as well as tissue handling (Wagey et al., 1999). Specifically, the effect of variations in the death to freezing interval for the activities and protein levels of PI 3-K, PKC, p70 S6K, PKB, Erk1and Erk2 were evaluated in rat brain and spinal cord tissues (Fig. 26 and 27). The tissues were studied following different death to freezing (DF) intervals ranging from 0, 0.5, 1, 3, and 6 hours. A reduction of about 20-30% in protein levels at 3 and 6 h was observed for PKC, PI 3-K and PKB from rat brain and spinal cord tissues. Protein levels of S6K were reduced by 50% in spinal cord tissue at 3 and 6 h DF intervals. Erk1 and Erk2 protein levels were stable for up to 6 h. The stability of the kinase activities were variable. For example, PKC activity was reduced by about 30% and p70 S6K activity decreased by approximately 25% at 6h. PI 3-K, PKB, Erk1 and Erk2 activities were also reduced by about 30% to 40% at 3 and 6 h, respectively.

These data demonstrated that protein levels of PI 3-K, PKC, PKB and Erk in rodent brain and spinal cord tissue are affected to some degree by different DF intervals, at least for up to 6 h. The activities of these kinases were also reduced with increasing death to freezing intervals. This finding indicate that protein kinase activities and protein levels in postmortem tissue could be artificially low due to the length of the delay between death to the freezing of the relevant tissue. Therefore, data from postmortem tissue should be cautiously interpreted and confirmed using other model systems. The findings that PKC and PI 3-K activities and/or protein levels are altered in postmortem tissues from ALS patients compared to controls provided an observation which directed further studies of these particular kinases in a different model system that could better serve to explain the possible contributions of PKC and PI 3-K in the mechanism of motoneuron dysfunction.

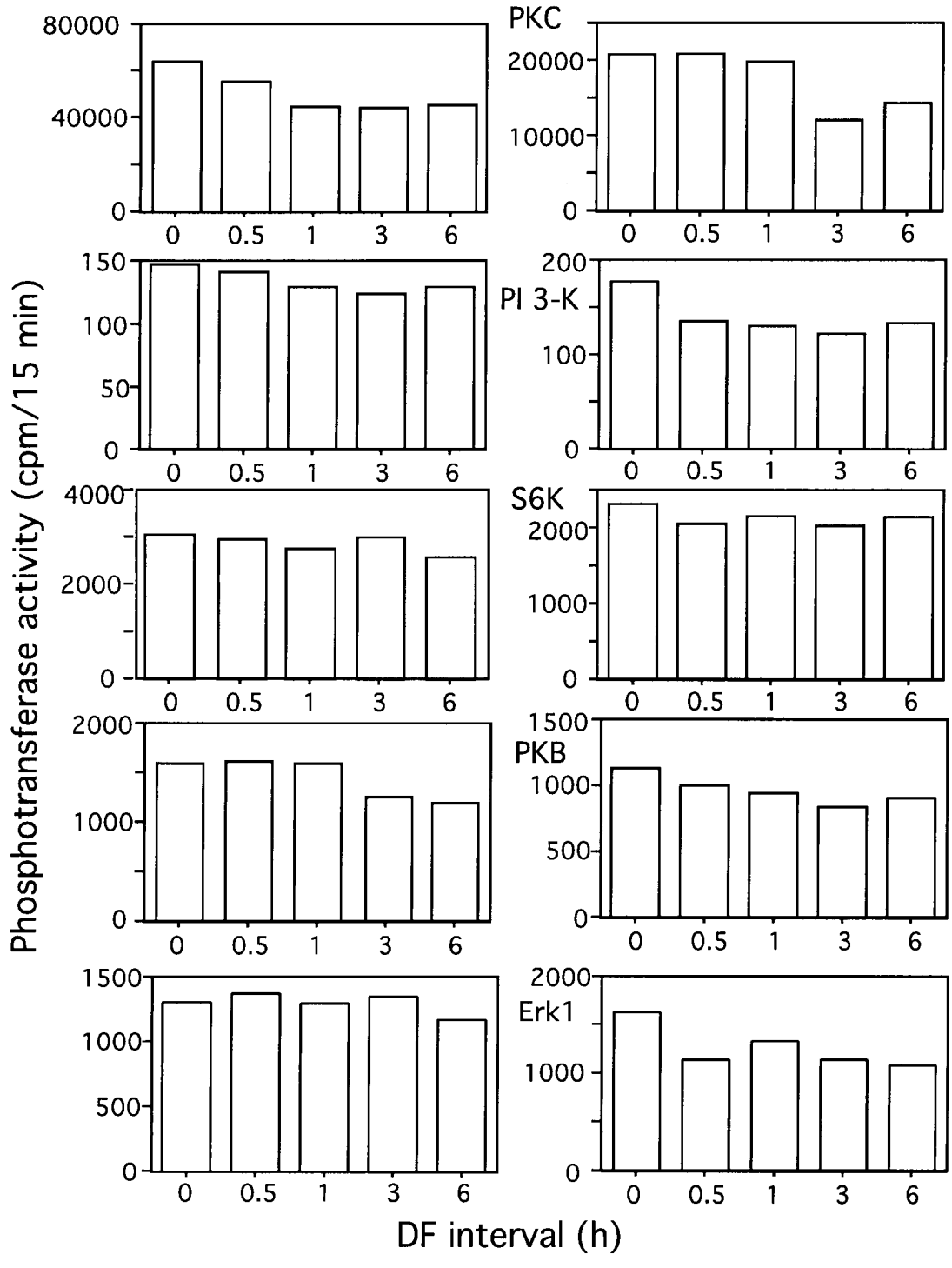

Fig. 26. Protein kinase activities in rat brain and spinal cord following exposure to different DF interval. Left side is the the activity in brain and right side is the activity in spinal cord.

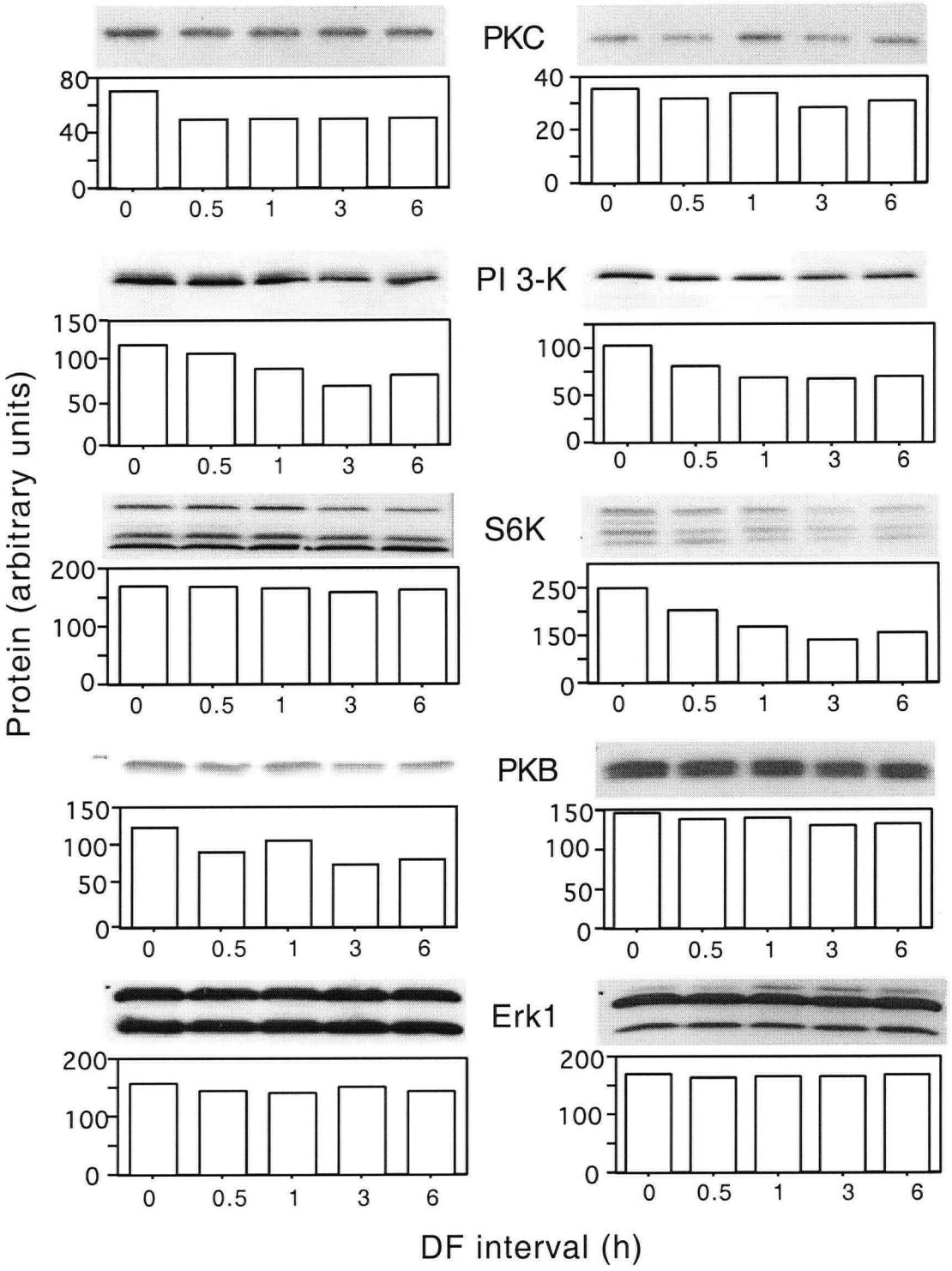

Fig. 27. Protein kinase expressions in rat brain and spinal cord following exposure to different DF interval. Left side is the protein level in brain and right side is the protein level in spinal cord.

Further examinaton of the role of PKC in cell death was made in a human cell line, HEK 293, transfected with NMDA receptor subunits. The transfected cells mimic some of the physiological responses observed in mammalian neurons (Raymond et al., 1994; 1996, Chen et al., 1999). By using this model system, it was possible to examine the role of PKC in NMDA-mediated cell death. The use of specific kinase inhibitors is a powerful tool to examine the role of a particular kinase in this model system. One strength of the HEK 293 cell line is that it can be transfected with specific NR1 and/or NR2 subunits according to the aim of the research. Once transfected, it can be used as a model to study glutamate-mediated toxicity since it possesses glutamate receptors. Use of HEK-293 cells permits the collection of large amount of cells for protein kinase analyses. However, it has several limitations. HEK-293 cells are a non-neuronal and a tumor derived cell line. Consequently, they may not exhibit all the characteristics of neurons in terms of their responsivity to certain stimuli. Furthermore, transfected HEK cells may not have the same properties as adult/aging neurons which neurons are the primary target of motoneuron dysfunction in ALS. Most cell lines do not go through developmental stages of maturation and aging such as in human neurons. The less differentiated state of HEK cells compared to neurons in vivo may influence the sensitivity of HEK cells to neurotoxic compounds compared to neurons in the human brain or spinal cord. Despite the advantages of HEK cells in being able to examine the effect of stimulation of only one particular subunit(s) of EAA receptor, in neurons there are many different subunit combinations of NMDARs and other types of glutamate receptors and these receptors likely co-exist and interact with each other. Therefore, the results from transfected HEK-293 cells should also be carefully interpreted with regards to mechanisms for cell death in

vivo. In this study, HEK293 cells were transfected with a combination of NR1A and/or NR2A, or NR1C and/or NR2A subunits. Under the experimental conditions used in this study, activation of Ca^{2+} -dependent isoforms of PKC potentiated NMDA-mediated cell death, whereas inhibition of PI 3-K and Erk did not show any effect.

As a different approach to evaluate the role of protein and lipid kinase activation in motoneuron dysfunction, an animal model of motoneuron degeneration, the pmn/pmn mouse was studied. There are several advantages in using an animal model such as pmn/pmn mice for analysis of protein kinases. First, fresh tissue can be obtained directly from the animal, eliminating the DF interval issue which occurs when using human tissue. Second, it is possible to obtain tissues from mice at different ages and at different stages of their disease state which is impossible to do using human tissues. This is important for understanding the pathogenic mechanism in the progression of a disease. Third, the mice can be treated with various treatments to evaluate the action of a specific agonist and/or inhibitor in the pmn/pmn mouse compared to controls. This approach can permit functional studies to be performed such as to study retrograde axonal transport in these mice. However, several issues need to be considered in evaluating the results from pmn/pmn mice. Since the pmn/pmn mouse phenotype arises through a spontaneous mutation, the cause of neuronal degeneration is unknown and may not be the same as in ALS patients. Furthermore, pathological features of the pmn/pmn mice resemble a "dying back" type of motor neuropathy (Schmalbruch et al., 1991) and may not have all of the characteristics of human ALS. Another aspect that was not evaluated in this study was the activity and expression of the catalytic subunit, p110, of PI 3-K in control and pmn/pmn mice. At the time this study was conducted, the monoclonal

p85 antibody from UBI was the only antibody that could immunoprecipitate PI 3-K. It was assumed that the p85 subunit was associated with the p110, thus one would pull down p110 with the immunoprecipitated p85 antibody. However, since p110 is the catalytic subunit of PI 3-K, it would be interesting to evaluate the activity and protein expression of this subunit of PI 3-K in control and pmn/pmn mice.

The study of pmn/pmn mice revealed that PI 3-K is reduced in spinal cord tissue of affected mice compared to controls at an age where these mice are impaired in locomotion and neurological function. Application of exogenous BDNF was necessary to activate the PI 3-K dependent pathways in pmn/pmn mice which mediates uptake and/or retrograde axonal transport, whereas in control mice the PI 3-K pathway was already maximally activated. BDNF also increased the number of labelled motoneurons in pmn/pmn mice. This data indicated that a deficiency in circulating growth factors such as BDNF may be associated with motoneuron dysfunction in this animal model. Application of BDNF was required in affected animals to improve the uptake and/or retrograde transport of fluorochromes by activation of PI 3-K signalling pathways.

Despite the limitations of each approach (human post-mortem tissue, HEK 293 cells and pmn/pmn mice), my data indicate that PKC and PI 3-K could play a role in motoneuron dysfunction. In analyzing the activities and protein levels of several protein kinases in humam post-mortem spinal cord tissue, I found elevations in the activities of PKC and PI 3-K in ALS tissue compared to control tissue. This elevated PKC and PI 3-K activity could be a compensatory response of the remaining cells to the motoneuron loss.

In pmn/pmn mice, PI 3-K activity is decreased at a stage when these mice have severe neurological dysfunction. This could mean that the response of motoneuron loss at the-end stage in human and mice could be different. Studies from tissues of pmn/pmn mice showed that PI 3-K activity is significantly decreased in the spinal cord particulate fraction of 28 day old pmn/pmn mice compared to control mice. A specific inhibitor of PI 3-K was able to partially influence the uptake and/or transport of fluorescent probes to motoneurons in control but not in pmn/pmn mice. This data indicated that altered PI 3-K activity plays a role in the impairment of motoneuron function in the pmn/pmn mice. From this study it is difficult to establish the role of PI 3-K in a functional motoneuron. Presumably, the ability of motoneurons to take up and/or transport a fluorescent marker reflects their function. In mice with motoneuron dysfunction, BDNF, a substance that increases PI 3-K activity in spinal cord tissue, is required to improve this functional property of motoneuron. The requirement of elevated PI 3-K in pmn/pmn mice was also supported by data showing that PI 3-K activity in pmn/pmn mice was augmented to a greater degree than control mice by application of BDNF. It is clear, that in mice with dysfunctional motoneuron, reduction of PI 3-K activity in the spinal cord is associated with impairment in the uptake and/or retrograde labelling of motoneuron. Two processes likely contribute to motoneuron death in motoneuron disease. First, an initial cellular insult such as mutant SOD or other factors on motoneuron could lead to motoneuron dysfunction. Secondly, there are "survival responses" which are likely mediated by several factors including growth factors or other substances. It is not known whether part of the initial insult which cause motoneuron death in pmn/pmn mice is related to a reduction in growth factor action. However, the observation that BDNF repaired the uptake and/or

retrograde transport of fluorochromes in pmn/pmn mice and that other neurotrophin improve motor symptoms in other animal model of motoneuron disease (Sagot et al., 1997; Haase et al., 1997) suggests that endogenous "survival responses" such as PI 3-K are not optimized in pmn/pmn mice. Whether altered PI 3-K regulation is preceded by cellular insult or acting together to cause motoneuron dysfunction remains unknown. It appears that some of the factors that control motoneuron survival is dependent on the PI 3-K pathway. To address this issue further, PI 3-K analysis should be done in younger animals before the appearance of the clinical symptoms.

In ALS patients, motoneuron loss causes muscle denervation. It is known that PI 3-K activation is dependent upon growth factors which could be increased due to muscle denervation in ALS patients. However, other stimuli such oxidative stress and/or unknown cytokines present in ALS patients could also lead to elevated PI 3-K activity. This response could occur at the end-stage of the disease in humans. However, in pmn/pmn mice, PI 3-K activity was not elevated in the older animals which had significant neurological dysfunction and muscle denervation (41 day of age). PI 3-K activity is decreased in pmn/pmn mice even at a stage when these mice have mild neurological dysfunction (28 day old). It is possible that at an early stage of motoneuron dysfunction, one of the pathogenic mechanisms includes a deficiency in growth factors which could deprive motoneurons from their target-derived neurotrophic factors. This could lead to motoneuron death and muscle denervation which is prominent in ALS patients. It could further alter "survival signalling" pathway including PI 3-K leading to impaired cellular processes such as uptake/retrograde transport and cytoskeleton assembly. At a later stage of the disease in humans, the

presence of damaged axons and motoneurons could trigger the remaining neurons and non-neuronal cells to increase their expression of certain growth factors. The data of increased PI 3-K activity in human postmortem tissue from ALS patients could be a response from surviving motoneurons or non-neuronal cells to elevate survival signals at the endstage of the disease. Thus, applying exogenous growth factors at an earlier stage of the disease could be beneficial to improve motoneuron dysfunction in ALS. Data obtained from human postmortem tissue and from tissue of pmn/pmn mice indicate that altered PI 3-K activity or expression could play a role in the events leading to motoneuron dysfunction.

PKC activity and protein levels were also altered in spinal cord tissues from ALS patients compared to control subjects. The activation of Ca^{2+} -dependent PKC isoforms potentiates NMDA-mediated cell death in HEK 293 cells. Downregulation of PKC in transfected HEK cells resulted in a reduction of NMDA-mediated cell death. There is increasing evidence that crosstalk between various pathways of kinases occur in the cell. The position of PKC in the signal transduction network is such that it converges multiple pathways and regulates various cellular functions. The postmortem data revealed increased PKC activity in spinal cord tissues from ALS patients compared to control subjects. One mechanism for the elevated PKC activity in postmortem tissues from ALS patients is due to elevated PI 3-K activity (Toker, 2000; Parekh et al., 2000). It has been shown that at least one isoform of the classical PKC (PKCβII) associates in vivo with PDK-1, an effector of the lipid product of PI 3-K (PI (3,4,5)-P_3). PDK-1 directly phosphorylates PKCβII (Dutil et al., 1998). Additionally, some novel and atypical isoforms of PKC (PKCε and ζ) can be directly phosphorylated and activated by PDK1- through a PI 3-K dependent pathway (Moriya

et al., 1996; Chou et al., 1998). Thus, the presence of elevated PI 3-K activity could cause PKC activity to be elevated as well in human postmortem tissues from ALS patients. PDK-1 is also considered an upstream kinase for PKB, p70 S6K and Rsk (MAPK family member) (reviewed in Toker et al., 2000). Therefore, the observation of an elevated protein level of PKB and p70S6K could also be stimulated by the elevated PI 3-K activity. However, the activity of PKB and p70S6K was not elevated in spinal cord tissues from ALS patients compared to controls. This data indicates that there may be an impairment in the signalling event from PI 3-K to these two downstream kinases in ALS patients. It may be possible that the elevated activity of PI 3-K possibly acting through PDK-1 was only conveyed to elevate PKC activity and not PKB and p70 S6K for unknown reasons. The actions of abnormal protein phosphatases could also contribute to altered activity and/or protein levels of PI 3-K and PKC in postmortem tissue form ALS patients. However, this is purely a speculative hypothesis with no evidence at present to support this view.

Another possibility for increased PKC activity is secondary to altered $[Ca^{2+}]i$ in ALS. The possible sources for increased $[Ca^{2+}]i$ include activation of glutamate receptors (including NMDA and AMPA receptors) due to excess glutamate present in ALS patients (Plaitakis, 1990; Rothstein et al., 1993). Influx of Ca^{2+} into the cell could also occur through voltage-dependent Ca^{2+} channels. This possibility is supported by the observation that sera from ALS patients can increase Ca^{2+} influx through N-type and P-type Ca^{2+} channels (Llinas et al., 1993; Smith et al., 1994). Further evidence of raised $[Ca^{2+}]i$ in ALS could be from mitochondrial release of Ca^{2+} (Hasham et al., 1994). Moreover, reports of decreased Ca^{2+} buffering capacity in human motoneurons (Ince et al., 1993; Alexianu et al., 1994) could also contribute to the

alteration of $[Ca^{2+}]i$ in ALS. Elevated $[Ca^{2+}]i$ could, in turn, lead to overactivation of PKC, activation of Ca^{2+}-dependent proteases, nucleases and lipases which result in cell death. Increased $[Ca^{2+}]i$ could also interact with altered Cu^{2+}/Zn^{2+} SOD to increase free radicals which cause cell death (Mattson, 1995). Some studies have found that oxidative stress could cause PKC hyperactivation (Gopalakrishna et al., 1989; Brawn et al., 1995). A recent report has showed that H_2O_2 causes tyrosine phosphorylation of the classical PKC isoforms and some isoforms of the novel PKC and atypical PKC classes, which lead to elevated PKC activity (Konishi et al., 1997). The finding that Ca^{2+}-dependent PKC isoforms potentiate NMDA-mediated cell death in NR1A/NR2A transfected Hek 293 cells demonstrate that activated PKC can contribute to cell death. Thus, increased PKC activity and/or protein due to increased $[Ca^{2+}]i$ or overactivation of glutamate receptors in the cell could further potentiate NMDA receptor-mediated cell death (Fig. 28). Inhibitors of PKC could be beneficial for motoneuron dysfunction.

Based on analysis of protein kinase activity and expression in relation to motoneuron dysfunction using three different approaches, it appears that abnormal regulation of PKC and/or PI 3-K in the nervous system could be a cause of motoneuron death. This process may act in concert with other altered cellular processes (eg.oxidative stress) to secondarily affect neurotransmitter receptor function leading to neuronal death in ALS.

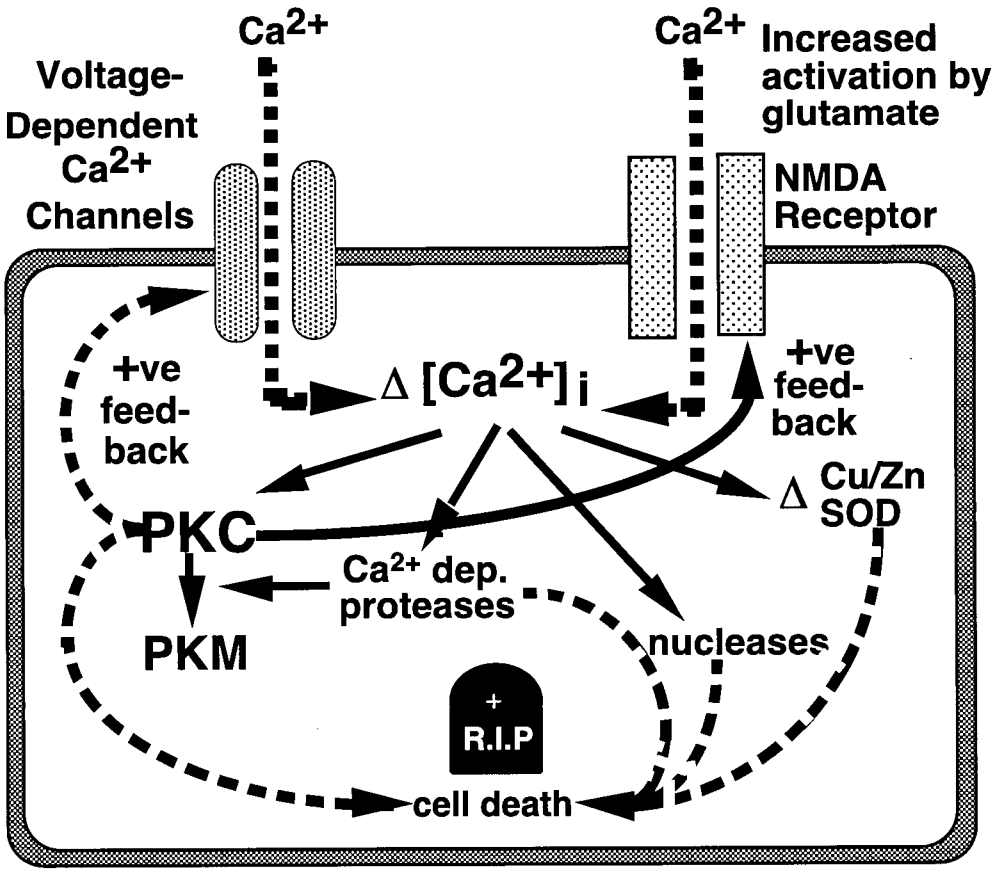

Fig. 28. A model of the possible mechanisms leading to cell death in ALS.

6.2. Conclusions

1. Studies of postmortem tissue showed that PKC activity was significantly increased in cytosolic and particulate fractions of spinal cord tissue from ALS patients compared to control subjects. The increased of PKC activity was partially due to an increase in PKC protein level in ALS spinal cord tissue. PKC activity was not significantly elevated in motor cortex, visual cortex, platelets and leukocytes from ALS patients compared to controls. PKC appeared to be altered only in the spinal cord, which is the region in the nervous system that is most affected by motoneuron loss in ALS patients.

2. PI 3-K activity and protein level was also elevated in the particulate fractions of spinal cord tissue from ALS patients compared to control subjects. The increase of both protein and activity of PI 3-K indicate no change in the specific activity of PI 3-K in the particulate fraction of spinal cord tissue from ALS patients. No changes in PI 3-K activity were present in the cytosolic fraction of spinal cord, or in motor and visual cortices from ALS patients compared to controls. Elevated protein levels of PKB and p70 S6K were found in ALS spinal cord tissue, but no differences in the activities of PKB, p70 S6K, Erk1 and Erk2 were present in spinal cord tissue from ALS patients compared to controls. Other region of the brain such as motor and visual cortex from ALS patients showed no differences in the activity and protein level of PI 3-K indicating that PI 3-K is only altered in the spinal cord region of ALS patients.

3. Phorbol ester, a PKC agonist, potentiates NMDA-mediated death and the PKC inhibitor, RO320432, was able to reduce NMDA-induced cell death of HEK cells transfected with NR1A/NR2A subunits of the NMDA receptor. The Ca^{2+} - and lipid-dependent PKC isoforms (cPKC) are the class of PKC that is responsible for the

toxicity of phorbol ester when added to HEK 293 cells in the presence of NMDA. NMDA stimulation caused an increase in the in vitro Ca^{2+} - and lipid-dependent PKC activity of NR1A/NR2A transfected HEK cells. It appears that an increase of PKC activity could lead to neurotoxic cell death in the presence of NMDA in NR1A/NR2A transfected HEK cells.

4. PI 3-K activity in the particulate fraction of spinal cord was significantly reduced in 28-day-old pmn/pmn mice, compared to similarly aged control mice. No change in total PI 3-K protein was found in the spinal cord particulate fraction indicating that there is a reduction in the specific PI 3-K activity in the pmn/pmn mice. The cytosolic fraction of spinal cord tissue in pmn/pmn mice demonstrated no significant differences in PI 3-K activity and protein level compared to control mice. Brainstem and cerebellum of pmn/pmn mice showed no alteration in PI 3-K activitiy and protein level compared to control mice. Analysis of activity and protein level of PKB, p70 S6K and Erk1 revealed no statistically significant differences in the activities of these three kinases. Protein level of p70 S6K was reduced in pmn/pmn mice, but no differences were found in PKB or Erk1 protein level between pmn/pmn and control mice. BDNF was able to augment PI 3-K activity of pmn/pmn and control mice, with a more pronounced effect observed in pmn/pmn mice. Inhibitors of PI 3-K alter motoneuron retrograde labelling in control mice but not in pmn/pmn mice. Thus, abnormalities in PI 3-K activity are present in pmn/pmn mice and are associated with an impairment of uptake and/or axonal transport in pmn/pmn mice. The effect of BDNF application on the uptake and/or motoneuron retrograde labeling in pmn/pmn mice was mediated through a PI 3-K pathway, but not the MEK pathway.

5. Abnormal regulation of PI 3-K and PKC pathways could be one mechanism leading to motoneuron death in ALS patients.

6.3. Future Studies

The signaling pathways located downstream of PI 3-K, PKC and Erk have not been thoroughly studied in relation to motoneuron death. Specifically, the stress activated protein kinase (SAPK) would be an interesting target to evaluate, since preliminary data from study of human postmortem tissue showed that stress activated protein kinase (SAPK) expression is elevated in the particulate fraction of spinal cord tissue from ALS patients compared to controls (Fig. 29). An immunohistochemical study has also demonstrated that SAPK is overexpressed in glial cells, but not in motoneurons from the spinal cord of ALS patients compared to control subjects (Migheli et al., 1997). Moreover, Virgo and colleagues (1995) have found induction of c-jun (which is phosphorylated by SAPK) in spinal cord tissue from ALS patients, with concurrent loss of the NR1 subunit of the NMDA receptor. The relevance of increased SAPK in ALS tissue is unclear. Further studies could be done in an animal model of ALS or in a primary neuronal culture model to determine the role of this kinase in relation to neuronal death.

The importance of scaffolding proteins to control the proper localization of proteins and protein-protein interactions within the cell has recently been under intense investigation. This field opens a new perspective on the specificity and regulation of proteins in the cell. The association between the NMDA receptor and protein kinases with associated proteins in the postsynaptic density was reported by Suen and colleagues (1998).

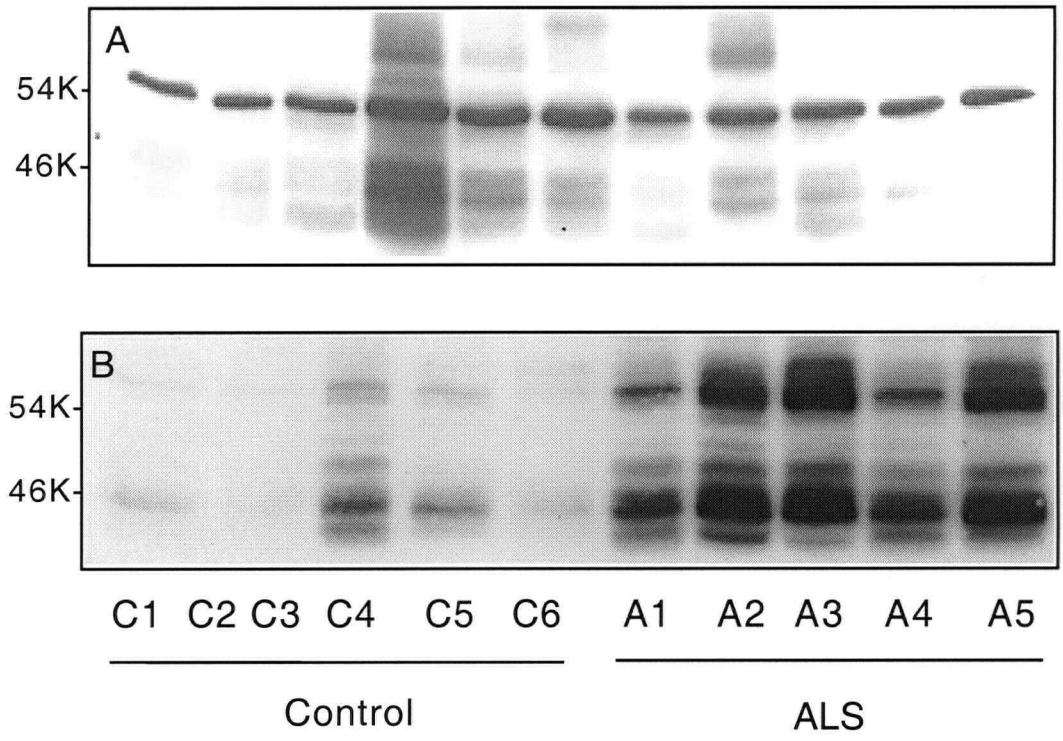

Fig. 29. Protein levels of SAPK in spinal cord tissue from ALS patients and control subjects. SAPK protein expression in the cytosolic (A) and particulate (B) fractions of spinal cord tissue from ALS patients and control subjects. A significant increase in the protein expression of the SAPK (54 kDa and 46 kDa) isoforms is observed in the particulate fractions of spinal cord from ALS patients compared to control subjects ($p < 0.005$).

They found that the NR1 subunit formed a complex with functionally active PKCγ and the major PSD protein (mPSD) through non-covalent interactions. Another study has reported that in cultured cortical neurons, the toxicity of NMDA receptor was associated with PSD-95 (Sattler et al., 1999). Suppresion of PSD-95 by antisense methods protects neurons from death, possibly by inhibiting the production of Ca^{2+} activated Nitric oxide (NO) by NMDA receptor without affecting the toxicity triggered by non-NMDA receptor or voltage-dependent calcium channels (Sattler et al., 1999). Sattler et al. (1999) concluded that by suppressing PSD95 expression, production of NO was uncoupled from NMDA receptor activation and neurons were protected against NMDA toxicity without affecting receptor function. In my study, the mechanism by which PKC potentiates NMDA toxicity was not fully addressed. It is likely that the PKC potentiation was not through direct phosphorylation of the NMDA receptor by PKC (see chapter 4). Thus, in future experiments it would be important to investigate the role of PSD proteins in the PKC potentiation of NMDA cytotoxicity and the role of other factors that could lead to motoneuron death. To address the role of PSD in cell death, a future experiment could be to co-transfect PSD and NMDA receptor subunits in HEK293 cells and treating the transfected cells with PMA or RO 320432 to evaluate cell death in the presence of PSD protein. PSD could be immunoprecipitated in the transfected cells and probed with either PKC or NMDA receptor antibodies to more fully define the interactions between these proteins. This experiment would be an initial one to address the potential role of PSD protein in NMDA-induced cell death.

The contribution of mutant SOD in motoneuron dysfunction should also be examined further (Gurney et al., 1994; Bowling et al., 1995; Roy et al., 1998). To

study the role of mutated Cu/Zn-SOD in ALS, it would be beneficial to obtain transgenic mice containing the mutant SOD gene. Protein kinase measurements in these mice at different ages and stages of their clinical symptoms would provide information as to whether there are some changes in protein kinases in these mice. If any changes in the activity and/or protein levels of a specific kinase is present in these mice, it is possible to determine whether abnormal regulation of the kinase occurred before, or after, the appearance of any clinical symptoms. To study NMDA cytotoxicity, cotransfecting wildtype or mutant SOD together with NMDA receptor subunits in HEK 293 cultures and measuring cell death could provide some insight in the extent of cytotoxicity caused by an interaction of a mutant SOD acting together with NMDA-mediated excitotoxicity.

Since the NMDA receptor is not the only type of receptor that could cause Ca^{2+} influx in the cell, it is also important to study the contribution of AMPA receptor subtypes in cell death and whether PKC plays a role in potentiating AMPA receptors in HEK 293 cells. Roy and colleagues (1998) have demonstrated in a primary culture of murine motoneuron Ca^{2+}-permeable AMPA receptor. Some types of AMPA receptor subunit are more permeable towards Ca^{2+} influx than others. Specific receptor subunits of AMPA are further regulated through mRNA editing, causing some differences in Ca^{2+} permeability. For instance the GluR2 subunit of AMPA receptors has reduced Ca^{2+} entry compared to the other subunits (Hollman et al., 1991; Burnashev et al., 1992). Thus, transfection of HEK293 cells with different subunit of AMPA receptor could be important to determine the role of the AMPA receptor subunit in causing cell death. Application of a PKC agonist or inhibitor to the

AMPA receptor-transfected cultures could reveal the role of PKC in AMPA receptor mediated cell death.

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