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THE ROLE OF CALPAIN IN EXCITOTOXIC NEURONAL CELL DEATH

by

Kimberley E. Hewitt

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Department of Cellular and Molecular Medicine
Faculty of Medicine
University of Ottawa
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ABSTRACT

The ubiquitous, Ca^{2+}-dependent, neutral proteases μ-calpain and m-calpain are heterodimeric regulatory enzymes consisting of an 80 kDa catalytic subunit and a 30 kDa regulatory subunit. In the central nervous system (CNS), calpain-mediated proteolysis of structural proteins and metabolic enzymes is a common feature of Ca^{2+}-dependent neuronal death in both *in vitro* and *in vivo* models of excitotoxicity and stroke. A number of inhibitor studies have lead to the conclusion that calpain activation is a necessary and contributory event in the death of neurons under ischemic or ischemic-like conditions.

Although the mechanism of calpain activation has been extensively studied *in vitro*, a precise understanding of the activation and regulation of the enzyme within cells remains elusive. In erythrocytes calpain activity appears to arise from a Ca^{2+}-dependent translocation of cytosolic calpain to membranes, followed by autolytic conversion of the catalytic subunit from an 80 kDa protein to a 78 or 76 kDa active form, and the regulatory subunit from a 30 kDa to a 17 kDa form. Whether this activation process seen in non-CNS cells is necessary for activation in neurons has, until the present study, not been directly tested. In particular, there is a paucity of information on the cellular process by which lethal increases in $[Ca^{2+}]_i$ through over-stimulated glutamate receptors brings about calpain activation in susceptible neurons. In this thesis we have examined the effects of lethal and non-lethal Ca^{2+} influx on the activation of μ-calpain, and the mechanism by which that activation occurs.

We have demonstrated that calpain activation in cultured neurons proceeds by a mechanism clearly distinct from the "traditional" route observed in erythrocytes and other non-CNS cells. Transiently exposing primary rat cortical neurons to lethal NMDA caused

protracted calpain activation, measured as increased endogenous spectrin hydrolysis, that was independent of translocation and autolysis of the protease. Calpain was largely membrane associated in cortical neurons and consequently neither translocation nor autolysis of the protease was observed following ionomycin or lethal NMDA treatment. By contrast, in rat erythrocytes calpain was largely cytosolic, and underwent rapid translocation and autolysis in response to ionomycin. An important finding in neurons was that calpain-mediated spectrin hydrolysis was specifically coupled to Ca^{2+} entry through the NMDA receptor. Thus, unlike that observed in erythrocytes, non-specific Ca^{2+} influx via ionomycin or KCl-mediated depolarization failed to activate the enzyme. Furthermore, while calpain activation was induced by both glutamate and AMPA, the effect of these compounds was prevented by NMDA receptor blockade. The data clearly demonstrated that calpain is selectively linked to the NMDA receptor, and that intracellular signals coupled to the NMDA receptor are responsible for activating calpain already associated with cellular membranes in cortical neurons.

One intracellular mediator of NMDA effects is protein kinase C (PKC). The rapid inactivation of PKC is a characteristic and necessary event in the excitotoxic death of neurons both *in vivo* and *in vitro*. We demonstrate that the NMDA-induced inactivation of PKC in cortical neurons contributes to calpain activation. The neurotrophin BDNF, which protects neurons from excitotoxicity by preventing the NMDA-induced inactivation of PKC, also blocked calpain activation. The use of PKC inhibitors showed this effect of BDNF on calpain activity to be mediated by the NMDA selective inactivation of PKC. Furthermore, sublethal NMDA receptor activation and PKC inhibition, treatments unable to activate calpain when applied separately, were in

combination able to promote calpain-mediated spectrin hydrolysis. The evidence suggests that calpain activation by NMDA requires at least two Ca^{2+}-dependent processes, one of which involves the inactivation of a PKC pool selectively coupled to the NMDA receptor. The possibility that the observed increase in spectrin hydrolysis by NMDA-mediated PKC inactivation was due, not to calpain activation, but to increased susceptibility of spectrin to calpain cleavage by dephosphorylation was examined and discounted. Similarly, the possibility that the dephosphorylation of calpain itself was a critical step in its activation was also eliminated. On the other hand, experiments revealed that the NMDA-mediated loss of the endogenous calpain inhibitor protein, calpastatin, from membranes could account for the concomitant activation of calpain in cortical cells under excitotoxic conditions. This loss of membrane calpastatin was found to be independent of PKC inactivation, suggesting that the two events necessary for calpain activation by NMDA were a PKC-independent inactivation of calpastatin, and a still undiscovered event dependent on the inactivation of a PKC pool juxtaposed to the NMDA receptor. Thus, the activation of calpain in cultured neurons is a tightly controlled event that appears to play a major role in excitotoxic processes, and as well, is likely important to the normal, physiological function of the NMDA receptor.

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INTRODUCTION

The adult human brain receives nearly 15% of total resting cardiac output, but on average comprises only 2% of the total human body weight (Cotran et al., 1989). This observation, combined with the highly perfused nature of the brain, attests to its exceptional energy requirement and underlies its intrinsic sensitivity to any change or interruption in blood flow. If circulation to the brain is completely blocked, a loss of consciousness occurs within seconds (Rossen et al., 1943). If maintained, a loss of blood flow will lead to irreversible pathological change within minutes. A serious threat to cerebral blood flow resulting in neuronal injury can occur under numerous circumstances, such as during a significant loss of blood (Zola-Morgan, Squire and Amaral, 1986), cardiac arrest, artery occlusion, or cerebral aneurysm (Pulsinelli, 1997). The neurological outcome following these events depends upon the nature, severity, duration, and localization of the insult (Pulsinelli, 1997).

The metabolic events that ensue in brain tissue following a compromise in cerebral perfusion have been the focus of much research attempting to delineate critical features contributing to neurological damage. By understanding neuronal function during a pathological insult, there is potential for the development of effective treatment for debilitating brain disease. Examination of the underlying mediators of cellular pathology is therefore an important endeavor from both a basic science and clinical perspective.

A major aspect of neuronal pathology in many disease states is thought to involve the excitatory amino acid neurotransmitter, glutamate. An over-activation of glutamate receptors resulting from excessive neurotransmitter release, and an ensuing increase in

intracellular Ca^{2+} and loss of ionic homeostasis, form the foundation of the excitotoxic hypothesis of cell death. These processes have been postulated to be a central feature of many neuronal pathologies such as stroke, epilepsy, traumatic brain injury, neurodegenerative diseases, and exposure to toxins (Meldrum, 1990; Meldrum and Garthwaite, 1990; Bradford, 1995; Zauner and Bulock, 1995).

This introduction will be limited to the current thinking on neurodegenerative mechanisms resulting from cerebral ischemia, stroke, and excitotoxicity at the cellular level. After examining the potential mechanisms contributing to excitotoxicity, the role of the Ca^{2+}-dependent protease, calpain, will be considered in detail. The regulation of this intracellular protease in neurons, its role in cell death, and its interaction with PKC, another known signaling effector involved in excitotoxic cell damage, are the central themes of this research thesis.

GLUTAMATE, EXCITOTOXICITY AND NEURONAL CELL DEATH

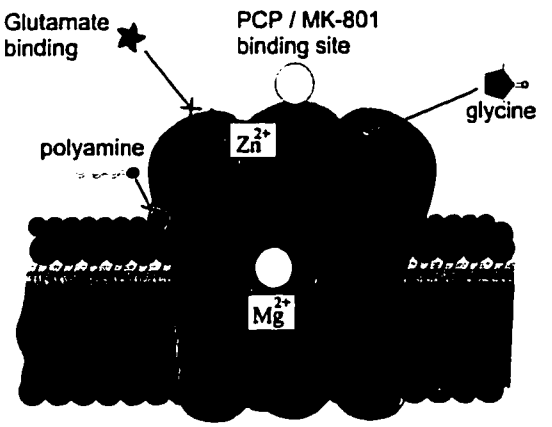
Glutamate is the principal excitatory neurotransmitter in the mammalian nervous system. When nerve terminals containing glutamate are depolarized, vesicular glutamate is released as a consequence of Ca^{2+}-influx through non-inactivating voltage-sensitive Ca^{2+} channels (N-type channels) at the terminal (Nicholls and Atwell, 1990; Nicholls, 1993). Once released, glutamate can interact with several distinct receptor subtypes. There are two broad categories of glutamate receptors, the ionotropic, channel-forming receptors, and the metabotropic, GTP-binding receptors linked to the activation of phospholipase C (PLC) or the inhibition or activation of adenylyl cyclases (AC). The

ionotropic class is further divided into three populations based on specific agonist responses: those activated by N-methyl-D-aspartic acid (NMDA), those activated by kainic acid (KA), and those sensitive to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA). The complexity of the glutamate neurotransmitter system is becoming more and more apparent as molecular biology demonstrates the profusion of receptor subunits that form these active receptor complexes. The potential for post-translational modification, and the multitude of intracellular signal transduction cascades that can be initiated by activation of these surface receptors adds to this complexity. The goal of much research has been to define the characteristics of these receptor subtypes in terms of their structure, physiology and pharmacology.

NMDA receptors

The NMDA receptor subtype of the ionotropic class of glutamate receptors is perhaps the most extensively characterized. This receptor possesses both voltage and ligand sensitivity due to its complex composition. In addition to its neurotransmitter binding site, the receptor consists of additional functional subcomponents with discrete ligand-binding domains. As shown in Scheme 1, there are at least five pharmacologically distinct sites through which receptor activity can be modulated - a transmitter binding site that binds glutamate, a regulatory site that binds glycine, a site within the channel that binds phencyclidine and related compounds, a voltage-dependent inhibitory Mg^{2+} binding site, and an inhibitory divalent cation site that binds Zn^{2+} (reviewed by Michaelis, 1998). Activation of the receptor complex requires both ligand binding and alleviation of the voltage dependent Mg^{2+} block (reviewed by Michaelis, 1998).

Scheme 1. Hypothetical cross-section of an NMDA receptor showing modulatory sites for activation and inhibition.



Adapted from Conley, 1996

Structurally, the receptor is formed by heteromeric pentamers or tetramers of NR1 and NR2 subunits which form cation permeable ion channels (Seeburg et al., 1994). The NR1 subunit consists of two extracellular domains (S1 and S2) which form the glutamate and glycine binding sites, respectively, three transmembrane domains (TM1, TM3 and TM4), and a hydrophobic domain (TM2) that constitutes the ion pore. There are four NR2 subunits (NR2A, NR2B, NR2C, and NR2D) (Conley, 1996). The combination of these two subunits determines the biophysical and pharmacological features of the NMDA receptor. The NR2 subunits confer single-channel conductance, kinetic properties, deactivation time constants, agonist affinity, and glycine, protein kinase C (PKC), Mg^{2+}, and antagonist sensitivity (Takahashi et al., 1996).

Relative to other glutamate receptor subtypes, NMDA receptors are five times more permeable to Ca^{2+} (Schneggenburger et al., 1993). This permeability is linked to an asparagine (N) residue in the TM2 region of the channel (Seeburg et al., 1994) and site-

directed mutations to a glutamine (Q) or an arginine (R) significantly decrease the Ca^{2+} permeability of the receptor (Burnashev et al., 1992). In addition, neuronal toxicity produced by NMDA depends on the Ca^{2+} permeability of the receptor complex, which is in part determined by the combination of receptor subunits present. Toxicity is greatest when NR1 and NR2A are co-expressed, less when NR2B is co-expressed with NR1, and not apparent when NR1 and NR2C are paired (Grimwood et al., 1996a,b; Grant et al., 1997). This toxicity profile correlates with enhanced Ca^{2+} permeation through the receptors formed.

AMPA/Kainate receptors

The structural and functional properties of both AMPA and kainate receptors have also been characterized in order to better understand the function of these receptors in neuronal signaling. Both ionotropic subtypes contain features that result in altered physiology depending on the subunit composition and post-translational modifications.

AMPA receptors are heteromeric pentamers or tetramers of GluR1, GluR2, GluR3 and GluR4 subunits. The major functional differences between these subunits arise from an arginine (R) residue in the GluR2 subunit in the TM2 pore region (Hume et al., 1991) which results from post-translational RNA editing of an adenosine to a guanosine (Yang et al., 1995). GluR1, GluR3, and GluR4 each have a glutamine (Q) at this site (Hume et al., 1991). Replacement of the GluR2 arginine with a glutamine confers Ca^{2+} permeability to the receptor complex (Boulter et al., 1990). In receptors with unedited GluR subunits the Ca^{2+} current is eight-fold greater than that of edited receptors (Seeburg, 1996). The majority of AMPA receptors contain at least one GluR2 subunit which confers Ca^{2+}

impermeability to the channel (Sommer et al., 1991). Nonetheless, Ca^{2+} permeable AMPA receptors have been reported *in vitro* (Jensen et al., 1998) and *in vivo* (Mahanty and Sah, 1998). There is also additional post-translational modification by RNA editing at an arginine (R) residue designated as the R/G site in GluR2, GluR3, and GluR4 (Lomeli et al., 1994). Editing of the arginine (R) to a glycine (G) modifies the kinetic behaviour of the AMPA channel (Lomeli et al., 1994). In addition, each of the four GluR subunits have two splice variants deemed flip and flop, which determine the desensitization characteristics of the channel (Sommer et al., 1990).

It should be noted that in addition to having ionotropic receptor function, the GluR1 subunit of the AMPA receptor has recently been shown to functionally couple with a G-protein (Gi_{α}) at its C-terminal, independent of its actions as a ionotropic receptor (Wang et al., 1997). Hayashi et al. (1999) have also demonstrated that AMPA receptors can signal by an interaction with the protein tyrosine kinase Lyn, through a Lyn-MAPK pathway. Their results showed that this pathway may contribute to neuroplasticity by increasing BDNF expression independently of AMPA's function as a ligand-operated ion channel. Collectively, these findings suggest that the AMPA receptor can possess both ionotropic and metabotropic-like functions.

Kainate receptors are composed of subunits deemed KA1, KA2, GluR5, GluR6 and GluR7. This receptor subtype is also subject to post-translational RNA editing in the TM2 (Q/R) site of subunits GluR5 and GluR6 (Lomeli et al., 1994). Unlike AMPA receptors where the editing of this site occurs at 100% efficiency, GluR5 and GluR6 editing is only 40% and 80% efficient, respectively (Paschen et al., 1995). GluR6

subunits can also undergo editing at two other sites - the (I/V) site where isoleucine (I) is edited to a valine (V), and the (Y/C) site in which a tyrosine (Y) is replaced by a cysteine (C) (Kohler et al., 1993). These modifications also serve to potentiate the Ca^{2+} permeability of the receptor (Kohler et al., 1993).

There is some evidence to suggest that kainate-"like" receptors can also function as metabotropic receptors, at least in avian and amphibian brain. In these species there are kainate binding proteins that have high homology to GluR and KA proteins (Gregor et al., 1989; Wada et al., 1989; Hollmann and Heinemann, 1994). When cloned and expressed, these proteins bind both kainate and domoate with high affinity, but do not appear to form either homomeric or heteromeric ion channels when expressed in frog oocytes. Antibodies raised against these proteins react against a 99 kDa protein in rat brain, thought likely to be one of the GluR proteins expressed in mammalian brain (Hampson et al., 1989). In goldfish, these proteins appear to interact with a PTX-sensitive G-protein (Willard et al., 1991; Ziegra et al., 1992). Purification experiments led to co-purification of a 35 kDa protein that was immunologically identical to the β subunit of G-proteins. Furthermore, kainate binding led to the stimulation of GTPase enzyme activity, indicating coupling to a functional G-protein.

Glutamate-Mediated Excitotoxicity and Cell Death

Excessive glutamate release and the activation of NMDA, AMPA and kainate receptor subtypes leading to excitotoxicity have been a major focus of much research on neurological disorders in the past 10 years. Glutamate-mediated neuronal injury is thought to result from excessive Ca^{2+} influx through glutamate ligand operated ion

channels and a loss of ionic homeostasis (Choi, 1987). The involvement of the NMDA and AMPA subtypes of ionotropic glutamate receptors in excitotoxicity and ischemia is supported by a collection of experimental evidence from both *in vivo* and *in vitro* experimental methodology.

The NMDA receptor initially gained major attention as a mediator for cell death following ischemia or excitotoxicity because of its high permeability to Ca^{2+}. The ensuing entry of Ca^{2+} through the ion pore formed during glutamate binding to the NMDA receptor appears central to subsequent neuronal pathology (Rothman and Olney, 1986; Gill, Foster, and Woodruff, 1988). In neuronal cultures, NMDA-triggered toxicity is dependent on extracellular Ca^{2+} (Choi, 1985, 1987), and prior application of NMDA receptor antagonists which block Ca^{2+} influx through the ion pore is neuroprotective (Chen et al., 1992; Black et al., 1995). Furthermore, Ca^{2+} entry through the NMDA receptor is more toxic than through non-NMDA receptors, VSCCs (Tymianski et al., 1993a; Durkin et al, 1996; Hewitt et al., 1998), or non-specific entry induced by Ca^{2+} ionophores (Durkin et al., 1996; Hewitt et al., 1998). This suggests that Ca^{2+} entry through the NMDA receptor is selectively linked to Ca^{2+}-dependent cell signaling effectors that directly mediate cell death.

Despite early promise, *in vivo* pharmacological therapies for cerebral ischemic brain damage based on NMDA receptor channel blockade have been, in general, disappointing. In fact no glutamate receptor antagonists have, to date, successfully survived the challenge of moving from animals studies to the human situation. Numerous investigators have reported significant neuroprotection using the NMDA receptor

antagonist MK-801 (Kochhar et al., 1991; Bielenberg and Beck, 1991; Dirnagl et al., 1990; Rod and Auer, 1989; Foster et al., 1988; Park et al., 1988; Gill et al., 1988; 1987) in various experimental models. Others have questioned the conclusion that these neuroprotective effects arise from NMDA receptor antagonism alone, since MK-801 can induce sufficient hypothermia with co-applied anesthesia in experimental models to provide neuroprotection (Sheardown et al., 1993; Nellgard and Wieloch, 1992; Buchan and Pulsinelli, 1990; Corbett et al., 1990). Early experiments using NMDA receptor antagonists were confounded by poor temperature control. Indeed, Colbourne and Corbett (1994) demonstrated that profound hypothermia is not necessary for neuroprotection, since lowering body temperature by just a few degrees promoted significant cell sparing. These results exemplified the strict requirement for temperature control in studies utilizing animal models of cerebral ischemia if valid interpretations of drug treatments were to be made. Nonetheless, a modest, but significant amount of neuroprotection by the NMDA receptor antagonist MK-801 was demonstrated in experiments that maintained normal body temperature during and following cerebral ischemia (Hewitt and Corbett, 1992, Gill and Woodruff, 1990). Similar findings have been reported with other antagonists of the NMDA receptor (Ibarrola et al., 1998; Miyabe et al., 1997; Jiang et al., 1996; Omae et al., 1996; Du et al., 1996; Palmer et al., 1995). These observations suggest that NMDA receptor activation is significantly contributing to ischemic neuronal injury.

More recently, the contributions of the AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionate) receptor to ischemic neuronal damage have been examined. Although the functional role of Ca^{2+}-permeable AMPA receptors is unclear, these

receptors do seem to contribute to glutamate toxicity and Ca^{2+} overload. Activation of AMPA receptors permits an influx of Na^{+}, which depolarizes the membrane, and acts to relieve the Mg^{2+} block of the NMDA receptor, thereby potentiating NMDA activity (Collingridge and Singer, 1990). In addition, membrane depolarization allows Ca^{2+} entry through VSCCs. The potent and selective AMPA receptor antagonist NBQX, protected CA1 hippocampal neurons when administered up to 12 (rat) or 24 (gerbil) hours after a transient global ischemic insult (Buchan et al., 1991a,b; Diemer et al., 1992; Nellgard and Wieloch, 1992; Buchan et al., 1993; Li and Buchan, 1993; Sheardown et al., 1993). AMPA receptor antagonism by NBQX was also shown to be protective against neuronal damage from permanent and temporary middle cerebral artery occlusion (Buchan et al., 1991b). However, like MK-801, NBQX has also been shown to induce hypothermia when administered to laboratory animals, and the compound lost its neuroprotective efficacy when brain temperature was maintained within physiological parameters (Nurse and Corbett, 1996). Moreover, the currently available AMPA receptor antagonists have been shown to possess significant and unacceptable side-effects under therapeutic conditions (Zivin, 1997), making them unlikely candidates for ischemic intervention.

A recent review by Colbourne et al. (1997) suggests that an examination of the downstream effects of hypothermia could provide important information for metabolic targets for neuroprotection. To date, there have only been a few studies that attempt to examine the metabolic features of hypothermia during a cerebral ischemic insult. Collectively, they suggest that hypothermia may be selectively targeting important downstream events in the excitotoxic cascade. Despite the fact that hypothermia would

primarily be expected to reduce metabolic demands, it does not appear to prevent the ischemia-induced loss of ATP. Using magnetic resonance spectroscopy, Sutherland et al. (1992) demonstrated that the levels of ATP did not differ before, during or after ischemia in control or hypothermic animals undergoing global forebrain ischemia. This suggested that the neuroprotective features of hypothermia did not involve the sparing of energy stores. Furthermore, intra-ischemic hypothermia did not to prevent the influx of Ca^{2+} observed during an ischemic insult (Kristian et al., 1992). Thus, hypothermia can induce significant cell sparing despite the fact that a loss of Ca^{2+} homeostasis still occurs. In this context, it appears that hypothermia may be targeting some downstream effector of intracellular Ca^{2+} entry. This is supported by the fact that hypothermia is neuroprotective if applied post-ischemically, and is still beneficial, even when delayed (Coimbra and Wieloch, 1994; Colbourne and Corbett, 1994).

The collection of findings examining the metabolic characteristics of cerebral ischemia and the efficacy of various interventions has resulted in a model in which cell death is viewed as a complex interaction of events. The focus on receptor-mediated toxicity has shifted to downstream signaling events that may offer more selective targets for intervention without disrupting normal cell function. This has led to examination of the underlying toxic events and signaling pathways that emanate from aberrant levels of Ca^{2+} influx. Ischemia-induced Ca^{2+} overload remains the rudimentary component of any approach to understanding cell death following ischemia or excitotoxicity.

Ca^{2+} Homeostasis and Ischemic Damage

Ca^{2+} has come to be considered a key mediator of cell death for a number of

reasons. First, many different types of cell injury *in vitro* fail to damage cells if extracellular Ca^{2+} is omitted from the preparation (Tymianski, 1996; Choi, 1985, 1987; and see above). Second, antagonists of ionotropic glutamate receptors or voltage-dependent ion channels, which allow Ca^{2+} influx, can ameliorate or prevent cell damage (Tymianski, 1996; Uematsu et al., 1991; Hewitt and Corbett, 1990; Rod and Auer, 1992; and see above). Because Ca^{2+} is known to play such an essential role as a second messenger, activating phospholipases, proteases, and endonucleases (Siesjo and Smith, 1991), and its rise is associated with the production of reactive oxygen species (ROS; such as $\cdot O_2^-$, H_2O_2, and $\cdot OH$) (Kristian and Siesjo, 1998, 1996; Kuroda and Siesjo, 1997), it is a reasonable hypothesis that the increases in Ca^{2+} observed lead to a pathological cascade of reactions which contribute to cell injury. This is the foundation for the Ca^{2+} hypothesis of ischemic cell death.

Under physiological conditions, Ca^{2+} is a highly regulated second messenger, transmitting information by its interaction with Ca^{2+} dependent response elements, and by regulating mitochondrial metabolism, protein synthesis, and gene expression (Miller, 1991) via its entry through transmitter and voltage dependent Ca^{2+} channels, and release from internal stores (the endoplasmic reticulum and calciosomes) (Berridge, 1993). Ca^{2+} influx through ion channels is usually counterbalanced by active efflux through a Ca^{2+}-activated ATPase and a $3Na^+/Ca^{2+}$ antiporter. This antiporter is driven by the Na^+ gradient, and therefore relies on the Na^+/K^+ ATPase and membrane potential. Release of Ca^{2+} from intracellular stores results from the activation of ligand receptors coupled to phospholipase C in which the generation of IP3 by hydrolysis of polyphosphoinositide

cause receptor gated channels of the endoplasmic reticulum to be activated, triggering the release of intracellular Ca^{2+} (Berridge and Dupont, 1994). This rise in intracellular Ca^{2+} is proposed to cause a "Ca^{2+}-induced Ca^{2+} release" from calciosomes which further increases free Ca^{2+} concentrations (Berridge and Dupont, 1994). Reuptake of Ca^{2+} from intracellular sources to the endoplasmic reticulum requires ATP. In addition, Ca^{2+} can be sequestered by mitochondria. Ca^{2+} accesses the mitochondrial matrix by a uniporter driven by the mitochondrial membrane potential, and is extruded by a Na^+/Ca^{2+} antiporter (Gunter et al., 1994). The Na^+ gradient is restored by Na^+/H^+ exchange (Gunter et al., 1994).

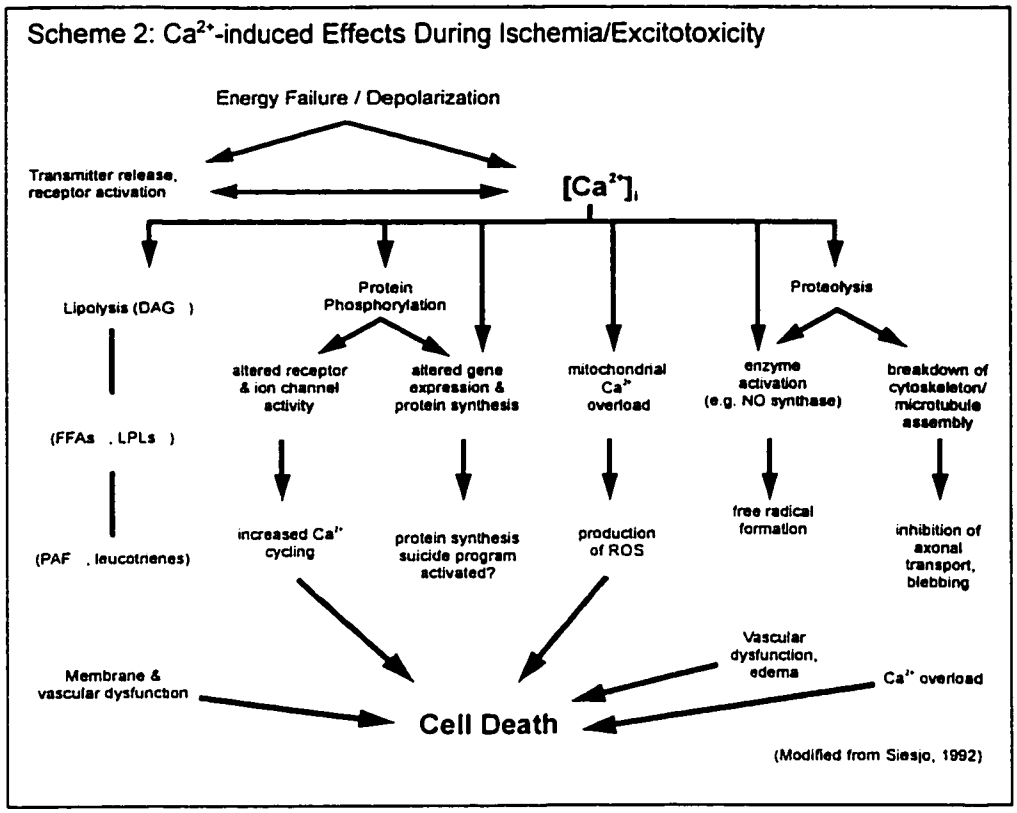
The Ca^{2+} hypothesis of ischemic/excitotoxic cell damage links the activation of glutamate receptors and an unphysiological rise in $[Ca^{2+}]i$ to numerous downstream biochemical processes that are pathological in nature. These include lipolysis, enhanced production of free radicals, mitochondrial Ca^{2+} overload, changes in protein phosphorylation/dephosphorylation, and enhanced proteolysis (Choi, 1988). The consequences of lipolysis, free radical production, and mitochondrial dysfunction due to Ca^{2+} entry will be discussed below. The importance of protein phosphorylation/dephosphorylation and enhanced proteolysis in cerebral ischemia will subsequently be discussed in further detail since these issues, and their potential interaction in ischemia/excitotoxicity, form an underlying basis for this thesis.

Lipolysis, Free Radical Formation, and Mitochondrial Ca^{2+} Overload

Lipolysis results in the production of free fatty acids (FFA) and lysophospholipids, which are potentially toxic (Farooqui et al., 1997; Katsura et al., 1993;

Siesjo and Katsura, 1992; Banik et al., 1987; Wieloch and Siesjo, 1982). The activation of phospholipase A_2 (PLA_2) can result in the accumulation of arachidonic acid, and through cyclooxygenase and lipooxygenase, the production of prostaglandins, leukotrienes, and thromboxane A_2. These processes can lead to the alteration of membranes, and damage to membrane phospholipids can result in uncontrollable Ca^{2+} influx as integrity of the bilayer is compromised (Farooqui et al., 1997).

Free radical production can also result from the activation of cyclooxygenase and lipooxygenase (Siesjo and Katsura, 1992; Wieloch and Siesjo, 1982). Lipolysis involving PLC can also lead to enhanced production of DAG, thereby potentially increasing PKC activity (Siesjo and Katsura, 1992; Berridge, 1993). These effects could influence membrane functioning through changes in ion channel and receptor activity. Both neurons and glia are potentially affected, resulting in cell edema due to loss of ionic homeostasis and the failure of glutamate uptake by glia (Schneider et al., 1992). Furthermore, during ischemia, the activation of phospholipases and the resulting production of reactive oxygen species (ROS) causes damage to microvasculature, with subsequent vascular dysfunction (Siesjo and Katsura, 1992). However, Ca^{2+}-dependent reactions that produce the largest source of ROS are thought to occur through the mitochondrial respiratory chain, where the consumption of oxygen through one-electron reduction produces $O_2^{\cdot}$ from O_2 (Halliwell, 1992; Floyd and Carney, 1992). Through the actions of superoxide dismutase present in the mitochondria, the final consequence is the excessive production of $O_2^{\cdot}$ plus H_2O_2, and the generation of highly reactive hydroxyl radical (.OH) and other oxidants (Halliwell, 1992).



When $[Ca^{2+}]i$ rises above a certain level, mitochondria start to compensate for this rise by accumulating Ca^{2+}. The accumulation of massive amounts of Ca^{2+} by mitochondria has been known for some time to cause substantial mitochondrial damage, in part through damage to the mitochondrial inner membrane via the actions of activated mitochondrial phospholipases (Gunter and Pfeiffer, 1990; Zoratti and Szabo, 1995). This causes a nonspecific increase in mitochondrial membrane permeability and a resulting loss of the ion gradient necessary for H^+ exchange (Gunter and Pfeiffer, 1990; Zoratti and Szabo, 1995). If prolonged, this can cause irreversible damage to mitochondria. Furthermore, once membrane permeability is enhanced, the accumulated Ca^{2+} is released, and the mitochondria are a source of ROS (Richter et al., 1995).

The combination and interaction of these processes (see Scheme 2 above) act to damage or impair integral cellular proteins, membrane lipids, the production of energy, and energy stores. In combination, these aberrant processes will undermine functional integrity and the potential recovery of ATP will be lost.

Protein Phosphorylation and Dephosphorylation in Excitotoxicity

In concert with lipolytic-induced changes to membranes, free radical induced damage, and mitochondrial dysfunction during ischemia, early and profound changes in protein kinase/phosphatase activity induced by excessive Ca^{2+} influx is thought to affect long term cellular functioning in a variety of ways. Neurons contain high concentrations of adenylate cyclase, phosphodiesterase, protein kinases, and phosphatases which regulate how chemical and electrical stimulation are translated by specialized synaptic and intracellular proteins (Zivin et al., 1990). The cellular functions modulated by protein phosphorylation include metabolism, excitability, Ca^{2+} permeability, neurotransmitter biosynthesis and release, receptor activity, and gene expression (reviewed by Nestler and Greengard, 1984). Protein phosphorylation/dephosphorylation can rapidly modulate ion channel activation or inhibition (Magnoni et al., 1991). On a longer time scale protein kinase cascades are ultimately responsible for transferring membrane signals to the nucleus, where specific genes responsible for cell regulation are activated or deactivated (Magnoni et al., 1991). Because phosphorylation plays such a major role in the regulation of numerous cellular functions, aberrant phosphorylation will have profound effects on cell fate. As such, changes in protein phosphorylation have been linked to a variety of neurodegenerative diseases including Alzheimer's Disease (Billingsley and Kincaid,

1997; Jin and Saitoh, 1995; Strong et al., 1995), ALS (Wagey et al.,1998; Krieger et al., 1996), and cerebral ischemia (Braunton et al., 1998; Au and Gurd, 1995; Babcock et al., 1995; Shackelford et al., 1995; Hu and Wieloch, 1994; Busto et al., 1994; Kindy, 1993; Yamamoto et al., 1992a; Cardell et al., 1991; Churn et al., 1990a,b; Zivin et al., 1990). The importance of protein phosphorylation as a regulator of apoptosis, although complex, is also well documented (Datta et al., 1997a,b; Ito et al., 1997; Jacobson, 1997; Yang et al., 1997).

Post-ischemic/excitotoxic changes in kinase/phosphatase-mediated signal transduction have been strongly correlated with irreversible cell damage and subsequent cell death (Braunton et al., 1998; Durkin et al. 1997; Au and Gurd, 1995; Babcock et al., 1995; Shackelford et al., 1995; Hu and Wieloch, 1994; Busto et al., 1994; Kindy, 1993; Yamamoto et al., 1992a; Cardell et al., 1991; Churn et al., 1990a,b; Zivin et al., 1990). For example, protein tyrosine phosphorylation, which normally mediates many of the actions of growth factors, has been observed to undergo alterations following cerebral ischemia or anoxia (Braunton et al., 1998; Au and Gurd, 1995; Hu and Wieloch, 1994; Campos-Gonzalez and Kindy, 1992). Increased TrkB protein tyrosine kinase receptor expression has also been shown to occur following brain injury (Merlio et al., 1993), suggesting an upregulation in tyrosine kinase phosphorylation-mediated signaling, at least with respect to this one receptor. Neurotrophins such as BDNF, which bind TrkB receptors and transduce tyrosine kinase phosphorylation, confer neuroprotection against ischemic and excitotoxic cell death (Tremblay et al., 1999; Ferrer et al., 1998; Kume et al., 1997; Cheng et al., 1997). On the other hand, the widespread inhibition of protein

tyrosine phosphorylation has been shown to protect hippocampal CA1 neurons from cerebral ischemia (Kindy, 1993).

The loss or inhibition of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) also occurs following ischemia, and these changes correlate with cell death (Babcock et al., 1995; Shackelford et al., 1995; Yamamoto et al., 1992a; Churn et al., 1990a,b). Because CaMKII is a substrate for calpain *in vitro* (Kwiatowski et al., 1989; Rich et al., 1990), it has been postulated that the loss of CaMKII during ischemia is mediated through increased proteolysis (Yamamoto et al., 1992a), however this relationship has not been directly addressed. In contrast, inhibition of CaMKII by KN-62 (1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine) was neuroprotective against NMDA toxicity and hypoxia/hypoglycemia-induced neuronal injury in cell cultures (Hajimohammadreza et al., 1995), suggesting CaMKII activity can have a detrimental role in cell survival.

Casein kinase II (CKII), a ubiquitously expressed serine/threonine protein kinase in eukaryotic cells, also shows decreased activity in vulnerable brain areas following transient forebrain ischemia (Hu and Wieloch, 1993). CKII activity increased in resistant brain areas (Hu and Wieloch, 1993) suggesting that preservation of the kinase's activity may be related to cell survival. However, there is little evidence for a role of CKII in ischemia beyond correlative studies of this kind.

More recently, PI3-kinase and an Akt signaling pathway have been implicated in promoting cell survival (Kaplan and Miller, 1997). PI3-kinase is a cytoplasmic signaling effector that is recruited to the membrane when growth factor receptors are activated

(Klippel et al., 1996). Akt is a serine/threonine kinase that is activated *in vivo* and *in vitro* by phosphatidylinositol 3,4-biphosphate (Franke et al., 1997), a second messenger produced by PI3-kinase activity. Over-expression of Akt was sufficient to promote cell survival of cultured cerebellar neurons in the absence of survival factors (Dudek et al., 1997). Activation of the PI3/Akt pathway mediated the survival response of these neurons to neurotrophic factors and prevented apoptosis (Dudek et al., 1997; Kulik et al., 1997). Following a focal ischemic insult, Lin et al. (1992) reported that there was a time-dependent decrease in the PI3 kinase activity, suggesting that this signaling pathway may be involved in cell fate during ischemia.

The protein phosphatase calcineurin also shows changes in response to ischemic (Hashimoto et al., 1998; Butcher et al., 1997; Drake et al., 1996; Yamasaki et al., 1992) or excitotoxic (Kikuchi et al., 1998; Ankarcrona et al., 1996; Dawson et al., 1993) insults. Calcineurin is a calcium/calmodulin-dependent protein phosphatase capable of modulating ion channel activity, neurotransmitter release, synaptic plasticity, and gene transcription (Yakel, 1997). Following cerebral ischemia, the immunoreactive profile of calcineurin was shown to be significantly altered (Hashimoto et al., 1998; Yamasaki et al., 1992). Following a transient decrease in the amounts of detectable calcineurin, the detection of the phosphatase was enhanced (Hashimoto et al., 1998; Yamasaki et al., 1992). Treatment with the calcineurin inhibitor FK506 in a rat model of transient ischemia significantly attenuated neuronal damage (Butcher et al., 1997; Drake et al., 1996). Similar protective effects of FK506 were found in retinal cell cultures and cerebellar granule cells treated with glutamate receptor agonists (Kikuchi et al., 1998;

Ankarcrona et al., 1996).

Protein Kinase C (PKC)

The above findings indicate that a variety of protein kinases and/or phosphatases may be contributing to a complex interaction of cell signaling events that ultimately lead to neuronal decline. Nonetheless, a specific role in neuronal survival has been attributed to protein kinase C (PKC), a multi-isoform protein kinase family that is involved in many physiological functions, and that is highly expressed in brain (Berridge, 1993). This family of kinases has been shown to undergo selective inhibition following an excitotoxic or ischemic insult (Durkin et al., 1997; Busto et al., 1994; Cardell et al., 1991; Zivin et al., 1990), and the inactivation of PKC appears to be a necessary step for cell death in excitotoxicity (Durkin et al., 1997). Because of its involvement in multiple cellular events, its prominence as a protein kinase system involved in cerebral ischemic brain damage, and its proposed interaction with calpain (see ***Calpain and Other Messenger/Effector Systems*** below), the role of PKC will be discussed in further detail here.

PKC isozymes are a ubiquitous class of enzymes which transduce a multitude of signals arising from phospholipid hydrolysis. PKCs are activated by receptor-induced stimulation of phospholipase Cs to yield diacylglycerol (DAG), or phospholipase D to yield phosphatidic acid which converts to DAG (reviewed by Dekker et al., 1995). In addition to regulation by DAG, conventional PKCs are also modulated by Ca^{2+}. Thus, these conventional PKCs are particularly sensitive to the hydrolysis of phosphatidylinositol biphosphate (PIP_2) since both DAG and IP3 are produced, allowing

activation of IP3 receptors, with the concomitant release of Ca^{2+} from intracellular stores (reviewed by Dekker et al., 1995).

PKCs have been categorized into three subclasses: conventional PKCs (α, β, and γ) which are regulated by DAG, phosphatidylserine, and Ca^{2+}; novel PKCs (δ, ε, η, and θ) which are regulated by DAG and phosphatidylserine, but not Ca^{2+}; and atypical PKCs (ζ, ι, and λ) whose activation can be stimulated by phosphatidylserine, but whose regulation has not been completely established (reviewed by Dekker et al., 1995). Structurally, PKCs are composed of regulatory and catalytic domains. The regulatory portion consists of an autoinhibitory domain which acts as a pseudosubstrate, and one or two membrane-targeting areas: the C1 domain (present in all isozymes) which binds DAG (except in atypical PKCs), and the C2 domain (present only in conventional and novel PKCs) which binds acidic phospholipids, and in conventional PKCs, Ca^{2+} (reviewed by Dekker et al., 1995). The catalytic domain of PKC is maintained in an inactive configuration by an interaction with the autoinhibitory domain (Orr and Newton, 1994a,b). Activation of PKC involves the removal of the pseudosubstrate from the catalytic site when DAG and phosphatidyl-L-serine bind to the two membrane-targeting domains, C1 and C2 (Newton, 1995). Binding of either ligand is enough to produce recruitment of PKC to the membrane, however it appears that both sites need to be bound for pseudosubstrate removal and maximal activation (Newton, 1997).

The activation of PKC in the CNS is a common and critical mediator of intracellular messages. PKC isozymes are involved in numerous important cellular

functions, including neurotransmitter release, synaptic plasticity, excitability, Ca^{2+} permeability, receptor activity, and the regulation of gene expression (Miller, 1986; Nishizuka, 1986a,b; Akers and Routtenberg, 1987; Nichols et al., 1987; Routtenberg, 1987; Leli et al., 1992). In addition to the physiological roles that PKC isozymes may play, changes in PKC activity have also been implicated in neurodegenerative processes. These include amyotrophic lateral sclerosis (ALS) (Krieger et al., 1993; Lanius et al., 1995), Alzheimer's Disease (Jacobsen et al., 1994; Wang et al., 1994; Fowler et al., 1995), and particularly cerebral ischemia (Louis et al., 1988; Cardell et al., 1990, 1991; Wieloch et al., 1991; Cardell and Wieloch, 1993; Busto et al., 1994; Murphy et al., 1994; Durkin et al., 1996).

Using a variety of animal models, numerous investigators have reported changes in PKC following cerebral ischemia. Yamaoka et al. (1993) measured protein kinase C isozymes following transient hypoxia in the rat hippocampus, and found that after 30 minutes of hypoxia, both PKC-α and PKC-γ were significantly reduced. In addition to a loss of kinase, others have reported a translocation of cytosolic PKC to the cell membrane, and a subsequent reduction in kinase activity (Cardell and Wieloch, 1993; Busto et al., 1994). This early and persistent loss of neuronal PKC *activity* is thought to be a fundamental characteristic of neuronal populations that will undergo cell death (Cardell et al., 1990; Crumrine et al., 1990; Zivin et al., 1990; Louis et al., 1991; Domanska-Janik and Zalewska, 1992; Cardell and Wieloch, 1993; Busto et al., 1994).

A suppression of PKC activity rather than a loss of enzyme suggests that PKC undergoes inhibition rather than degradation following an ischemic insult (Kochhar et al.,

1989; Busto et al., 1994). *In vitro* examination of PKC following excitotoxic or ischemic insults supports this notion. Domanska-Janik and Zalewska (1992) demonstrated that the decrease observed in PKC activity was not affected by protease inhibitors, suggesting that a decrease in PKC-mediated phosphorylation was not due to the loss of intact kinase via hydrolysis. Durkin et al. (1996) demonstrated that the loss of PKC activity in primary cortical neurons in response to glutamate, NMDA, and AMPA occurred in the absence of any detectable change in the *amount* of PKC present, measured either by immunological or conventional enzyme assay techniques. Furthermore, this loss was recoverable, eliminating irreversible hydrolysis as an explanation for the loss in kinase activity (Durkin et al., 1996).

The suppression of PKC activity by glutamate and glutamate analogues also depends on the presence of extracellular Ca^{2+} during the insult, and the influx of Ca^{2+} through specific ion channels (Durkin et al., 1996). Application of Ca^{2+} ionophores or neuronal depolarization through KCl-mediated activation of VDCCs did not induce a loss of PKC activity or cell injury, despite the fact that Ca^{2+} entry via these means was equal to or greater than that observed with excitotoxin application (Durkin et al., 1996). This suggests that the loss of PKC activity is tightly coupled to glutamate receptor activation, and results from the same Ca^{2+}-dependent cell signaling pathway that mediates the death response of these neurons. Furthermore, the inhibition of PKC in "immature" cortical neurons during excitotoxin exposure makes these normally resistant neurons (Didier et al., 1994; Eimerl and Schramm, 1994; Choi et al., 1987) succumb to excitotoxic incursion (Durkin et al., 1997). This observation suggests that the long-term inactivation of PKC

following excitotoxicity or ischemia may be *more than* an indicator for neuronal injury. It suggests that the suppression of PKC activity is directly contributing to the mechanism by which these cells die in response to glutamate. In support of this, pretreatment with the neurotrophin BDNF prevented excitotoxin-induced cell death and the loss of PKC activity, despite the fact that NMDA receptor distribution, function, and ion permeability appeared not to be affected (Tremblay et al., 1999). Under these experimental conditions, the subsequent application of any number of PKC inhibitors reversed the BDNF neuroprotection, suggesting that the attenuation of NMDA-induced PKC inactivation was the crucial step mediating BDNF's neuroprotective action (Tremblay et al., 1999). Thus, determining the downstream targets and reactions mediated by the suppression of PKC following excitotoxic or ischemic insults may prove valuable in identifying the essential features of cell signaling that are contributing to neuronal death.

Enhanced Proteolysis

In addition to the changes described above, cerebral ischemia and excitotoxicity are also characterized by increased proteolysis. The degradation of specific protein substrates during ischemia and excitotoxicity could significantly contribute to deleterious cell function, since the modification of a protein by hydrolysis is a decisive event by virtue of its irreversibility. Determining the critical cellular substrates which undergo proteolysis in the cell death process has been a major research endeavor in recent years, particularly with respect to the actions of caspases and calpains, two families of proteases highly implicated as principal mediators of neuronal damage.

Caspases are cysteine proteases with high homology to Ced genes from

Caenorhabditis elegans (Yuan et al., 1993) that have been highly implicated in apoptotic processes. Ellis et al. (1991) demonstrated that ced-3 and ced-4 were vital for cell death observed in C. elegans during development, where 131 of the 1090 cells generated die by apoptosis. The Ced-3 protein has marked sequence homology to mammalian caspase-1 (ICE) (Yuan et al., 1993). Since this initial observation, at least 10 more mammalian caspases have been identified, each being numbered in order of their discovery (Cohen, 1997). Caspases are inactive proenzymes which require hydrolysis at specific aspartate cleavage sites for activation (Cohen, 1997). Apoptosis-inducing signals initiate signal cascades that lead to cleavage of specific caspases and their subsequent activation. The specific proteases involved in the cleavage and activation of caspases have yet to be defined for most cell types. However, in T lymphocytes granzyme B has been shown to directly cleave caspase-3, and perhaps caspase-9, which then proteolytically activates caspase-3 (Darmon et al., 1995; Quan et al., 1996; Cohen, 1997). It is likely that similar proteolytic cascades mediated by these or other proteases are present in all cell types, poised to activate caspases in response to specific cell signals. Caspase-3 (in conjunction with upstream caspases) has been purported to be the key executioner of apoptosis in mammalian cells, with its activation being a late event in the apoptotic signaling cascade (Pike et al., 1998b).

Caspase activation appears to contribute to the apoptotic component of cell death following lethal excitotoxin exposure and cerebral ischemia (Chen et al., 1998; Ma et al., 1998; Nath et al., 1998; Namura et al., 1998; Tenneti et al., 1998; Du et al., 1997). In support of this, Sengpiel et al. (1998) demonstrated that an inhibitor of upstream caspase-

l attenuated NMDA-induced cell death in cultured rat hippocampal neurons. Tenneti et al. (1998) recently demonstrated that the caspase inhibitors V-ICEinh and Z-VAD had no effect on Ca^{2+} entry or mitochondrial dysfunction following NMDA application, but ROS production and lipid peroxidation were completely blocked, as was cell death. Furthermore, caspase inhibitors have been shown to produce synergistic neuroprotective effects when combined with the NMDA receptor antagonist MK-801, in a mouse model of ischemia (Ma et al., 1998).

Endogenous caspase substrates include, but are not limited to, the DNA repair enzyme PARP (poly(ADP-ribose) polymerase) (D'Amours et al., 1998; Rosen and Casciola-Rosen, 1997; Gu et al., 1995), tau (Canu et al., 1998), actin (Mashima et al., 1997), PKC (Porter et al., 1997), and spectrin (Wang et al., 1996a; Nath et al., 1998, 1996b). These substrates are significant because, with the exception of PARP, all have been reported as substrates for calpain. Which of these or other substrates are important in mediating cell death for either protease remains to be elucidated.

While caspases are strongly implicated in apoptotic cell death, calpains are thought to participate in processes mediating necrosis (Pang et al., 1998). Both necrotic and apoptotic pathways may be stimulated in the same cells under neurodegenerative conditions. For example, Nath et al. (1998) recently reported the appearance of a caspase-mediated 120 kDa breakdown product of spectrin, in addition to calpain-mediated fragments, in rat primary cortical neurons and rat cerebellar granule cell cultures treated with NMDA, AMPA or kainate. In these same experiments oxygen-glucose deprivation also caused the appearance of the 120 kDa spectrin fragment, suggesting that both

caspases and calpains could be activated by excitotoxicity and ischemia. Both calpain and caspase activity have also been observed in cerebellar granule cells undergoing apoptosis in response to potassium and serum deprivation (Canu et al., 1998), in chicken and rat dorsal root ganglion neurons, chicken spinal motor neurons, and rat thymocytes in response to trophic deprivation (Villa et al., 1998), and in hippocampal cultures exposed to β-amyloid (Jordan et al., 1997). Pang et al. (1998) have also demonstrated that the inhibition of succinate dehydrogenase with the mitochondrial toxin 3-nitropropionic acid (3NP) induced acute excitotoxic necrosis and delayed apoptosis in primary rat hippocampal neurons. In this study specific caspase inhibitors failed to protect against apoptosis or necrosis, even though nuclear fragmentation was prevented (Pang et al., 1998). By contrast, calpain inhibition did protect neurons from acute necrotic cell death (Pang et al., 1998). Calpain inhibition has also been shown to be sufficient to protect hippocampal neurons from β-amyloid, staurosporine and NMDA-induced cell death (Jordan et al., 1997), and dorsal root ganglion neurons, chicken spinal motor neurons, and rat thymocytes from trophic deprivation (Villa et al., 1998). Calpain inhibition therefore appears crucial for cell survival following some types of injury, despite concurrent caspase activation. Activation of this protease following an ischemic or excitotoxic insult appears to be a central feature of neuronal cell death.

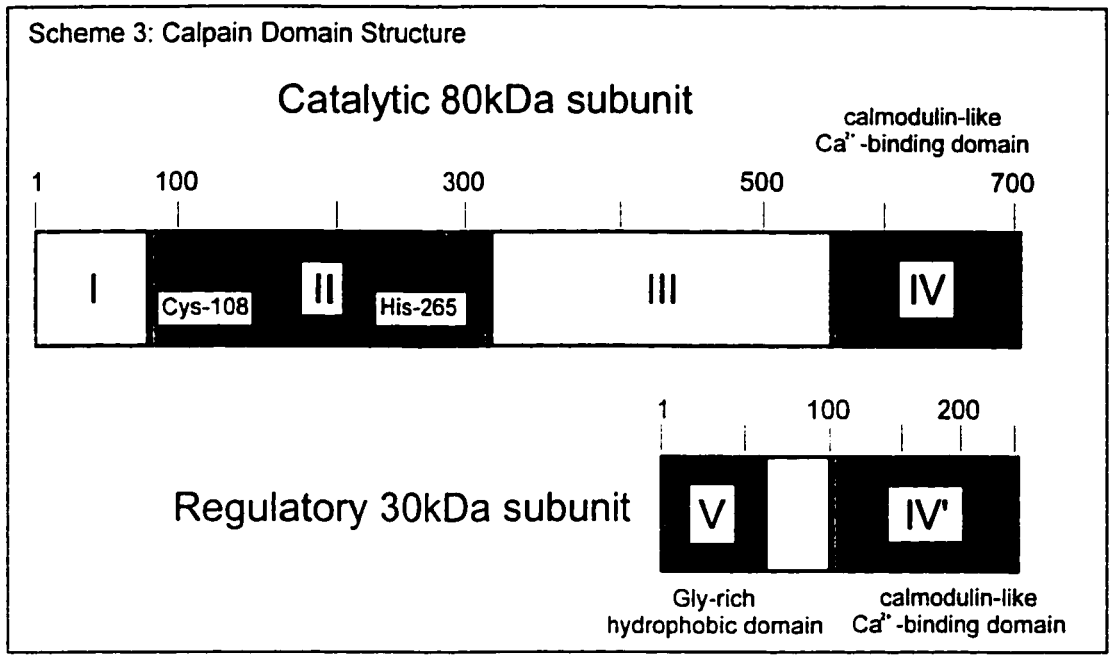
CALPAIN

Calpain is a major Ca^{2+}-dependent, intracellular protease found not only in

vertebrates, but also in *Drosophila* and *Schistosoma* (Sorimachi et al., 1994). The protease is considered a major mediator of Ca^{2+}-dependent cell signaling (Suzuki et al., 1992; Ono et al., 1998). Rather than causing extensive protein degradation, calpain generally cleaves its neuronal substrates in a limited manner at one or two specific sites (Guroff, 1964; Harris and Morrow, 1988). This selective cleavage often leads to functional changes in a substrate, as opposed to processing for further degradation (Kawasaki and Kawashima, 1996; Frangioni et al., 1993; Oda et al., 1993; Takai et al., 1977).

Multiple forms of vertebrate calpain have been reported. There are two ubiquitously expressed isozymes, μ- and m-calpain (Mellgren, 1980; Dayton et al., 1981), a form which expresses intermediate Ca^{2+} requirements in comparison to the ubiquitous forms, μ/m-calpain (Ohno et al., 1984), as well as the recently characterized tissue specific isoforms from stomach (nCL-2), and skeletal muscle (p94 or nCL-1) (Sorimachi et al., 1989). In addition, Dear et al. (1997) reported the existence of two additional forms of calpain in vertebrates, Capn5 and Capn6, which lack the calmodulin-like Ca^{2+} binding domains of conventional calpains (see below). Most information on calpains comes from studies on the two ubiquitous forms of the protease, the only isoforms identified in brain tissue (Sorimachi et al., 1997a; Malik et al., 1983; Zimmerman and Schlaepfer, 1982). Both consist of a large 80kDa subunit (consisting of four domains, I - IV), encoded by separate genes, and an identical 30 kDa regulatory subunit (consisting of two domains) (Suzuki et al., 1987) (see Scheme 3). Domain II of the 80 kDa subunit possesses the

catalytic site. By examining the selective reaction between calpain's essential thiol group



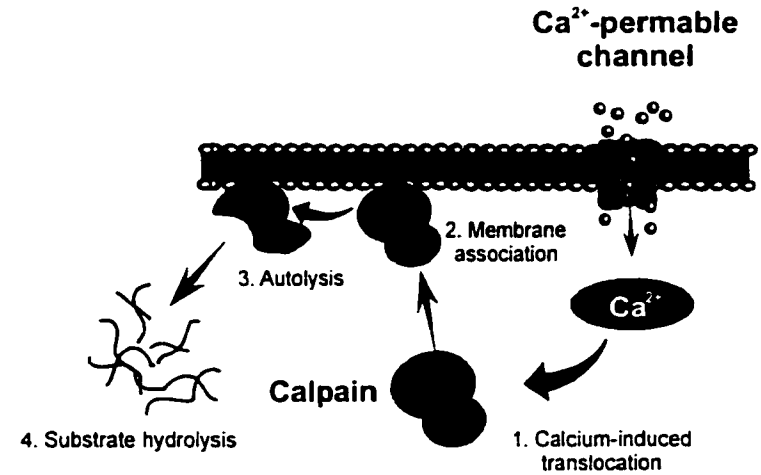
and two-hydronic–state time-dependent inhibitors, Mellor et al. (1993) suggested that the active site of calpain consisted of a Cys-His pair with an ionizing modulatory group, highly analogous to active sites in monomeric non-Ca^{2+}-activated cysteine proteases. More recently, Arthur et al. (1995) suggested that the active site of calpain likely consists of a Cys-His-Asn triad since site-directed mutations of Cys108, His265, and Asn286 resulted in calpain mutants that were devoid of catalytic activity.

Regions outside the catalytic site are responsible for binding Ca^{2+}. Domain IV of calpain has at least four EF-hand calmodulin-like Ca^{2+}-binding regions (reviewed by Wang and Yuen, 1994). The function of domain III is unclear, but may involve the transduction of the Ca^{2+} binding signal from domain IV to domain II to induce proteolytic activation (Vilei et al., 1997). The regulatory subunit of μ- and m-calpain has a glycine-rich portion on its N-terminal, which promotes interaction with membrane phospholipids

(Suzuki et al., 1992; Suzuki et al., 1988). This interaction is thought to reduce the Ca^{2+} requirements for proteolytic activation. The C-terminal half of the regulatory subunit also possesses four EF-hand calmodulin-like Ca^{2+}-binding sites (Wang and Yuen, 1994). Ubiquitous calpains exhibit a strict requirement for Ca^{2+}: 3 - 50 μM for half-maximal *in vitro* activation of μ-calpain, and 200 - 1000 μM for m-calpain (Zalewska, 1996).

Models of calpain activation are based largely on *in vitro* studies of purified enzyme, or studies conducted on platelets and erythrocyte ghosts. In these non-neuronal cell systems, inactive calpain resides mainly in the cytosol, but upon Ca^{2+} binding it translocates to membranes, interacts with membrane phospholipids, and undergoes an autolytic conversion. This conversion reduces the large subunit to a 78 or 76kDa form, and the small subunit to 17kDa (Suzuki et al., 1992, 1988, 1981a,b). The autolytic species of calpain is widely viewed as the active form of the protease (Baki et al., 1996; Suzuki et al., 1992; 1988; 1981a,b) (see Scheme 4 below). Baki et al. (1996) examined the kinetics

Scheme 4: Conventional Model of Calpain Activation by Translocation and Autolysis



of autolysis and activation of μ-calpain using microtubule-associated protein 2 (MAP2) as a substrate. The initial rate of MAP2 hydrolysis was a linear function of the autolysed 76 kDa form of the μ-calpain. This finding supported the view that native μ-calpain was an inactive proenzyme and that activation required autolysis. The autolytic conversion also reduced the Ca^{2+} requirement approximately threefold.

There is however, some dispute as to whether or not autolysis is necessary for protease activity. There are reports that indicate that substrate hydrolysis can occur without the autolytic conversion of calpain, and that the intact protease is an active enzyme (Molinari et al., 1994). Molinari et al. (1994) targeted unautolyzed μ-calpain to the erythrocyte membrane by increasing, in a controlled way, the Ca^{2+} concentration in the cells. This was achieved by incubating erythrocytes with the Ca^{2+} ionophore A23187 and fixed Ca^{2+} concentrations. Isolation of erythrocyte membranes demonstrated that calpain remained bound to the membrane in the 80-kDa unautolyzed form in the presence of low Ca^{2+} concentrations (1.75 microM), and that under these conditions substrate hydrolysis did occur. This hydrolysis was confirmed to be a consequence of calpain activity since it was sensitive to a calpain inhibitor and required Ca^{2+}. It was proposed that the mechanism for activation of calpain relied upon translocation to the membrane rather than on autolysis. We have recently shown similar results in neurons (Hewitt et al., 1998), and these results are outlined in detail in Chapter 3 of this thesis. Thus, while autolysis does occur (at least in erythrocytes and platelets), its function is not truly known.

Calpains are also regulated by an endogenous inhibitor, calpastatin. Conventional models of protease activation generally ignore this aspect of the calpain system, but the presence of an endogenous inhibitory protein suggests that calpain activation is more complex than outlined above. The major obstacle in the calpain field remains to be a complete understanding of the regulation and activation of calpain in intact cells.

Calpastatin: The Endogenous Calpain Inhibitor

The hydrolysis of endogenous substrates by calpain is an irreversible event, and therefore must be strictly regulated. As discussed above, calpain activities are regulated in part by an absolute requirement for Ca^{2+}. It is also likely that spatial sequestration or localization of calpain confers selectivity of action within cells by regulating its association with substrates. In addition, the presence of a calpain inhibitor protein is thought to provide further regulation of the protease *in vivo*.

Calpastatin is the only known specific, endogenous inhibitor of calpain, and it appears to be co-expressed together with calpain in all cell types examined (Murachi, 1984). It is typically isolated from soluble extracts but, its association with membrane fractions has also been reported (Inomata et al., 1989; Kawasaki et al., 1993). Calpastatin binds to acidic phospholipids of membranes via its N-terminal region (Mellgren et al., 1989a). This region is deleted in erythrocyte calpastatin, perhaps explaining the inhibitor's exclusive cytosolic localization under basal conditions in these cells (Mellgren et al., 1989b). While, this protein is believed to play an essential role in the regulation of calpain activity within cells, the true nature of this modulation in intact cells remains to be fully understood.

While cDNA analysis of calpastatin gives a predicted molecular weight of 72-77 kDa (depending on the species), the protein migrates anomalously on SDS-PAGE with an apparent molecular weight of 115-130 kDa (Killifer and Koohmaraie, 1994). In rat heart and kidney two 110 kDa forms of calpastatin have been reported (Salamino et al., 1994b), while in human hematopoietic cell lines 118 kDa and 116 kDa forms were predominant (Adachi et al., 1991). Western blot analysis of calpastatin from rat optic nerve showed four bands of 107, 68, 43, and 27 kDa (Shields et al., 1997). This variability of calpastatin in molecular weight within species has been attributed to different start sites in translation of the protein and multiple mRNAs (De Tullio et al., 1998).

Calpastatin contains four internal repeat sequences (domains I-IV) of about 140 amino acids, each capable of exhibiting inhibitory activity (Emori et al., 1987, 1988), and a unique N-terminal region, domain L (Imajoh et al., 1987). Each calpastatin repeat contains a central, highly conserved inhibitory sequence (TIPPXYR) which interacts with the active site of calpain, and is absolutely required for inhibitory activity (Kawasaki et al., 1989). Flanking regions surrounding this central inhibitory sequence associate with the EF-hand domains of calpain, and prevent membrane binding of the protease (Takano et al., 1995; Ma et al., 1994; Yang et al, 1994, Nishimura and Goll, 1991). Recombinant calpastatin forms, deduced from rat brain mRNAs (De Tullio et al., 1998), were shown to have different inhibitory properties depending on the number of repetitive inhibitory domains (Melloni et al., 1998a). The most effective form was a truncated calpastatin consisting of domain L and a single inhibitory domain (Melloni et al., 1998a). All forms

of calpastatin were more effective against μ-calpain, but were preferentially degraded and inactivated by m-calpain (Melloni et al., 1998a).

Calpastatin is also highly regulated posttranslationally by phosphorylation (Adachi et al., 1991; Pontremoli et al., 1992; Salamino et al., 1994a,b; Salamino et al., 1997). Two forms of calpastatin representing a single phosphorylated/dephosphorylated protein were demonstrated in skeletal muscle (Salamino et al., 1994b). The phosphorylation could be induced *in vitro* by PKA, while dephosphorylation occurred following exposure to alkaline phosphatase (Salamino et al., 1994a). The unphosphorylated form of calpastatin was reported to have maximal inhibitory activity against μ-calpain, while phospho-calpastatin was specific for the inhibition of m-calpain (Pontremoli et al., 1992; Salamino et al., 1994a). Surprisingly, an increase in the phosphorylated form of the inhibitor left the detected levels of unphosphorylated calpastatin unmodified (Salamino et al., 1994b). Nonetheless, this "conversion" was suggested to provide a mechanism to efficiently modulate the activity of m-calpain.

In intact human hematopoietic cells, phosphorylation was shown to increase the proportion of calpastatin associated with membranes (Adachi et al., 1991). In this study, calpastatin phosphorylation in cells was induced by TPA, but not the Ca^{2+} ionophore A23187, forskolin, or db-cAMP, indicating that phosphorylation of calpastatin was likely due to PKC activity. Phosphopeptide mapping demonstrated that phosphorylation *in vivo* of calpastatin occurs primarily on serine residues, with no detection on tyrosine residues (Adachi et al., 1991).

These data collectively suggest that calpastatin phosphorylation regulates its ability to inhibit calpain in response to cell signaling events, either by regulating its subtype specificity, or targeting its subcellular localization. These processes may be different in different cell types, depending on the forms of calpain present (erythrocytes have only the μ-calpain isoform, for instance), and the requirements of the cell during signaling events.

Calpain Substrates

The list of known and putative calpain substrates is extensive. In terms of membrane and cytoskeletal proteins, calpain has been shown to cleave the α_1-adrenergic receptor (Lynch et al., 1986), the NMDA receptor (Bi et al., 1998c,d), the AMPA receptor (Bi et al., 1998a,b; Bi et al., 1997a,b; Gellerman et al., 1997; Bi et al., 1996; Bi et al., 1994), L-type Ca^{2+} channels (Hell et al., 1996), filamin (Fox et al., 1985), adaptins, tubulins, clathrin light chain (Sato et al., 1995), annexin I (Wang and Creutz, 1994), β-adducin (Scaramuzzino and Morrow, 1993), band 3, band 4.1 (a and b), spectrin (α and β) (Croall and DeMartino, 1986), Ca^{2+}-ATPase (James et al., 1989, Wang et al., 1988), the EGF receptor (Cassel and Glaser, 1982; Gregoriou et al., 1994), fibronectin (Elamrani et al., 1993), $G_o\alpha$ (Greenwood and Jope, 1994), integrin β4 (Potts et al., 1994), the IP_3 receptor/Ca^{2+} channel (Magnusson et al., 1993), MAP-2 (Johnson and Foley, 1993), neurofilament proteins (Greenwood et al., 1993; Raabe et al., 1995), talin (Fox et al., 1985), paxillin (Raabe et al., 1995), tau (Litersky et al., 1993), and vimentin (Fischer et al., 1986).

Enzymes shown to be susceptible to modulation by calpain-mediated hydrolysis include Ca^{2+}-dependent cyclic nucleotide phosphodiesterase, MLC kinase (Ito et al., 1987), calcineurin (Tallant et al., 1988), glycogen synthetase (Belocopitow et al., 1965), phosphorylase kinase *b* (Huston and Krebs, 1968), phosphorylase phosphatase (Mellgren et al., 1979), phosphotyrosine phosphatase 1B (Frangioni et al., 1993), phospholipase C β3 (Banno et al., 1995), polycation-stimulated protein phosphatase (Waelkens et al., 1985), pp60src (Oda et al., 1993), protein kinase C (Takai et al., 1977; Inoue et al., 1977; Kishimoto et al., 1989), pyruvate kinase (Dahlqvist-Edberg and Ekman, 1981), the regulatory subunit of cAMP-dependent protein kinase (Beer et al., 1984), tryptophan hydroxylase (Hamon and Bourgoin, 1979), and tyrosine hydroxylase (Togari et al., 1986).

Other substrates include β-crystalline (David and Shearer, 1993), connectin (Kim et al., 1995), the estrogen receptor (Puca et al., 1977), fos, jun (Carillo et al., 1994; Hirai et al., 1991), insulin receptor substrate (IRS-1) (Smith et al., 1993), myelin basic protein (Tsubata and Takahashi, 1989), myofibrillar proteins (Dayton et al., 1976, 1981), the progesterone receptor (Vedeckis et al., 1980), and troponin (T and I) (DiLisa et al., 1995).

Of these substrates, only a small portion have been examined in intact cells, making an evaluation of "true" enzyme/substrate interactions difficult. References to proteins as calpain substrates evaluated with purified calpain, purified substrate, in the absence of a membrane phospholipid environment, and with unphysiological concentrations of Ca^{2+} may not be applicable to an *in vivo* setting. In addition, this supposed multitude of potential substrate proteins makes it difficult to ascertain the

functional significance of calpain activation in a general sense. Nonetheless, calpain may hydrolyze different substrates in response to different cell signaling events, and may selectively alter but a few substrates in any given cell type. Determining the *in vivo* substrates for calpain activation during physiological cell functioning is a difficult task. Nonetheless, following experimental cerebral ischemia or excitotoxicity two calpain substrates have been shown to consistently undergo selective cleavage, and have been used extensively to indicate calpain activation following an ischemic or excitotoxic insult. These substrates are α-spectrin and the microtubule associated protein, MAP-2 (Fukuda et al., 1998; Alexa et al., 1996; Bahr et al., 1995; Yokota et al., 1995; del Cerro et al., 1994; Mastesic and Lin, 1994; Roberts-Lewis et al., 1994; Felipo et al., 1993; Johnson and Foley, 1993; Roberts-Lewis and Simon, 1993; Saido et al., 1993b; Johnson and Jope, 1992; Arai et at., 1991; Johnson et al., 1991; Siman et al., 1989; Seubert et al., 1988, 1989; Siman and Noszek, 1988). Because spectrin hydrolysis is most often used to measure calpain-mediated hydrolysis in brain tissue, it will be discussed in some detail here.

Spectrin

Most of what is known about spectrin is based on information obtained from erythrocytes and erythrocyte ghosts. Erythrocyte spectrin is a heterodimer composed of α and β subunits (also known as band 1 and band 2 proteins) with apparent molecular weights of approximately 240 kDa and 220 kDa, respectively (Winkelmann and Forget, 1993). The α spectrin subunit consists of 22 segments that are primarily 106 amino acid

homologous repeats. However, at segment 10 a deviation occurs where the repeat is considerably shorter and consists of a highly conserved region with homology to SH3 domains of the src protein family (Wasenius et al., 1989; Drubin et al., 1990). The function of this area is unknown, but given its SH3 homology, it may well bind proline rich regions of tyrosine kinases (Koch et al., 1991; Musacchio et al., 1992). Amino acid inserts in segments 20 and 21, and reduced sequence homology of segment 22 relative to other spectrin segments, gives this region of the protein specialized properties (Winkelmann and Forget, 1993). These regions, along with the C-terminus, show high homology to the C-terminus of α-actinin, a homodimeric actin-binding protein (Baron et al., 1987). This C-terminal region of erythrocyte α-spectrin also contains EF-hand Ca^{2+} binding regions, but there is little evidence that erythrocyte spectrin directly binds Ca^{2+} under physiological conditions (Winkelmann and Forget, 1993).

Erythrocyte β spectrin also has several characteristic structural features, including an N-terminal region containing an actin-binding domain (Winkelmann and Forget, 1993). The binding of β-spectrin to actin also requires the presence of the α subunit, and is enhanced 10- to 100-fold by band 4.1 protein (which also binds to the N-terminus of β spectrin) (Cohen and Foley, 1984; Becker et al., 1990). The β subunit consists of 17 typical spectrin repeats with variable sequences within segments 1 and 2. Like segments 20 through 22 of the α subunit, these areas exhibit homology with α actinin (Byers et al., 1989; Winkelmann et al., 1990). β spectrin also has an ankyrin binding site localized to segments 15 and 16 (Kennedy et al., 1991), and is phosphorylated in intact erythrocytes

near the C-terminus (Harris and Lux, 1980; Mische et al., 1990). The functional significance of phosphorylation is unclear, but may affect αβ subunit association (Mische et al., 1989).

The functional unit of erythrocyte spectrin is the αβ heterodimer. The C-terminal region of β spectrin and the N-terminal region of α spectrin are involved in self-association, with α spectrin contributing a single α helix from its N-terminal and β spectrin contributing two α helices of its C-terminal to form a triple helical structure folded in a manner typical of a spectrin repeat (Speicher et al., 1993). The subunits associate side-to-side in an antiparallel manner, forming a flexible rod-like dimer that forms the basic structural element of the erythrocyte membrane (Winkelmann and Forget, 1993). These αβ heterodimers undergo a head-to-head association to form a tetramer, which is the physiologically relevant form of spectrin in the erythrocyte membrane (Goodman and Zagon, 1984). Tetramers assemble with numerous other proteins, forming the complex erythrocyte cytoskeleton.

In brain, αβ spectrin is highly similar to erythrocyte spectrin, exhibiting immunochemical cross-reactivity, similar structural appearance, similar tetramer formation, and an ability to bind actin, protein 4.1, and ankyrin (Bennett, 1990). However, there are some significant differences in these spectrin forms - unlike erythrocyte spectrin, brain spectrin exhibits high affinity calmodulin binding, increased tetramer stability, different affinities for its associated proteins, and unique peptide maps (Bennett, 1990; Harris et al., 1986). The calmodulin binding site is localized to segment

11 of brain α spectrin (Leto et al., 1989; Sri Widada et al., 1989,1990; Harris et al., 1988), and calmodulin binding alters the susceptibility of α spectrin to calpain hydrolysis (Harris et al., 1989; Seubert et al., 1987). There is also evidence that the EF-hand Ca^{2+} binding domains of brain spectrin are not vestigial as they are in erythrocyte spectrin, and can directly bind Ca^{2+} (Wallis et al., 1992).

While in erythrocytes spectrin functions as a mediator of membrane shape, its role in brain is less obvious. Like erythroid spectrin, brain spectrin cross-links actin filaments, and in association with ankyrin, is able to bind ion channels and pumps. Unlike erythrocyte spectrin, brain spectrin is not uniformly distributed along cell membranes, and the segregation of spectrin has been proposed to underlie the localization of specialized membrane proteins (Bennett, 1990). High concentrations of spectrin have been observed in the post-synaptic densities of neurons (Carlin et al., 1983), and the post-synaptic membrane of neuromuscular junctions, where it is associated with receptor clustering (Bloch and Morrow, 1989). More recently, spectrin has been shown to directly bind the NMDA receptor, selectively to the C-terminal domains of NR1A, NR2A, and NR2B (Weschler and Teichberg, 1998). This binding is regulated by phosphorylation, calcium and calmodulin (Wechsler and Teichberg, 1998). Thus, in brain it appears that spectrin acts to maintain the spatial organization of specialized membrane proteins, and mediate their attachment to the cytoskeleton. Spectrin in neuronal cells may function to structurally link signals from the extracellular milieu via membrane proteins to intracellular effector molecules. Disruption of this system would have serious

implications for normal cell functioning. Indeed, the homozygous mutation of brain α spectrin in Drosophila is lethal (Coyne et al., 1989).

It has been proposed that calpain activation fragments the spectrin network in areas of localized Ca^{2+} influx, producing structural changes in these regions (Lynch and Baudry, 1984). Under pathological conditions such as those occurring during ischemia, it is possible that the activation of calpain results in a slow and irreparable disruption of the cytoskeletal matrix. Given the apparent role of spectrin in the cytoarchitectural integrity of neurons, it is clear that an increasing degree of structural disruption in the spectrin cytoskeletal architecture would lead to a progressive decline in cell function and viability.

Calpain and Cell Death

Calpain may well play a central role in a number of pathologies including Alzheimer's Disease (Tsuji et al., 1998; Yamazaki and Ihara, 1998; Nixon et al., 1994; Saito et al., 1993), muscular dystrophy (Hussain et al., 1998; Topaloglu et al., 1997), cataract (Shearer et al., 1995; Lampi et al., 1992; Azuma and Shearer, 1992; Marcantonio and Duncan, 1991), traumatic brain injury (Pike et al., 1998a; Zhao et al., 1998; Kampfl et al., 1996a; 1997; Posmantur et al., 1996a,b, 1997; Arrigoni and Cohadon, 1991), arthritis (Yamamoto et al., 1992b), and cerebral ischemia (Bartus et al., 1994a,b; Hiramatsu et al., 1993; Rami and Krieglstein, 1993; Arlinghaus et al., 1991; Lee et al., 1991). In each of these conditions it has been proposed that aberrant calpain activation leads to the hydrolysis of substrates essential for cell function.

In cerebral ischemia, a profound increase in calpain activity is an early and prognostic feature of neuronal damage (Seubert et al., 1989; Lee et al., 1991; Ostwald et

al., 1993; Rami and Krieglstein, 1993; Saido et al., 1993b; Bartus et al., 1994a,b; Roberts-Lewis et al., 1994; Hong et al., 1994b; Yokota et al., 1995; Neumar et al., 1996). For example, a rapid and persistent increase in calpain-generated spectrin fragments in the vulnerable CA1 pyramidal cell region, but not in more resistant brain areas, has been observed following cerebral ischemia in the gerbil (Roberts-Lewis et al., 1994; Saido et al., 1993b). This accumulation of degraded spectrin occurred well before the first signs of morphological damage (Roberts-Lewis et al., 1994; Saido et al., 1993b).

Inhibitors of calpain have proven to be neuroprotective in various *in vivo* models of cerebral ischemia (Bartus et al., 1994a,b; Lee et al., 1991; Rami and Krieglstein, 1993; Hong et al., 1994a) suggesting that persistent calpain activation is a crucial feature of the cell death process initiated following such an insult. For example, the calpain inhibitors leupeptin and calpain inhibitor I reduced CA1 damage following forebrain ischemia in the gerbil (Lee et al., 1991) and the rat (Rami and Krieglstein, 1993). Systemic administration of the calpain inhibitor MDL28170 was also neuroprotective against focal ischemic brain damage in the rat (Hong et al., 1994a). A relatively new class of potent calpain inhibitors, the peptidyl α-keto amides (Li et al., 1996b; Li et al., 1993), have also been shown to be neuroprotective in focal models of ischemia (Bartus et al., 1994a,b). Both of the α-keto amides, AK295 and AK275, reduced ischemia-associated brain damage in a dose-dependent manner when peripherally administered in a rat model of focal ischemia (Bartus et al., 1994a,b).

The proteolytic cleavage of spectrin by calpain during ischemia is likely a

response to glutamate receptor activation since direct excitotoxin administration to brain produces changes in calpain-mediated spectrin hydrolysis similar to that seen in ischemia. For example, intracerebral excitatory amino acid (EAA) application to the dorsal hippocampus caused spectrin degradation which preceded the onset of pyramidal cell loss (Roberts-Lewis and Siman, 1993; Siman et al., 1989), and only NMDA or kainate concentrations that resulted in cell death produced spectrin proteolysis (Siman et al., 1989). Thus, as in ischemia, an early and persistent increase in calpain activity was a defining characteristic of lethally injured cells. In addition, intraventricular application of EAAs has been shown to cause spectrin degradation that mirrors the peptide maps produced by proteolytic cleavage of spectrin by calpain *in vitro*, but not that produced by other proteases (Siman and Noszek, 1988), suggesting that the pattern of spectrin proteolysis observed following glutamate receptor activation is directly resulting from the activation of calpain.

The relationship between ischemia, excitotoxicity and calpain activation has also been examined in *in vitro* slice models, and the data closely parallel that observed *in vivo*. In hippocampal slices, brief periods of hypoxia caused proteolysis of cytoskeletal proteins, and calpain inhibitors (such as calpain inhibitor I, leupeptin, and MDL28170) improved recovery of synaptic transmission and cell viability following an hypoxic or ischemic insult (Arai et al., 1990, 1991; Lee et al., 1991). Similarly, an excitotoxic insult with NMDA to hippocampal slices caused sustained calpain activation that was Ca^{2+} - dependent, and which was attenuated by MK801 (del Cerro et al., 1994). The translational suppression of calpain in cultured hippocampal slices by antisense

oligonucleotides directed against mRNA encoding for calpain I was also shown to provide neuroprotection from NMDA (Bednarski et al., 1995). Excitotoxicity induced by AMPA application to cerebellar slice preparations (Caner et al., 1993; Wang et al., 1996a) also caused calpain activation, and the resulting neurotoxicity was reduced by the calpain inhibitors MDL28170 (Caner et al., 1993) and PD150606 (Wang et al., 1996a).

This *in vitro* approach has also been extended to cell culture preparations. Primary cortical neurons have been used to examine the effects of calpain inhibition on both hypoxia/hypoglycemia and excitotoxicity. For example, cytotoxic hypoxia using NaCN caused a loss in cell viability that was reduced following treatment with calpain inhibitor I or leupeptin (Rami and Krieglstein, 1993). The induction of hypoxia/hypoglycemia by atmospheric gas and glucose manipulation caused a loss in cell viability and an increase in spectrin hydrolysis that could be attenuated by PD150606 (Wang et al., 1996a). Pre-treatment with calpain inhibitor I also reduced kainate-induced cell damage in primary cortical cultures (Cheng and Sun, 1994). Similarly, a variety of calpain inhibitors have been shown to protect cultured Purkinje (Brorson et al., 1994; Wang et al., 1996a), hippocampal and cerebellar (Brorson et al., 1995; Rami et al., 1997) cells against excitotoxic insult.

Collectively, these findings suggest that calpain activation is a common feature of Ca^{2+}-dependent cell death in *in vitro* and *in vivo* models of excitotoxicity and stroke. A significant role for increased calpain activity and subsequent substrate proteolysis in cell death following cerebral ischemia and excitotoxicity appears clear. However, there are a number of problems with this interpretation of calpain activation as a causal feature of

cell death. First of all, with perhaps the exception of the α-keto amides, calpain inhibitors are notoriously non-specific for calpain. Many inhibit other cysteine proteases. Furthermore, dose-dependent relationships between calpain inhibition and neuronal survivability are rarely reported (but see Bartus et al., 1994a,b). It is essential to demonstrate that the concentration of the inhibitor at which neuroprotection is first achieved is the same concentration at which substrate hydrolysis is affected. This would seem essential if a true cause and effect relationship were to be demonstrated between calpain activation, cell viability, and inhibitor efficacy/specificity. Indeed, Manev et al. (1991) examined the calpain inhibitor leupeptin in granule cell cultures treated with glutamate, and demonstrated that while spectrin hydrolysis was dose-dependently attenuated with increasing concentrations of the inhibitor, cell death was not. More recently, Brana et al. (1998) reported that even though calpain-mediated spectrin hydrolysis was restricted to subfields of the hippocampus destined to undergo cell injury following ischemia in organotypic hippocampal slice cultures, the detection of spectrin hydrolytic products and labeling with propidium iodide (PI; a marker for membrane integrity and cell death) 24 hours after the insult did not correlate. Most PI positive cells did not have spectrin breakdown products. Furthermore, the calpain inhibitor MDL28170 was not neuroprotective if pre-applied, although it did attenuate spectrin hydrolysis. Conversely, if applied post-ischemically the compound did confer neuroprotection.

Based on these conflicting observations, the relationship between calpain activation and ischemic/excitotoxic brain damage is unclear. In addition, the mechanistic features of calpain activation linked to neuronal death and its relationship to other known

mediators of excitotoxicity have not been elucidated.

Calpain and Other Messenger/Effector Systems

In addition to cytoskeletal proteins, changes in signaling proteins have been attributed to calpain activation. Of particular interest is the relationship between PKC and calpain. As described above, PKC is significantly affected by cerebral ischemia and excitotoxicity. It has been postulated that PKC is an *in vivo* substrate for calpain, and that a loss of PKC-mediated phosphorylation is indicative of hydrolysis of the kinase in neurons and other cell types (Tanabe et al., 1998; Dwyer-Nield et al., 1996; Shea et al., 1995, 1996; Hong et al., 1995; Patel et al., 1994; Al and Cohen, 1993; Savart et al., 1992; Adachi et al., 1990; Young et al., 1987; Kajikawa et al., 1983). μ-Calpain reportedly cleaves PKC to form an "irreversibly" active, Ca^{2+}-phospholipid independent product, PKM (Kishimoto et al., 1983). Phorbol ester-induced degradation of PKC suggests that the activated membrane-bound kinase may be more susceptible to calpain I proteolysis, and degradation of the kinase may be linked to calpain activation (Croall and Demartino, 1991). Phorbol ester-induced degradation of PKC can be blocked by an antibody raised against the hinge-portion of PKC that is susceptible to μ-calpain degradation in permeabilized glioma cells (Young et al., 1987). The increased rate of PKC degradation was directly linked to a lowering in the steady-state PKC levels in these experiments (Young et al., 1987). It has been suggested that an interaction between calpain and PKC could account for the reduction in PKC levels sometimes observed following ischemia, however, this conclusion is not entirely supported by experimental evidence.

There is ample evidence to suggest that PKC is not a physiological substrate for calpain-mediated hydrolysis, and that PKC is not hydrolyzed by calpain in instances of excitotoxicity and ischemia. The kinase activity of PKM has been shown to be transient, and immunoblot analysis does not demonstrate a major pool of PKM following phorbol ester treatment (Croall and Demartino, 1991). It has also not been established that PKM is a necessary intermediate of PKC resulting from calpain hydrolysis (Croall and Demartino, 1991). Furthermore, as noted above, it is the reversible suppression of PKC activity that is associated with cell death from ischemia or excitotoxicity in neurons (Durkin et al., 1996). Since this loss of PKC activity is reversible, PKC is likely being affected by an inhibitory factor, rather than an *irreversible* proteolytic cleavage (Durkin et al., 1996). Furthermore, no significant shift in the molecular weight of the kinase is observed following ischemia or excitotoxicity, suggesting that hydrolysis of intact protein is not occurring (Durkin et al., 1996). Thus, it is unlikely that changes in PKC activity can be accounted for by the mechanism of calpain-mediated hydrolysis, and that PKC acts as a substrate for calpain under conditions of excitotoxicity or ischemia in neuronal tissue. Nonetheless, both calpain and PKC are two Ca^{2+}-dependent cell signaling effectors that are heavily implicated in excitotoxic neuronal death. Examining the functional relationship between these two Ca^{2+} dependent effectors in the detrimental signaling cascade following an insult could lead to a better understanding of signaling events following an excitotoxic incursion.

AIMS

The aims of this research involved a number of inter-related issues concerning the relationship between glutamate receptor activation, calpain activation and regulation, calpain's relationship with other signaling events in the pathological cell signaling cascade, and cell death. With this in mind, we attempted to answer a number of questions by carrying out the following:

1. An analysis and characterization of the features of calpain activation under conditions of excitotoxicity.

2. Determining how glutamate-receptor mediated increases in intracellular Ca^{2+} lead to the activation of calpain in neurons, and how this protease was regulated in intact neuronal cells.

3. Determining the relationship between calpain inhibitors, calpain activation, and cell death.

4. Examining the relationship between calpain, and another cell death mediator during excitotoxicity, specifically, the loss of PKC activity.

Chapter 1. Measuring changes in calpain activity from intact cells

A variety of assay techniques have been described for the determination of calpain activity. For the most part, these involve the *in vitro* activation of calpain that has either been purified from a tissue source, or commercially obtained. These types of assays are useful for conducting kinetic analyses or determining structure/activity relationships, and have been used extensively to verify calpain extraction from biological sources (Sasaki et al., 1984), the activity of purified calpain against synthetic substrates (Sasaki et al., 1984), and the efficacy of inhibitors in preventing calpain activation *in vitro* (Mallya et al., 1998). Such *in vitro* methods are restricted however, by their inability to ascertain endogenous calpain activity or regulation as they exist in intact cells. Knowledge of endogenous calpain activation and regulation, its physiological substrates in various cell systems, and its response to specific cell signaling mechanisms remain relatively obscure. This lack of knowledge is recognized to be the largest obstacle in the calpain field (Molinari and Carafoli, 1997) and arises in part from the difficulty of capturing calpain activation *in situ*.

The objectives of these early studies were to compare various calpain assays for the purpose of choosing one or more that would effectively support the body of the thesis study, namely, the elucidation of the mechanism of action of calpain in neuronal excitotoxicity.

1.1 BACKGROUND

1.1.1 Antibody-Based Approaches

Calpain activation is widely believed to result from the translocation of inactive protease from the cytosol to the membrane, where it subsequently undergoes autolysis, and for this reason antibodies that recognize the autolytic fragments of calpain have been utilized to detect calpain activation *in situ*. Saido et al. (1993a) were the first to apply this approach to intact cells using immunoelectron microscopy. It was observed that A23187-induced Ca^{2+} entry caused calpain translocation from the cytosol of platelets to the membrane, where it underwent autolysis. It was assumed that autolysis was necessary for protease activation, and therefore that the autolytic species of calpain observed represented the active form (Suzuki et al., 1981a,b; Saido et al., 1992; Brown and Crawford, 1993; Molinari and Carafoli, 1997).

More recently however, data from both erythrocytes and primary neuronal cultures suggest that the translocation and autolytic conversion of calpain can no longer be viewed as absolute indicators of protease activation (Molinari et al., 1994; Hewitt et al., 1998). Using endogenous substrate hydrolysis of the Ca^{2+}-ATPase as an *in situ* indicator of calpain activation, Molinari et al. (1994) demonstrated that calpain-mediated hydrolysis in erythrocytes occurred at much lower Ca^{2+} concentrations than that required for autolysis. They were able to show that the intact form of the protease was active, and as such autolysis itself was not a proper indicator of protease activity. As will be shown (see Chapter 3), studies embodied in this thesis (Hewitt et al., 1998) have demonstrated that in primary cortical neurons calpain exists as a membrane-associated protease, and it

does not undergo autolytic processing during activation. These data indicate that the protease can be differentially regulated in various cell types, and that autolysis can not be reliably used as a measure of protease activity in all cell types (Hewitt et al., 1998; see Chapter 3).

1.1.2 Fluorogenic-Based Assays

The use of fluorogenic substrates as tools for measuring calpain activity offers the advantage of following protease activity determinations temporally. This approach has been successfully applied *in vitro*. The fluorogenic substrate Suc-Leu-Tyr-AMC was found to be an effective substrate for μ- and m-calpain (Sasaki et al., 1984). More recently, Mallya et al. (1998) have recently described a fluorogenic assay that allows continuous measurement of calpain activity using the synthetic substrate Suc-LY-MNA as a tool for measuring protease inhibitor efficacy. The suitability of such *in vitro* assays for determining *in situ* activation of calpain is compromised by selectivity issues, since these types of substrate conjugates have been reportedly cleaved by other proteolytic enzymes present in whole cell preparations (Seol et al., 1989; Woo et al., 1989). Whether changes in calpain activity mediated *in situ* can be successfully quantitated by such *in vitro* assays remains to be determined.

Cell permeable fluorogenic substrates seem more favourable as candidates for effective *in situ* measurement of calpain activity since their use does not require cell lysis. Moreover, these substrates have the potential to detect calpain activity in cells directly in response to cell signaling events (Rosser et al., 1993). The selectivity of these substrates to calpain hydrolysis may be an issue since there are potentially many proteases in the

intact cell that could induce cleavage. Nonetheless, Rosser et al. (1993) demonstrated increased hydrolysis of the fluorogenic substrate Boc-Leu-Met-AMC by calpain in hepatocytes in response to P_2-receptor stimulation with ATP. This non-fluorescent, membrane permeant substrate enters cells and conjugates to glutathione through the aminocoumarin moiety via the action of glutathione S-transferase. The association with glutathione renders the substrate impermeant and allows it to accumulate in the cell. Proteolytic cleavage between the Met and AMC-thiol moieties liberates and unquenches the highly fluorescent, membrane impermeant AMC-thiol conjugate. Using this approach, Rosser et al. (1993) demonstrated in hepatocytes that the hydrolysis of the compound was selectively mediated by calpain, since it was induced by increases in intracellular Ca^{2+}, and could be blocked by the calpain inhibitor Cbz-Leu-Leu-Tyr-CHN_2, but not inhibitors to serine proteases (TAME), extracellular proteases (aprotinin or α_2-macroglobulin), a general inhibitor of lysosomal proteases (monensin), or an inhibitor of cathepsin L (Cbz-Phe-Ser(Obz)-CHN_2). The application of such substrates to capture *in situ* calpain activation in neurons has not yet been reported.

1.1.3 Spectrophotometric-Based Assays

Moss et al. (1991) described a spectrophotometric assay for calpain in both rat and rabbit brain homogenates using azocasein as a substrate. Activity was detected as the amount of liberated azo chromophore remaining in solution after protein precipitation by TCA. This assay was shown to work for both fresh and frozen brain homogenates, and the *in vitro* hydrolysis of azocasein was attributed to calpain since it was Ca^{2+} dependent and sensitive to calpain inhibitors (Moss et al., 1991). However, no attempts were made

to examine differences in calpain activity induced in these tissues prior to homogenization, making this assay very similar to *in vitro* purified calpain assays, only with a cruder source of protease. Assays of this kind have no possibility of measuring calpain activity in unlysed cells since the substrate has little or no cell permeability.

1.1.4 Calpain Assays Based on Endogenous Substrate Hydrolysis

The most reliable indicator of calpain activation *in vivo* or *in situ* appears to be an indirect determination based on the hydrolysis of known *endogenous* calpain substrates. Such methods have particularly been utilized in brain tissue and neuronal cells (Simon and Noszek, 1988; Roberts-Lewis et al., 1994; Hong et al., 1994b; Manev et al., 1991). Because calpain-mediated cleavage of its cellular substrates is typically limited to only one or two sites, the determination of calpain-mediated hydrolytic products has been a widely used protocol for examining enzymatic activation following application of excitotoxins (Benarski et al., 1995; Brorson et al., 1995; Manev et al., 1994), traumatic brain injury (Posmantur et al., 1997; Kampfl et al., 1996a), and cerebral ischemia (Roberts-Lewis et al., 1994; Hong et al., 1994b) *in situ*. Spectrin has surfaced as the substrate of choice since calpain hydrolysis of brain α-spectrin results in hallmark hydrolytic products with apparent molecular weights of 155 and 150 kDa (Roberts-Lewis et al., 1994). Commercial spectrin antibodies are readily available that recognize both intact spectrin and its hydrolytic products. However, these antibodies can not distinguish between spectrin fragments produced by calpain and other proteases, such as caspases, that cleave spectrin into similar molecular weight fragments (Posmantur et al., 1998; Pike et al., 1998a,b; Smirnova et al., 1998; Wang et al., 1998; Nath et al., 1996b). Nonetheless,

because the exact hydrolytic site of brain α-spectrin by calpain has been determined (Harris and Morrow, 1988), it has allowed the production of antibodies that selectively recognize the peptide sequences flanking the calpain cleavage site (Roberts-Lewis et al., 1994). These antibodies selectively react against calpain-mediated spectrin fragments, and not those produced by other proteases (Roberts-Lewis et al., 1994), thus providing a selective tool for measuring calpain activation in brain tissue.

Our early goal was to examine possible methodologies for studying calpain in intact neurons, and develop protocols that would allow for the reliable detection of calpain activity *in situ*. The challenge in developing experimental assays to determine calpain activation in cells remains the specificity and validity of the assay. Principally one must be assured that calpain is responsible for the observed activity, and that it is not mediated by other proteases. In addition, if we are to understand calpain activation and regulation in intact neurons, the response observed must be attributable to cellular activity in response to a stimulus rather than being artificially induced by the assay conditions (such as activation resulting from the addition of exogenous Ca^{2+} to the assay preparation).

1.2 METHODS

1.2.1 Reagents

Dulbecco's phosphate-buffered saline, Eagle's minimum essential medium (MEM), and 5-fluoro-'2-deoxyuridine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Heat-inactivated fetal bovine serum (FBS) and L-glutamine were

purchased from GIBCO Laboratories (Grand Island, NY, USA), and heat-inactivated horse serum (HS) was from Hyclone Laboratories (Logan, UT, USA). NMDA and MK801 were purchased from Research Biochemicals International (Natick, MA, USA), ionomycin, from Calbiochem (La Jolla, CA, USA), and protein reagent from Bio-Rad Laboratories (Richmond, CA, USA). Suc-Leu-Tyr-AMC was from Bachem (King of Prussia, PA), and Boc-Leu-Met-AMC was purchased from Molecular Probes Inc. (Eugene, OR). All other chemicals were purchased from Sigma Chemical Co.

1.2.2 Cell/Animal Models

1.2.2.1 Primary Neuronal Cultures

Cultures of E18 rat cortical neurons were prepared as previously described (Durkin et al., 1996). Briefly, timed-pregnant Sprague-Dawley rats (Charles River Canada, St. Constant, Quebec, Canada) were anesthetized using halothane anesthesia and killed by cervical dislocation following 18 days of gestation. Fetuses were removed and the cortical region of their brains dissected. Dispersion of cortical neurons was accomplished by trituration with a 10 ml pipette, and the cells centrifuged at 250 *g* for 5 min at 4°C. Cells were plated on poly-L-lysine coated, 35-mm-diameter tissue culture dishes (Du Pont-Life Technologies, Burlington, Ontario, Canada) at 10^5 cells/cm^2 in plating medium consisting of 80% MEM, 10% FBS, 10% HS, and 2 mM L-glutamine, and incubated at 37°C in 5% CO_2 and 95% air. Cultures were treated with 15 μg/ml 5-fluro-2'-deoxyuridine and 35 μg/ml uridine on day 4 of culture for 2 days to minimize glial cell growth. Following exposure to mitotic inhibitors, one half of the medium was

removed and replaced with growth medium consisting of 90% MEM and 10% HS. Unless stated otherwise, experiments were performed on cells after 15-18 days in culture.

1.2.2.2 Cell Lysis of Neuronal Cultures

Unless stated otherwise, cells were washed once with a controlled salt solution (CSS; 120 mM NaCl, 5.4 mM KCl, 0.8 mM $CaCl_2$, 15 mM glucose, buffered with 25 mM Tris, pH 7.4), suspended in a hypotonic lysis buffer containing 50 mM Tris HCl, 2 mM EDTA and 2 mM EGTA, pH 7.4, and briefly sonicated. Cell extracts were sedimented at 600 g for 5 min to remove nuclei and any unlysed cells, and the resulting supernatant was separated into membrane and cytosolic fractions by centrifugation at 100,000 g for 10 min in a Beckman model TL-100 ultracentrifuge.

1.2.2.3 Two-Vessel Occlusion Forebrain Ischemia with Hypotension

A reproducible, standard operating procedure for two-vessel occlusion (2-VO) in the rat has been developed in the laboratory of Dr. Howard Lesiuk (Loeb Medical Research Institute), and was utilized throughout these experiments. Male, Sprague-Dawley rats, weighing 150-300 g were randomly assigned to treatment groups. Animals were denied access to food 6 hours prior to surgery. After pretreatment with atropine (0.5 mg/kg, ip), general anaesthesia was induced with sodium pentobarbital (65 mg/kg, ip). Supplemental doses of sodium pentobarbital (15-30 mg/kg, ip) were administered, if needed, to maintain adequate anaesthesia. Once anaesthesia was induced, the trachea was intubated with a #16 Teflon iv catheter under direct vision with a laryngoscope.

Mechanical ventilation (using a Model 665 small animal ventilator, Harvard apparatus, South Natick, MA) was adjusted according to arterial blood gas (ABG) determinations to maintain normocapnia (pCO_2 = 35-45 torr) and normoxia (pO_2 = 90-120 torr), throughout the operative procedure. A thermocouple probe was placed at the tympanic membrane into the middle ear to allow continuous monitoring and adjustment of head temperature. Temperature was maintained at 37.5°C ($\pm$ 0.5) using a heat lamp for the duration of the surgery, unless animals belonged to an experimental hypothermic group (see below).

A #24 Teflon iv catheter, was charged with heparinized (10 u/ml) normal saline, and inserted through the tail artery into the descending aorta for: continuous blood pressure monitoring, aspiration of blood to induce controlled hypotension (see below), and arterial blood sampling for ABG, haematocrit, and serum biochemical determinations. For blood pressure monitoring, the cannula was connected to a pressure transducer, and blood pressure was sampled 40 times a second by an analog to digital converter, and stored on a computer disc.

Once blood gases, blood pressure, and temperature were within the desired ranges, the common carotid arteries were exposed bilaterally through a midline, ventral, cervical incision. Forebrain ischemia was induced through bilateral carotid artery occlusion with temporary cerebral aneurysm clips and coincident controlled hypotension (mean BP = 50 $\pm$ 2 torr) induced by temporary, partial exsanguination. This was achieved by the aortic cannula, with blood withdrawn into a syringe containing 0.5 ml of heparinized (10 u/ml) saline. After 10 minutes of ischemia, the clips were removed and the blood was reinfused over 30 seconds. Repeat ABG and haematocrit determinations

were obtained following neck suture; the arterial cannula was removed, and the incision closed. Ventilatory support was continued until the animal was breathing well on its own. Postsurgically animals were maintained under a heating lamp coupled to a rectal probe, capable of maintaining body temperature within ±0.2°C of the preset value. Temperature was maintained post-operatively until the animal was completely awake. Food and water were allowed *ad lib* following surgery.

For hypothermia experiments, animals were treated identically as described above, however intra-ischemic temperature was maintained at 33°C (± 0.5). Post-operatively, body temperature was quickly recovered by the use of heat lamps, and maintained at a normal level until animals were fully awake and capable of self-regulated body temperature.

1.2.2.4 Hippocampal and Cortical Tissue Dissection

Animals were lethally injected with sodium pentobarbitol (100 mg/kg; ip), and when unresponsive to pain, were decapitated. Brains were rapidly removed and dissected on ice. The dorsal hippocampus and/or cortex was isolated and immediately frozen in sample vials with dry ice. Samples were stored at –80°C until time of assay.

1.2.3 Calpain Assay Methods

1.2.3.1 Azocasein Spectrophotometric Assay

This assay procedure was conducted essentially as described by Moss et al. (1991). Rat cortical tissue was homogenized on ice (20% w/v) in a buffer containing 20

mM Tris HCl, 1 mM EDTA, 5 mM 2-mercaptoethanol (2-ME), 15 mM cysteine HCl, and 250 mM sucrose, pH 7.4. Azocasein stock solutions were made by dissolving 40 mg/ml azocasein in deionized water, with the addition of 5 mg of sodium bicarbonate once in solution, and adequate assay buffer (containing 50 mM Tris HCl and 15 mM 2-ME, pH 7.4) to make a final stock of 20 mg/ml. A 50 mM $CaCl_2$ solution in assay buffer served as a source of Ca^{2+} in the reaction mixture, and was added as required to exact the Ca^{2+} concentrations indicated. These final Ca^{2+} concentrations were determined by the software program EQCAL, which determines the necessary Ca^{2+} addition to provide final concentrations in the presence of EDTA and/or EGTA (BIOSOFT, Cambridge, UK).

Reactions were prepared in triplicate, in a final reaction volume of 300 µl containing 20 µl of azocasein stock, $\pm$ $CaCl_2$ (as indicated), and 300 µg of tissue homogenate. Reactions were performed in triplicate for 1 hour at 26°C. Following 1 hour of incubation, Ca^{2+} concentrations were normalized for all samples, and 0.15 ml of 15% TCA was added to each reaction mixture. Samples were placed on ice for 30 minutes, and then centrifuged at 14,000 rpm for 10 minutes in an ependorf benchtop centrifuge. The supernatant was collected and combined with 90 µl of NaOH (0.5 M) and the resulting colorimetric product was read at 440 nm.

1.2.3.2 Boc-Leu-Met-AMC *In situ* Fluorogenic Assay

Boc-Leu-Met-AMC (20 µM) was added to mixed neuronal cultures plated on glass coverslips (protocol for plating exactly as described above (section 1.2.2.1) for

plastic dishes) in 1 ml of buffer containing 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 0.8 mM $MgCl_2$, 10 mM HEPES, and 3 mM glucose, pH 7.4. Measurements were made at room temperature after positioning the coverslip in a holding chamber mounted on the stage of an Olympus IMT-2 inverted microscope fitted with a 40X oil immersion objective, NA 1.3 (Olympus Corp., Lake Success, NY). This was coupled to an M1 imaging system (Imaging Research Inc., St. Catherines, ON), and measurements were acquired at 20 sec intervals for 10-15 minutes with excitation at 360 nm, and emission at 410 nm, while using a 390 dichroic filter.

1.2.3.3 *In situ* Measurement of Calpain-Induced Spectrin Hydrolysis

A calpain-specific anti-spectrin antibody was prepared based upon the methodology described by Roberts-Lewis et al. (1994). An oligopeptide corresponding to the peptide sequence on the NH_2 terminus of the COOH-terminal fragment (GMMPRDGC) of the calpain cleavage site of brain α spectrin was purchased from Research Genetics, Inc. (Huntsville, AL). The peptide was conjugated to keyhole limpet hemocyanin (Calbiochem, La Jolla, CA) using *m*-maleimidobenzoyl-*N*-hydrosuccinimide (MBS; Calbiochem, La Jolla, CA) as a crosslinker. The peptide-carrier conjugate was dialyzed against phosphate buffered saline (PBS) with four changes overnight to remove any uncoupled peptide. The peptide-carrier solution was combined with complete Freund's adjuvant and vortexed. Prior to injection with antigen, male, New Zealand white rabbits (2.5 kg; Charles River, St. Constant, Que.) were mildly anesthetized with Atravet (10 mg/kg; CDMV, St. Hyacinth, Que.), and 5 cc of blood was withdrawn from the ear

vein to obtain pre-immune serum. Animals received a total 1.5 ml volume of the antigen, delivered over multiple intramuscular injection sites. Two boost injections with incomplete Freund's adjuvant were delivered subcutaneously at 14 day intervals following the initial antigen exposure, and antibody-containing serum was collected 5 days following the final boost.

The resulting antibody was tested in rat brain (male Sprague Dawley rats, Charles River, St. Constant, Que.) homogenate prepared in 10 volumes of buffer containing 50 mM Tris, and 2 mM PMSF, pH 7.4. To remove nuclei and unlysed cells, the samples were spun at 600 g for 5 min and the resulting supernatant collected as a post-nuclear fraction. Equal amounts of protein were incubated at 26°C for 1 hour in the presence or absence of 1 mM $CaCl_2$ (final free Ca^{2+} calculated by EQCAL) and 10 units of purified μ-calpain (Calbiochem) in an assay buffer containing 20 mM Tris, 1 mM EDTA, 5 mM 2-mercaptoethanol, 15 mM L-cysteine, and 250 mM sucrose, with a final reaction volume of 700 ul. Following the incubation period, samples were combined with an equal volume of SDS electrophoresis buffer, and prepared for Western blot analysis (see section 1.2.4). The proteolytic products produced under these conditions were detected using the calpain-specific spectrin breakdown product (Cal-SBDP) antibody, and were compared to a commercially available spectrin antibody (240/235E, Chemicon, Temecula, CA) which detects intact spectrin as well as multiple hydrolytic products produced by a variety of proteases.

1.2.4 SDS-PAGE Gel Electrophoresis and Western Blot Analyses

1.2.4.1 Reagents

Acrylamide and methylene bisacrylamide were purchased from Pharmacia Biotech (Baie D'Urfe, QC). Bovine serum albumin (BSA), N,N,N',N'-tetramethylethylenediamine (Temed), Tris, glycine, Tween-20, laural sulfate (SDS), nitro blue tetrazolium (NBT), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), glycerol, and bromophenol blue were from Sigma Chemical Co. (St. Louis, MO). Sodium chloride (NaCl) was purchased from BDH Inc. (Toronto, ON). Ammonium persulfate (APS) was from Biorad (Hercules, CA), and PVDF Western blotting membranes were from Boehringer Mannheim (Laval, QC).

1.2.4.2 SDS-PAGE Electrophoresis

For gel preparation a stock solution containing 40% acrylamide and 0.4% methylene bisacrylamide was diluted to a desired final concentration using a 2X gel-casting buffer containing 0.75 M Tris-HCl and 0.2% SDS, pH 8.8, and an appropriate amount of water. The solution was de-gassed, and combined with 10 µl of the polymerizing agents temed and APS (50% stock solution in water). This solution was used in combination with a Biorad mini-protean gel system, and once polymerized a 4% stacking gel was prepared using the same acrylamide stock solution combined with a 2X stack-casting buffer containing 0.25 M Tris-HCl and 0.2% SDS, pH 6.8, and the appropriate amount of water.

Protein determination was by a modified Lowry assay (Biorad). Samples containing equal amounts of protein solubilized in SDS electrophoresis buffer (20% glycerol, 10% β-mercaptoethanol, 6% SDS, and 1 mM Tris-HCl, pH 6.8 with enough bromophenol blue to colour) were added to each well, and ran at a constant voltage of 200 mV using a Biorad Model 3000xi electrophoresis power pak. The reservoir buffer contained 25 mM Tris , 192 mM glycine, and 1% SDS, and power was applied until the dye front reached the bottom of the gel. Standardized, pre-stained high or low range molecular weight markers (Biorad) were used for the determination of apparent molecular weights.

Electrophoresed proteins were transferred from the gel to PVDF Western blotting membranes (Boehringer Mannheim, Laval, QC) using the same power pak as above, connected to a Hoeffer Scientific Instruments TE Series Transphor Electrophoresis Unit (purchased from Pharmacia Biotech, Baie D'Urfe, QC), at a constant current of 300 mA, in a buffer containing 25 mM Tris-HCl, 192 mM glycine, and 20% MeOH, pH 8.2 for 1.5 hours.

1.2.4.3 Western Blot Development

Membranes were placed on a rotating belly dancer (Stovall Life Science, Inc., Greensboro, NC) in a TBST solution (Tris-buffered saline with tween-20; containing 10 mM Tris-HCl, 150 mM NaCl, and 0.05% Tween-20, pH 8.0) containing 1% BSA for 30 min to block non-specific binding. Following this blocking step, an antibody solution was prepared in TBST, and blots were incubated for a minimum of 90 min. Three 10 min

wash steps followed in TBST, and then an appropriate secondary antibody (either alkaline phosphatase conjugated anti-rabbit IgG or anti-mouse IgG; 1:3300) in TBST was added for 45 min. Following exposure to the secondary antibody, blots were washed three times as above, and were then reacted with an alkaline phosphatase developer solution containing 100 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$, 120 μM NBT, and 540 μM BCIP, pH 9.5.

1.2.5 Cell Death Assays

Primary cortical neurons in 35 mm dishes were transiently exposed to 50 μM NMDA in 1 ml of conditioned media for 5 minutes, washed with 1 ml of CSS, and returned to 1 ml of conditioned media for the times indicated before being lysed (see section 1.2.2.2), and fractions collected for Western blotting (as described in section 1.2.4). In addition, the induction of apoptosis in primary cortical cells was achieved by exposing neurons to 1 μM staurosporine in conditioned media for six hours. Following exposure, cells were washed with CSS, and lysed as described (section 1.2.2.2).

For the measurement of spectrin hydrolysis, samples were run on a 10% SDS-PAGE gel for Western blot analysis (as described in section 1.2.4).

1.3 RESULTS

1.3.1 Azocasein Assay

An analysis of the *in vitro* assay of calpain using azocasein as a substrate produced results similar to that reported by Moss et al. (1991). The hydrolysis of the azo chromophore exhibited enzyme dependency in that, the extent of substrate hydrolysis increased linearly over the concentration of the calpain-containing rat cortical extract tested (Figure 1.1A). The assay also demonstrated the characteristic Ca^{2+}-dependency reported for calpain activity (Figure 1.1B). Substrate hydrolysis increased linearly in the presence of 0 to 750 μM Ca^{2+}, reached a plateau between 750 and 1000 μM Ca^{2+}, and increased again in a linear fashion at greater Ca^{2+} concentrations. This biphasic response to Ca^{2+} was consistent with the existence of both the low (μ) and high (m) calcium requiring forms of calpain in rat brain homogenates. The increase in substrate hydrolysis in response to Ca^{2+} was attributable to calpain activity since two calpain inhibitors, MDL 28,170 (Figure 1.1C) and calpain inhibitor I (Figure 1.1D) decreased the production of free azo chromophore in the reaction mixture in a dose-dependent manner. Thus, as previously reported (Moss et al., 1991), this assay effectively measured calpain activation in rat brain homogenates in the presence of Ca^{2+}, and the presence μ- and m-calpain, which could be differentiated on the basis of Ca^{2+} concentration present in the assay.

Using optimized protein (1000 μg) and Ca^{2+} (800 μM, for measuring μ-calpain) concentrations determined from the above experiments, the assay was used to examine whether *in situ* activation of calpain could be detected from ischemic rat brain. Following incubation, homogenates of isolated dorsal hippocampi from ischemic rats did not show demonstrable differences in calpain activity at any of the time points examined when

compared to controls (Figure 1.2). These results indicated that either this assay was ineffective at detecting *in situ* changes in calpain activity, or that calpain activity was not changing under the experimental conditions. Given the abundant literature that such changes in calpain do occur, we concluded that such an assay was not going to serve our immediate or long term needs.

1.3.2 Boc-Leu-Met-AMC Fluorogenic *In situ* Assay

The difficulties of examining biochemical changes *in vivo*, and the labour intensive nature of animal surgery in relation to sample yield promoted the change to a more simplified model of excitotoxicity in order to assess potential assays for examining calpain activation *in situ*. Attempts were made to replicate the findings of Rosser et al. (1993) using the cell permeable fluorogenic substrate Boc-Leu-Met-AMC in hepatocytes exposed to ATP, but using primary cortical cells exposed to NMDA. When neuronal cultures were exposed to the fluorescent compound, a comparison of photomicrographs of cells under phase bright (neurons) and fluorescent imaging revealed that glial cells, but not neurons, exhibited increased, time-dependent fluorescence (Figure 1.3A). Despite many efforts in altering the assay conditions, fluorescence was never detected in cultured neuronal cells. The fluorescent signal from glial cells was also not ablated by chelating extracellular Ca^{2+} from the treatment conditions (Figure 1.3B), suggesting that the protease responsible for the observed hydrolysis was not dependent on Ca^{2+} signaling or entry from extracellular sources.

1.3.3 *In situ* Measurement of Calpain-Induced Spectrin Hydrolysis

Western blot analyses of rat brain homogenate using a commercially available anti-spectrin antibody (240/235E, Chemicon, Temecula, CA) revealed that incubation in the presence of 1 mM Ca^{2+} and 10 units of purified calpain resulted in the hydrolysis of intact spectrin to breakdown products with apparent molecular weights of 155 and 150 kDa (Figure 1.4A). Furthermore, inhibition of calpain using calpain inhibitor I (6.5 μM) largely reversed this hydrolysis (Figure 1.4A). The Cal-SBDP antibody developed in our laboratory (see section 1.2.3.3) was compared to the commercial antibody in order to assess its ability to selectively detect these same calpain mediated spectrin breakdown products. Figure 1.4B demonstrates that the antibody was selective for the same 155 kDa hydrolytic product detected by the commercial antibody against spectrin, following *in vitro* digestion with calpain. Furthermore, as described by Roberts-Lewis et al. (1994), the resulting antibody did not recognize intact spectrin, and only reacted to its antigen when exposure of the peptide sequence occurred upon calpain cleavage (Figure 1.4B).

Exposure of primary cortical neurons to the excitotoxin NMDA, resulted in the same spectrin breakdown product as that produced by purified calpain (Figure 1.4C). This NMDA-induced hydrolysis of spectrin was attenuated in a dose-dependent manner by treatment of the cells with the calpain inhibitor, calpeptin (Figure 1.4D), confirming calpain was responsible for the hydrolysis observed. These results were the first indication that calpain activation was part of the signaling cascade initiated by the over-activation of NMDA receptors in primary cortical neurons.

Recent reports suggest that caspases also cleave spectrin to produce molecular weight fragments very similar to that mediated by calpain, in addition to producing a 120 kDa fragment. To ensure that the Cal-SBDP antibody was a selective tool for the detection of calpain and not caspase activity, we compared the spectrin fragments produced by NMDA toxicity with that produced by high dose staurosporine exposure, a known inducer of caspase activation and apoptosis in primary cortical neurons (MacManus et al., 1997). The commercial spectrin antibody indicated that toxic levels of both NMDA and staurosporine produced molecular weight fragments of 155 and 150 kDa (Figure 1.5), six hours post-treatment. In addition, staurosporine exposure resulted in a 120 kDa product, not evident following NMDA. When the same samples were probed with the Cal-SBDP antibody, only NMDA treatment resulted in a detectable spectrin fragment. Thus, these results indicated that spectrin hydrolysis during excitotoxicity and apoptosis is mediated by different proteases, and that calpain activation is associated with NMDA receptor activation and not staurosporine induced toxicity. Furthermore, caspase-3 seems to be selectively activated under conditions of staurosporine toxicity, without the involvement of calpain. The Cal-SBDP antibody therefore selectively detected calpain-mediated spectrin hydrolysis, and represented a selective tool for measuring activation of the protease induced *in situ*.

1.3.4 *In situ* Calpain-Induced Spectrin Hydrolysis in Cerebral Ischemia

Once the Cal-SBDP antibody was validated as a selective tool for measuring calpain activity, it was important to ensure that the spectrin hydrolysis observed

following excitotoxic exposure to cell cultures was representative of that induced *in vivo* by cerebral ischemia. In this way, we could confirm that the phenomenon under investigation was common to both the *in vivo* setting and the *in vitro* model, validating the use of primary neuronal cultures as a model system to study calpain activation.

Dorsal hippocampal homogenates from untreated control rats (n = 2) and rats subjected to 10 minutes of forebrain ischemia (n = 4) were compared 48 hours after the insult. Western blot analyses showed that spectrin hydrolysis was significantly increased following ischemia (Figure 1.6). Furthermore, intraischemic hypothermia attenuated calpain-mediated spectrin hydrolysis (Figure 1.6). These results suggest that the prevention of cytoskeletal damage by calpain early in the pathological cascade may be a process whereby hypothermia exacts its neuroprotective action. Increased spectrin hydrolysis also preceded signs of morphological cell damage. Photomicrographs of CA1 from control rats, and ischemic rats 48 hours and 7 days post-insult, demonstrated that morphological signs of cell death occurred >48 hours after ischemia (Figure 1.7). Thus, an early increase in spectrin breakdown may be taken to indicate an active proteolytic processing of cell cytoskeleton prior to overt signs of morphological damage, suggesting that calpain activation during ischemia is a pre-death event.

These differences in the degree of calpain-mediated spectrin hydrolysis in normothermic and hypothermic animals were not attributable to any physiological parameters other than hypothermia. Figure 1.8 demonstrates that with the exception of blood glucose, physiological parameters did not differ between normothermic and hypothermic rats undergoing ischemia. Blood pO_2 neither differed pre- or postoperatively

within groups, nor between groups (Figure 1.8A). pCO_2 levels did significantly increase ($p < 0.05$) post-operatively for both groups, but these changes were equivalent for both normothermic and hypothermic animals such that between group comparisons were not significant ($p > 0.05$) (Figure 1.8B). Blood pH was similar for both groups, and did not vary from pre-operative recordings (Figure 1.8C). There was a statistically significant difference between blood glucose recordings taken pre-operatively (Figure 1.8D). As a group, hypothermic rats had a mean blood glucose level of 4.4, while normothermic rats averaged 5.05. Figure 1.9 demonstrates that throughout the ischemic period, intraischemic body temperature was tightly maintained at 37.5°C for normothermic rats, and 32.5°C for hypothermic rats. Thus, despite the fact that low blood glucose would act to exacerbate the insult (Auer, 1988), animals treated with intraischemic hypothermia had less calpain-mediated spectrin breakdown which could only be attributed to hypothermia itself.

1.4 DISCUSSION

In order to examine and understand calpain activation and regulation in intact neurons, it must be possible to evaluate protease behaviour in response to specific stimuli. We have examined a variety of ways to capture *in situ* calpain activation using colorimetric, fluorogenic, and endogenous substrate hydrolytic assays. The hydrolysis of endogenous spectrin by calpain and the application of a calpain-specific anti-spectrin antibody was clearly superior to any of the methods utilizing exogenous substrates.

The hydrolysis of brain spectrin by calpain cleaves the protein roughly in half, producing two hydrolytic products with apparent molecular weights of 155 kDa and 150 kDa (Harris et al., 1988; Roberts-Lewis et al., 1994). Incubation of rat brain homogenate with purified calpain in the presence of Ca^{2+} resulted in this characteristic pattern, as detected by a commercially available spectrin antibody that recognizes both intact spectrin and its hydrolytic forms. The resulting Cal-SBDP antibody produced by conventional polyclonal antibody production protocols effectively and exclusively recognized the 155 kDa breakdown product of spectrin produced by calpain. Furthermore, this same fragment was detected in cell cultures treated with NMDA, and dose-dependently inhibited by the calpain inhibitor calpeptin, when present during NMDA application. This fragment was identical in size to that produced in the dorsal hippocampus of rats subjected to global forebrain ischemia, suggesting that calpain activation both *in vitro* and *in vivo* is likely a response to excitotoxic processes initiated by glutamate receptor activation. Furthermore, *in vivo* calpain activation in response to ischemia was an early event, detectable prior to overt morphological damage. The application of intra-ischemic hypothermia, a known neuroprotectant, was able to attenuate calpain activation. This suggests that the prevention of calpain-mediated hydrolysis may be sufficient to rescue vulnerable neurons following an ischemic insult. It further supports the idea that hypothermia is targeting essential Ca^{2+}-dependent signaling mechanisms during ischemia, thereby providing its neuroprotective effects.

The application of both the Cal-SBDP antibody and the commercial antibody against spectrin in primary cell cultures treated with NMDA or staurosporine not only

revealed that the Cal-SBDP antibody was specific for calpain, but also elucidated that different protease cascades were initiated by the different neurotoxins. Caspase-3, the principal executioner of apoptosis in mammalian cells (Jacobson et al., 1996; Nicholson et al., 1995), was selectively activated in cells treated with staurosporine, but not NMDA. This finding is consistent with those of others which suggest that staurosporine application in primary cortical cells induces apoptosis, while glutamate receptor agonists induce necrosis (MacManus et al., 1997). However, others have suggested that NMDA application to primary neurons can lead to apoptosis and caspase activation (Nath et al., 1998; Bonfoco et al., 1995). Observations that staurosporine can induce both calpain and caspase-mediated spectrin hydrolysis in septo-hippocampal cell cultures and that it can be blocked with calpain inhibitors (Pike et al., 1998b), further conflicts with our current finding. However, these differences may be accounted for by cell type (septo-hippocampal cultures versus primary cortical cells), severity of the insult (Bonfoco et al., 1995), or the time at which protease-mediated substrate hydrolysis was assessed (Nath et al., 1998). Under our experimental conditions it was shown that, independent of calpain activation, staurosporine-induced caspase-3 activation hydrolyzes spectrin into fragments with apparent molecular weights of 155, 150, and 120 kDa. NMDA receptor activation appears to be restricted to the activation of calpain, resulting in spectrin breakdown products of 155 kDa and 150 kDa, with the 155 kDa selectively detected by the Cal-SBDP antibody. The utilization of both antibodies allowed for the determination and separation of which proteolytic events were initiated in primary neurons in response to specific stimuli.

The alternative methods utilized for the detection of *in situ* calpain activation were less successful than the examination of spectrin hydrolysis. The azocasein assay was able to determine calpain activity induced *in vitro* by the addition of Ca^{2+}, but was not effective in detecting changes in calpain activity initiated *in situ*. This may have resulted from a variety of factors. *In situ* activation may have been terminated upon freezing of the tissue samples during isolation. Alternatively, the addition of 800 μM Ca^{2+} to both control and ischemic tissue samples may have activated calpain to equivalent levels *in vitro*. This application of exogenous Ca^{2+} and resulting hydrolysis would reflect the availability of calpain for activation *in vitro*, rather than how much had been activated *in situ*. Because there is no reason to suspect differences in the amount of protease present between control and ischemic rats, the addition of equal amounts of Ca^{2+} would be expected to result in equal levels of activity. This is indeed what was observed. Despite its failure as a method for capturing *in situ* calpain activity, the assay did confirm the presence of both Ca^{2+}-requiring forms of calpain in rat brain. The hydrolysis of azocasein could also be completely blocked by calpain inhibitors, suggesting that it acted as a selective substrate for the protease under the assay conditions.

The possibility that calpain activation could be determined directly in unlysed cells using a cell permeable, fluorogenic substrate was an attractive prospect. Rosser et al. (1993) had previously demonstrated calpain activation in hepatocytes using Boc-Leu-Met-AMC. We attempted to use this same substrate in primary cortical neurons to examine the effects of NMDA on calpain activation. However, application of the compound revealed that only glial cells (confirmed by GFAP staining, Dr. J. Tauskela)

were positive for fluorescence. Initially it was thought that glia were selectively taking up the compound, but subsequent work conducted by Dr. J. Tauskela at the NRC revealed that neurons lack sufficient levels of glutathione S-transferase to conjugate Boc-Leu-Met-AMC to the necessary glutathione residue. Thus, while the substrate was likely getting into neurons, these cells did not accumulate the compound. Because glial cells are not deficient in glutathione-S-transferase, the compound was selectively concentrated in these cells following incubation (manuscript in preparation, Dr. J. Tauskela). Because of its remarkable selectivity, the application of this compound as a marker for glial cells in mixed culture preparations is being investigated as an alternative to other procedures such as GFAP staining (Dr. J. Tauskela, personal communication).

In conclusion, we have developed a selective tool for the *in situ* capture of calpain activation. By using an antibody raised against the peptide sequence of the calpain cleavage site of spectrin, we have confirmed that the spectrin hydrolysis induced in brain tissue in response to incubation with purified protease yields the same fragment initiated following NMDA application and cerebral ischemia. Thus, calpain activation is a common feature of both cerebral ischemia and excitotoxin induced cell death in primary cultures. Furthermore, this antibody has previously been shown to selectively detect spectrin fragments produced by calpain, but not cathepsin B, cathepsin L, elastase, chymotrypsin, trypsin, thrombin, plasmin, or urokinase (Roberts-Lewis et al., 1994). We have also now confirmed that it does not react with spectrin fragments produced by caspase-3. The measurement of spectrin hydrolysis using this specific antibody is a selective, valid, and powerful tool for the determination of calpain activity in neuronal

cells. Based on these evidences, the spectrin-based calpain assay was the principal tool used throughout this thesis to investigate the role that calpain plays in excitotoxic-induced neuronal injury in primary cortical cells.

Figure 1.1 The application of a colorimetric assay for calpain activation in rat brain cortical homogenates using azocasein as a substrate. **A**: The protease dependency of azocasein hydrolysis in rat brain homogenate in the presence of 500 μM $CaCl_2$. Protein determination was by a modified Lowry method (Biorad). **B**: The Ca^{2+}-dependency of azocasein hydrolysis using 300 μg of rat brain homogenate. **C**: The ability of the calpain inhibitor, MDL 28,170 to attenuate azocasein hydrolysis in rat brain homogenate, in the presence of 800 μg of protein and 500 μM $CaCl_2$. **D**: The ability of the calpain inhibitor, calpain inhibitor I, to inhibit azocasein hydrolysis in rat brain homogenate, in the presence of 800 μg of protein and 500 μM $CaCl_2$. Rat brain tissue preparation and general assay procedures were as described in Methods (sections 1.2.2.4 and 1.2.3.1, respectively). The levels of Ca^{2+} indicated in each figure represents the final free Ca^{2+} concentration added to the EDTA-containing buffer, as determined by the Ca^{2+} determination software EQCAL. Points represent the mean of three separate experiments (n = 9) $\pm$ standard deviation.

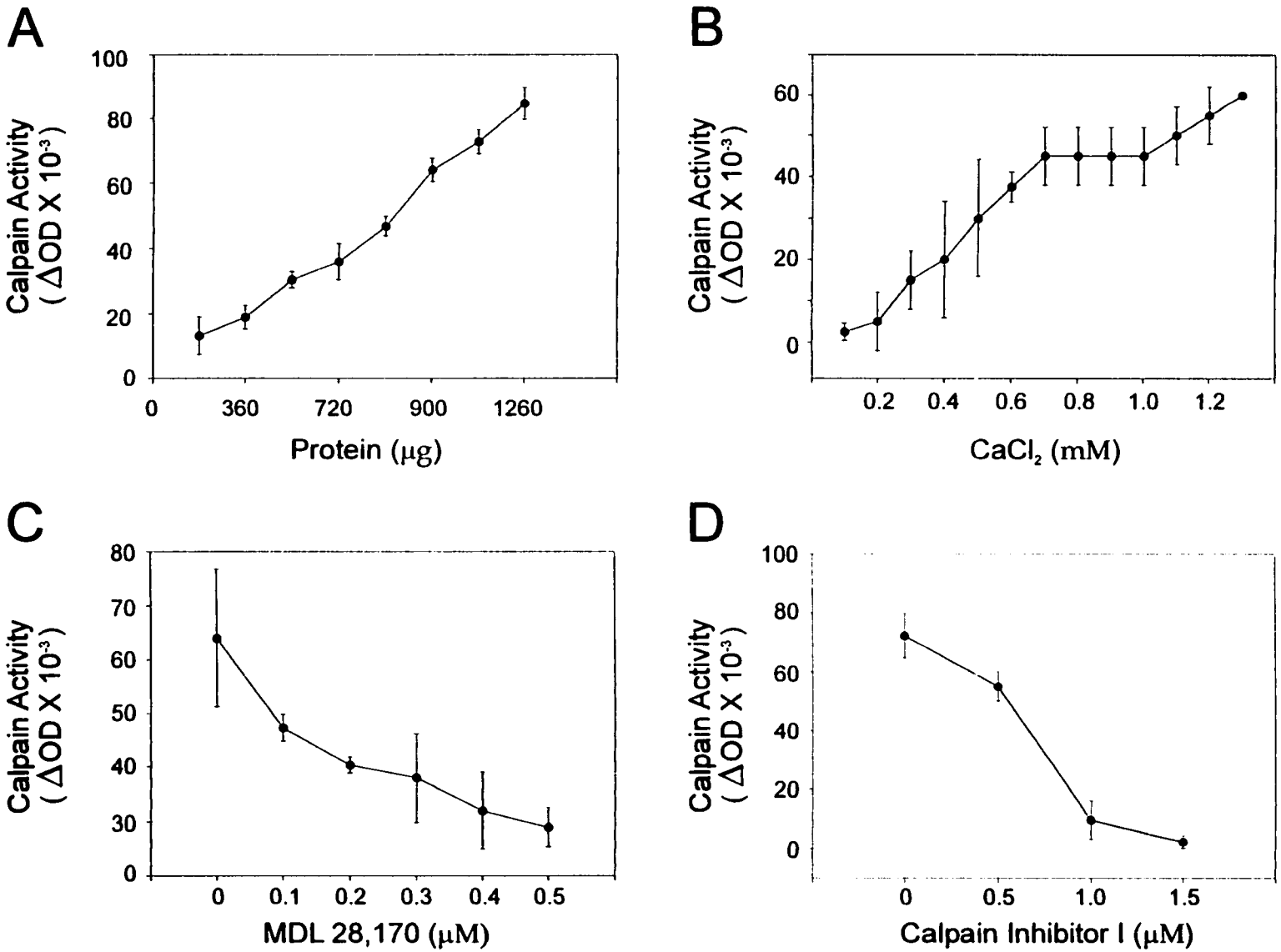

A
Calpain Activity
(ΔOD X 10-3)
100
80
60
40
20
0
0
360
720
900
1260
Protein (μg)
B
Calpain Activity
(ΔOD X 10-3)
60
40
20
0
0.2
0.4
0.6
0.8
1.0
1.2
CaCl2 (mM)
C
Calpain Activity
(ΔOD X 10-3)
80
70
60
50
40
30
0
0
0.1
0.2
0.3
0.4
0.5
MDL 28,170 (μM)
D
Calpain Activity
(ΔOD X 10-3)
100
80
60
40
20
0
0
0.5
1.0
1.5
Calpain Inhibitor I (μM)

Figure 1.2 The ability of the azocasein assay to detect changes in calpain activation induced *in situ* by global forebrain ischemia. One, 24, and 48 hours following 10 minutes of forebrain ischemia, the dorsal hippocampus was dissected (as described in Methods section 1.2.2.4) and homogenized on ice in a buffer containing 20 mM Tris HCl, 1mM EDTA, 5 mM 2-ME, 15 mM cysteine HCl, 250 mM sucrose, pH 7.4 (20% w/v). A concentration of 1000 μg of hippocampal homogenate and 800 μM $CaCl_2$ were added to the assay, with reactions carried out as described in section 1.2.3.1 of the Methods. Bars represent the mean $\pm$ standard deviation of multiple determinations from two animals. Statistical analysis with ANOVA revealed no significant main effect between control (unoperated) and ischemic rat hippocampal samples ($p > 0.05$).

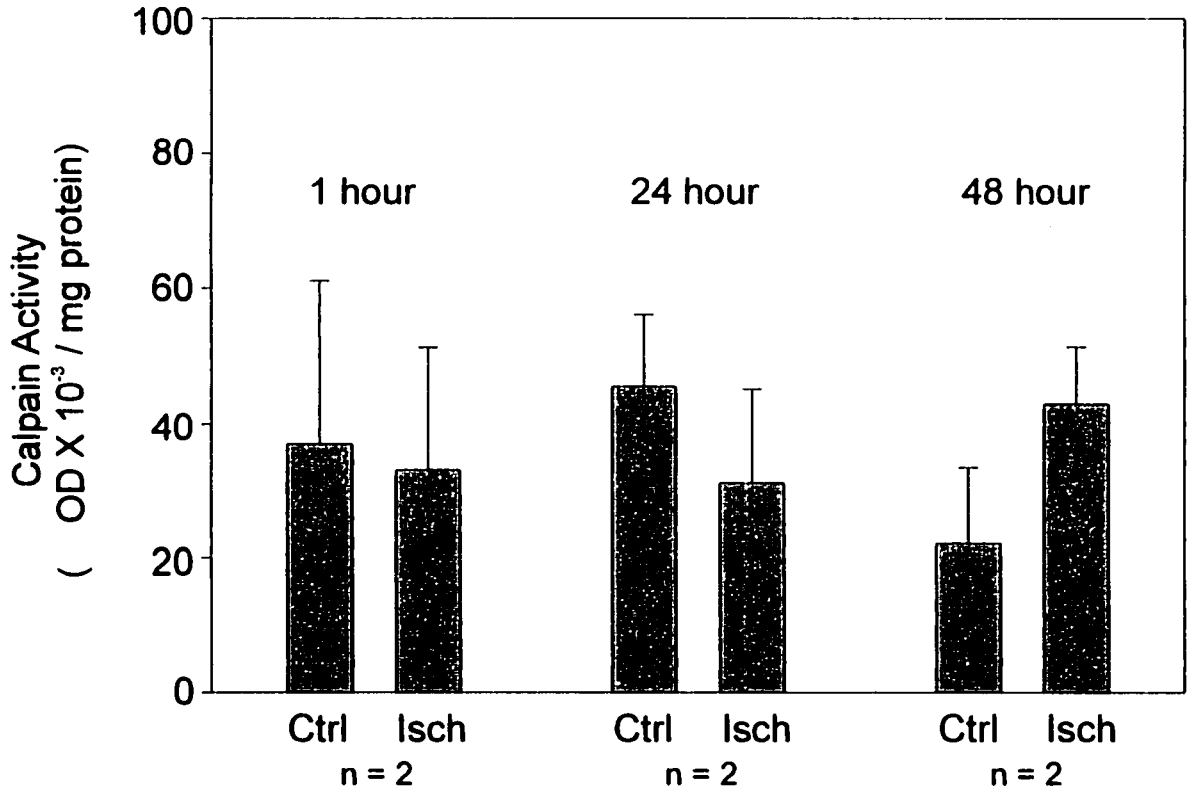
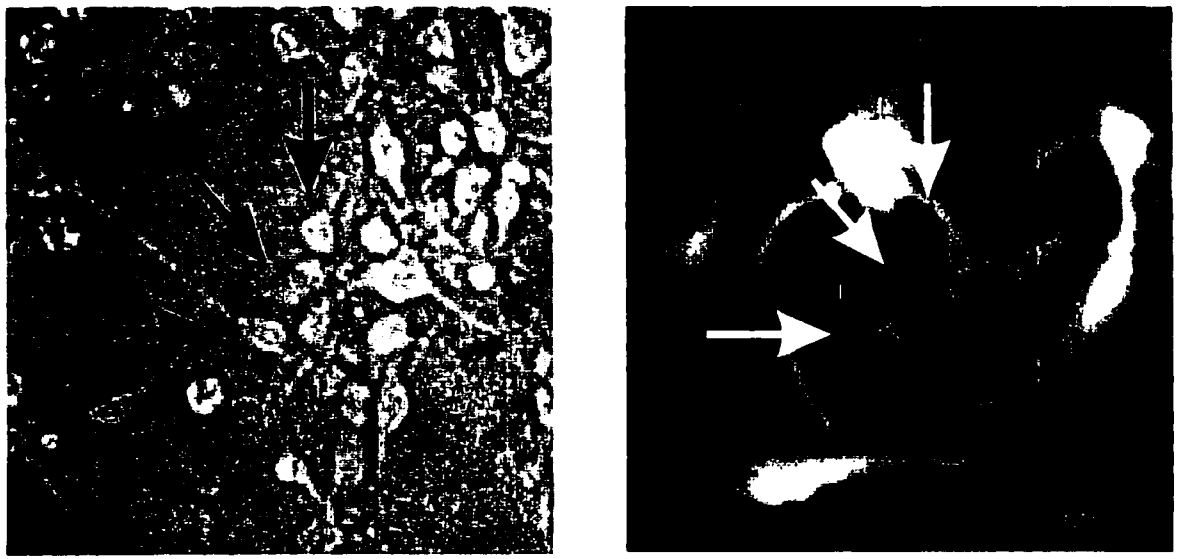

Calpain Activity
(OD X 10-3 / mg protein)
100
80
60
40
20
0
1 hour
24 hour
48 hour
Ctrl
Isch
Ctrl
Isch
Ctrl
Isch
n = 2
n = 2
n = 2

Figure 1.3 Fluorescent labelling of primary cortical cultures with Boc-Leu-Met-AMC. **A**: Representative phase contrast image showing neuronal cells, and fluorescent imaging of the same microscopic field showing the localization of Boc-Leu-Met-AMC fluorescence. Arrowheads indicate neurons in the mixed culture preparation, as determined from phase-bright imaging. Fluorescence-positive cells correspond to glial cells (confirmed by GFAP-labelling by Dr. J. Tauskela, NRC). Phase contrast images were obtained at the outset of the experiment, while fluorescent images were obtained 10-15 minutes after addition of the substrate. **B**: Fluorescence change in primary cultures in the presence (2 mM) or absence (5 mM EGTA added to treatment buffer) of extracellular Ca^{2+}. Fluorescence was expressed as slope, obtained from curves plotting the change in fluorescence intensity over time. Treatment conditions and recording were as described in Methods (section 1.2.3.2). Bars represent the mean of determinations from 4 separate experiments (n = 5) $\pm$ standard deviation (ANOVA, $p > 0.05$).

A


Phase contrast
CMAC

B

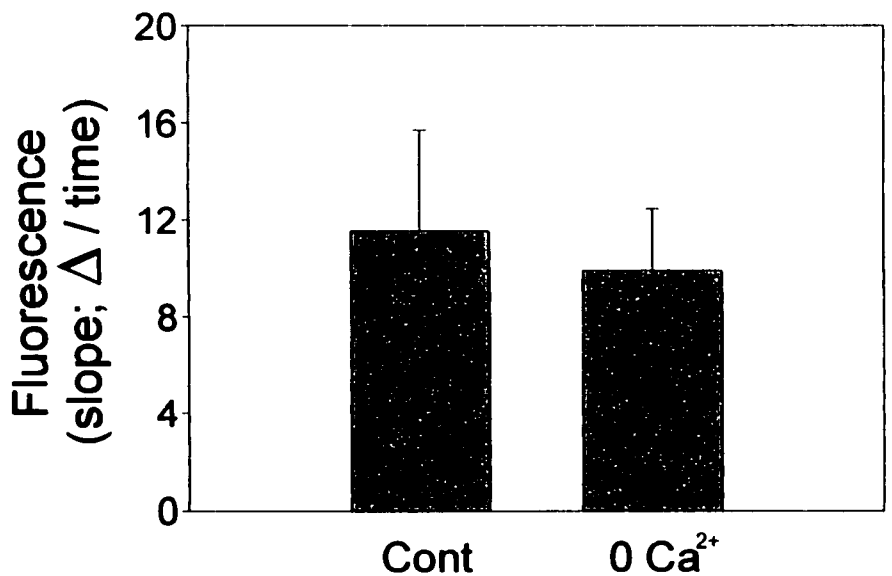

Fluorescence
(slope; Δ / time)
20
16
12
8
4
0
Cont
0 Ca2+

Figure 1.4 Characterization of the Cal-SBDP antibody and its selectivity for calpain-mediated spectrin breakdown products. **A**: Hydrolysis of spectrin in rat brain homogenates by purified calpain, and its attenuation by CPI I on Westerns probed with an anti-spectrin antibody (240/235E) which reacts against intact spectrin and its hydrolytic products. Brain homogenates were prepared in 10 volumes of buffer containing 50 mM Tris, and 2 mM PMSF, pH 7.4. Nuclei and unlysed cells were removed by centrifugation at 600 g for 5 min, and the resulting supernatant was incubated at 26°C for 1 hr in the presence or absence of 1 mM $CaCl_2$, 10 units of purified μ-calpain, and 13.5 μM CPI I in an assay buffer containing 20 mM Tris, 5 mM 2-ME, 15 mM L-cysteine, and 250 mM sucrose. Western blots were as described in Methods (section 1.2.4). **B**: A comparison of hydrolytic products detected with 240/235E (1:1000) and Cal-SBDP (1:1000) antibodies in brain homogenates incubated as in A, with purified calpain in the presence of Ca^{2+}. **C**: The hydrolytic product of spectrin produced two hours after a transient NMDA treatment (50 μM for 5 min) in primary cortical neurons (as in Methods section 1.2.5), on Westerns probed with the Cal-SBDP antibody (1:1000). **D**: The effect of the calpain inhibitor, calpeptin, on NMDA-induced spectrin hydrolysis, on Westerns probed with the Cal-SBDP antibody (1:1000). The indicated concentrations of calpeptin were applied one hour prior to NMDA application, and remained in the media until time of harvesting for Western blot analysis (2hrs post-treatment with NMDA). NMDA application and Western blot analysis were as described in sections 1.2.4 and 1.2.5 of Methods, respectively. Data are representative of at least three separate experiments.

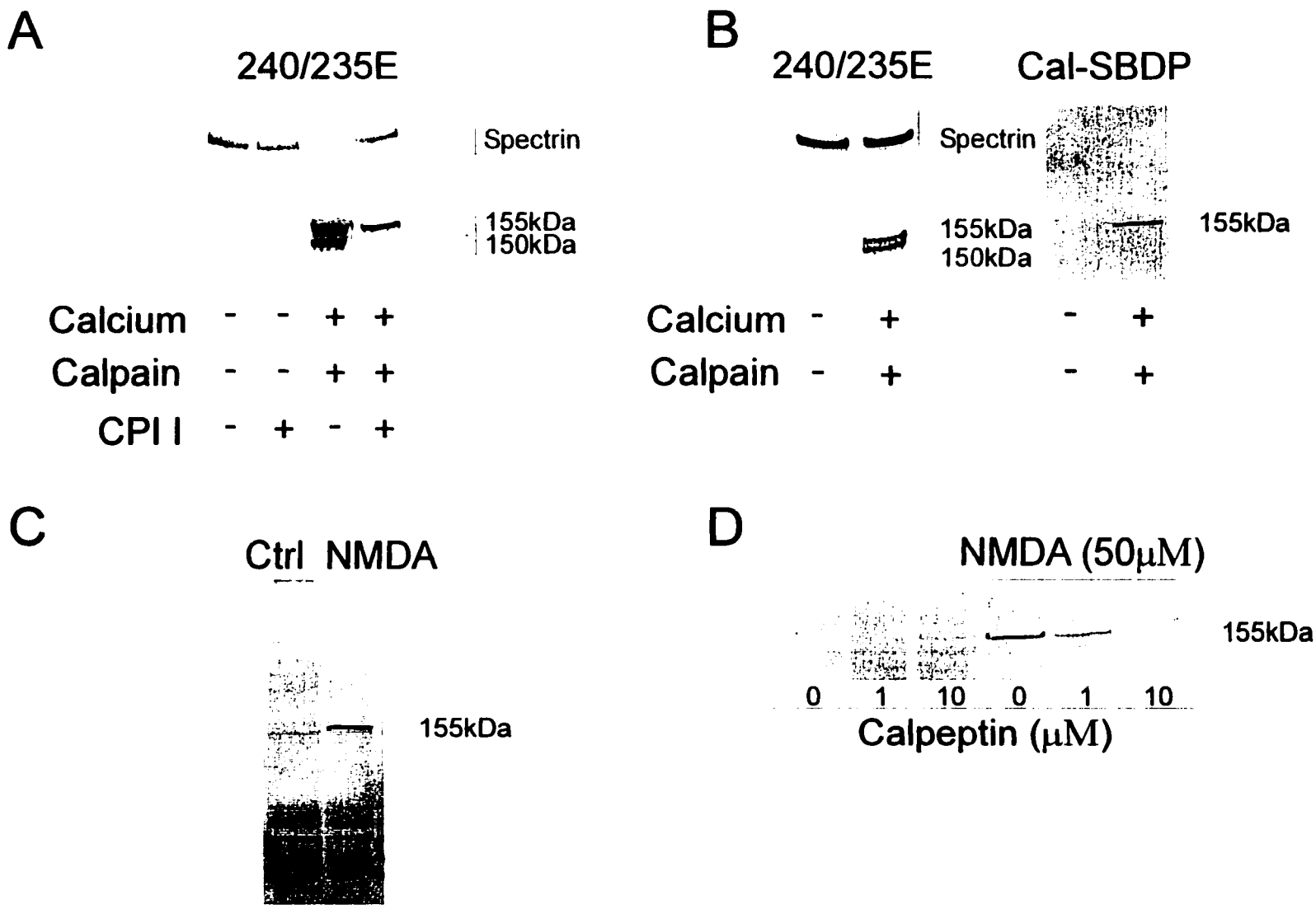

A
240/235E
Spectrin
155kDa
150kDa
Calcium - - + +
Calpain - - + +
CPI I - + - +
B
240/235E
Cal-SBDP
Spectrin
155kDa
150kDa
155kDa
Calcium - + - +
Calpain - + - +
C
Ctrl NMDA
155kDa
D
NMDA (50μM)
155kDa
0 1 10 0 1 10
Calpeptin (μM)

Figure 1.5 A comparison of the effects of NMDA and staurosporine on calpain-mediated spectrin hydrolysis. **A**: Detectable spectrin fragments produced by NMDA (50 μM for 5 min in conditioned media) and staurosporine (1 μM in conditioned media for 6 hours) using the Cal-SBDP antibody (1:1000), as described in Methods (section 1.2.5). Cells were harvested for Western blot analysis 6 hours post-treatment for both NMDA and staurosporine. **B**: The same samples in A reacted against the commercial spectrin antibody (240/235E; 1:1000) that recognizes intact spectrin, and the hydrolytic products of the protein produced by various proteases. Data are representative of three separate experiments.

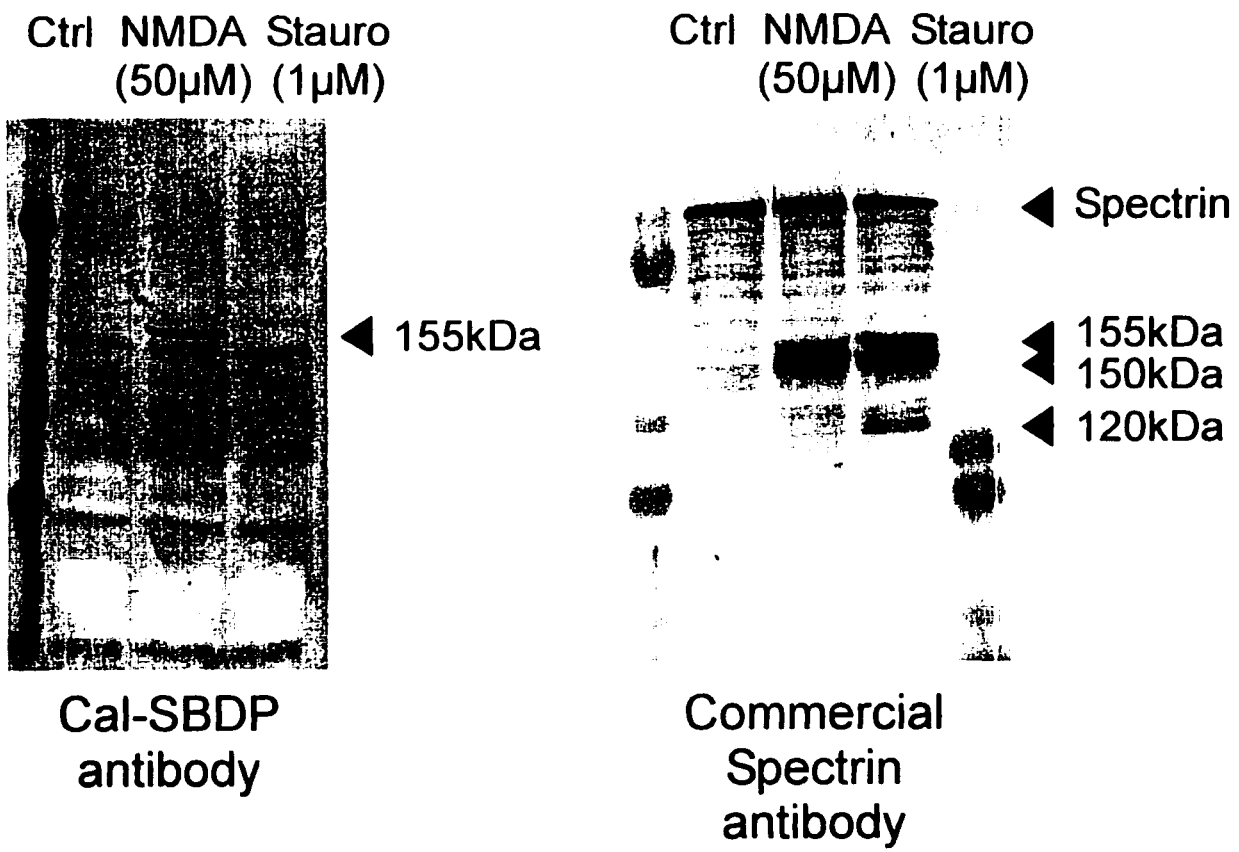

Ctrl NMDA Stauro
(50μM) (1μM)
155kDa
Cal-SBDP
antibody
Ctrl NMDA Stauro
(50μM) (1μM)
Spectrin
155kDa
150kDa
120kDa
Commercial
Spectrin
antibody

Figure 1.6 An examination of calpain-mediated spectrin hydrolysis in rat dorsal hippocampus 48 hours post-ischemia, with or without intra-ischemic hypothermia. Each lane represents an individual rat (unoperated controls, n = 2; ischemia (37°C), n = 4; and ischemia with hypothermia (33°C), n = 4). Two-vessel occlusion surgery (section 1.2.2.3), tissue dissection (section 1.2.2.4), and Western blot analyses (section 1.2.4) using the Cal-SBDP antibody (1:1000) were as described in Methods. Tissue was homogenized in a buffer containing 20mM Tris HCl, 1 mM EDTA, 5 mM 2-ME, 15 mM cysteine HCl, and 250 mM sucrose, pH 7.4, centrifuged at 600 g for 5 min to remove unlysed cells and nuclei, and the resulting supernatant separated into cytosolic and membrane fractions.

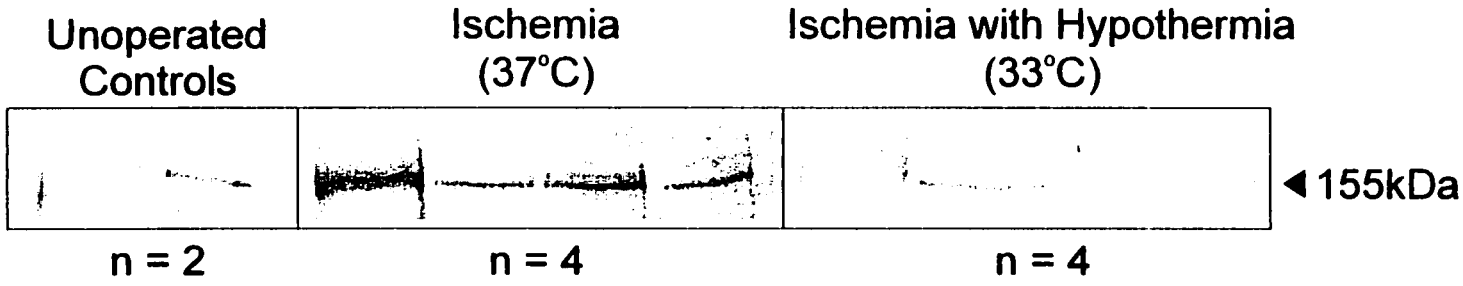

Unoperated Controls
Ischemia (37°C)
Ischemia with Hypothermia (33°C)
155kDa
n = 2
n = 4
n = 4

Figure 1.7 Hematoxylin and eosin stained brain sections demonstrating the time course of neuronal cell death in hippocampal CA1 following transient forebrain ischemia. Two and seven days following a sham operation (i.e., all manipulations described in section 1.2.2.3 except exsanguination and artery occlusion) or a 10 min 2-vessel occlusion with hypotension (see section 1.2.2.3), rats were lethally injected with sodium pentobarbitol (100 mg/kg, ip), and were transcardially perfused with heparinized saline, followed by 4% paraformaldehyde. Brains were removed, and sent to the histological lab at the Ottawa Civic Hospital for paraffin embedding, sectioning (7 μM sections), and histological staining with hematoxylin and eosin. Photomicrographs (40X) of histological sections were taken at the level of the dorsal hippocampus to show the morphological effects of global forebrain ischemia over time. **A.** Sham-operated control seven days post-surgery; **B.** transient global forebrain ischemia induced by 2-VO with hypotension, two days post-surgery; and **C.** transient forebrain ischemia induced by 2-VO with hypotension, seven days post-surgery. Arrows indicate approximate CA1 boundaries.

(All surgeries for this experiment were performed by M. Todd, M.Sc., in the laboratory of Dr. H. Lesiuk).

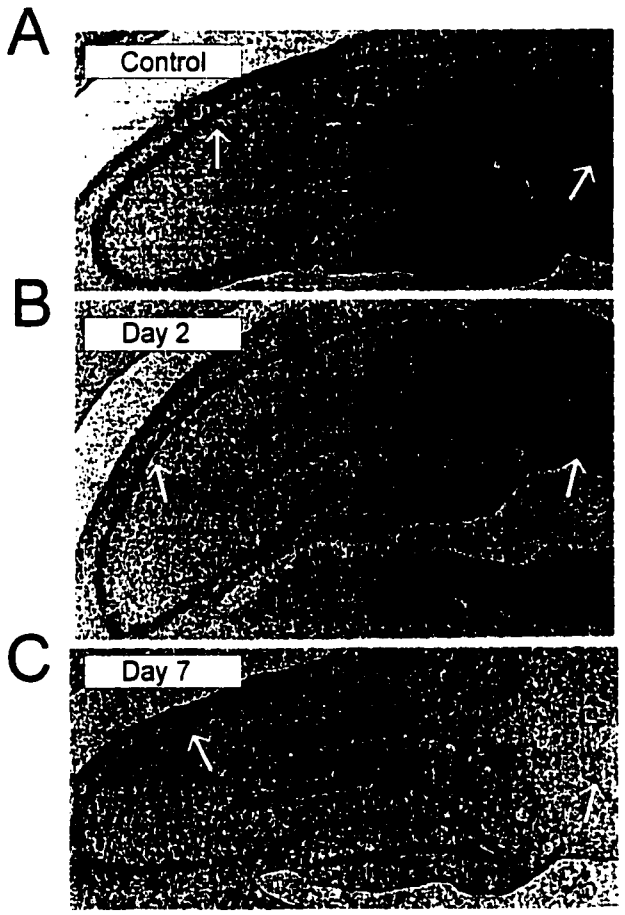

A
Control
B
Day 2
C
Day 7

Figure 1.8 The physiological parameters recorded in rats undergoing transient forebrain ischemia as described in Methods (section 1.2.2.3). **A:** Measurement of pO_2 in both normothermic and hypothermic rats demonstrated that O_2 levels were comparable between treatment groups ($p > 0.05$), and did not vary post-operatively in response to ischemia ($p > 0.05$). Normal pO_2 range is between 90 – 120 torr. **B:** Blood gas determinations of pCO_2 showed a post-operative increase above normal levels for both normothermic and hypothermic rats ($p < 0.05$), but these changes were equivalent for both groups such that blood CO_2 content did not differ between the treatment groups either pre- or post-operatively ($p > 0.05$). Normal pCO_2 values range between 35 – 45 torr. **C:** Blood pH determinations did not differ between or within groups pre- or post-operatively ($p > 0.05$). **D:** Blood glucose determination prior to occlusion did indicate that as a group, hypothermic rats had a significantly lower blood glucose level (ANOVA, $p < 0.05$). Normothermic rats had mean blood glucose values of 5.05 ($\pm$0.8) while hypothermic rats averaged 4.4 ($\pm$0.8). Bars represent the means $\pm$ standard deviation for each set of parameters (for A – D, n = 11 rats per group).

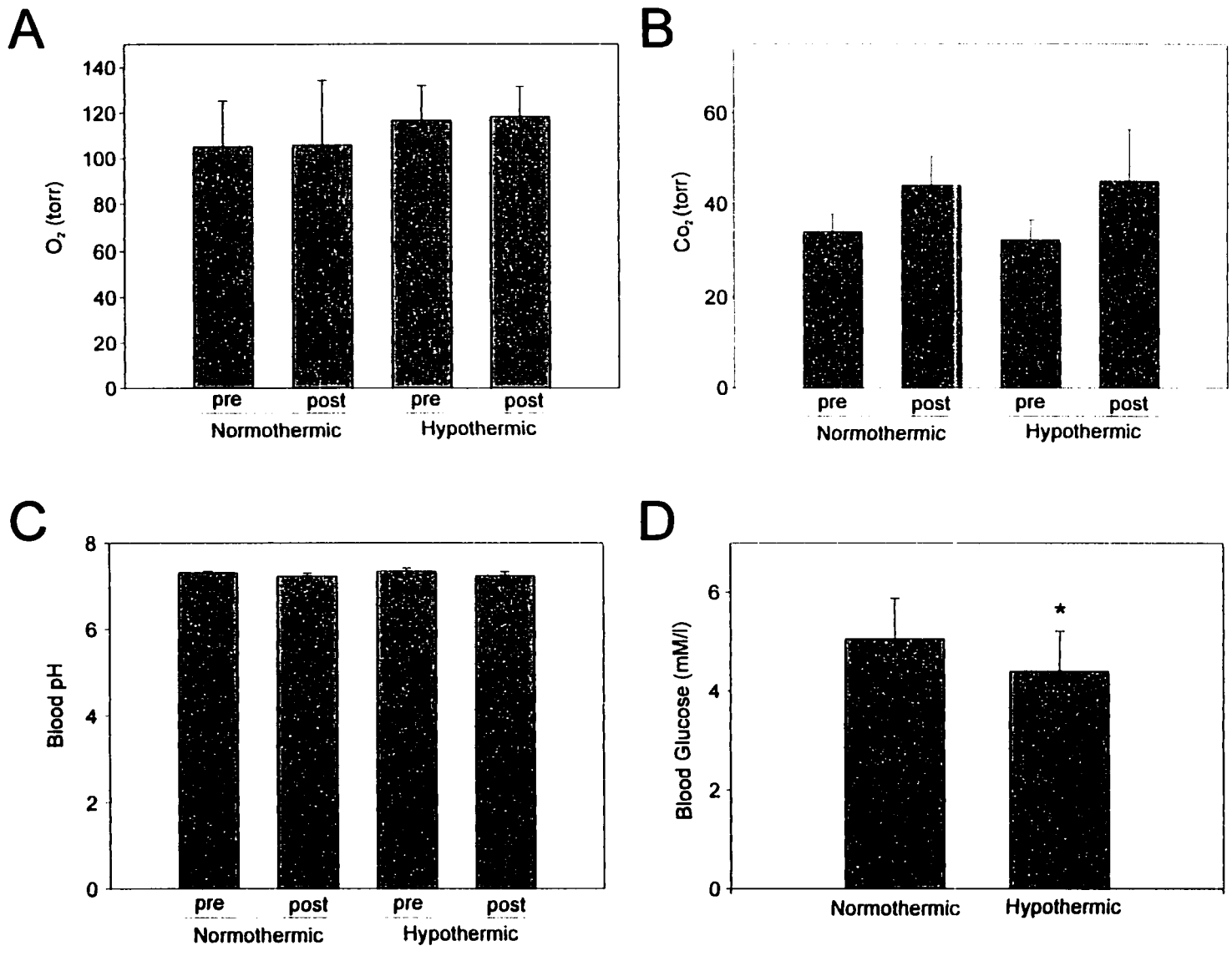

A
O_2 (torr)
140
120
100
80
60
40
20
0
pre
post
pre
post
Normothermic
Hypothermic
B
Co_2 (torr)
60
40
20
0
pre
post
pre
post
Normothermic
Hypothermic
C
Blood pH
8
6
4
2
0
pre
post
pre
post
Normothermic
Hypothermic
D
Blood Glucose (mM/l)
6
4
2
0
*
Normothermic
Hypothermic

Figure 1.9 Intraischemic body temperature measured at the tympanic membrane in rats undergoing 2-VO with hypotension. Normothermic rats had average recorded temperatures of 37.5°C, while hypothermic rats were maintained at 32.5°C. Bars represent the mean of mean intraischemic body temperature measurements (n = 11 rats per group) $\pm$ standard error of the mean. Analysis of Variance revealed that this difference in intraischemic body temperature was statistically significant ($p < 0.05$).

Intraischemic Temperature (°C)
38
36
34
32
30
Normothermic
Hypothermic
*

Chapter 2. Characterization of Calpain Activation in Primary Cortical Neurons, and its Specificity to NMDA Receptor Activation

2.1 Background

It is well established that calpain activation occurs following an ischemic or excitotoxic incursion in neuronal tissue. However, there is a considerable lack of information on the underlying mechanism(s) by which lethal increases in $[Ca^{2+}]_i$ through over-stimulated glutamate receptors bring about calpain activation in susceptible neurons. As described in the General Introduction, calpain activation has been linked to glutamate receptor activation in rat brain (Siman et al., 1989). However, generalized increases in $[Ca^{2+}]_i$ induced by ionophores induce calpain activity in a variety of cell types (Hayashi et al., 1991; Molinari et al., 1994; Michetti et al., 1996; Saido et al., 1993a; Nagao et al., 1994; Shea et al., 1996; Fujitani et al., 1997; Shea, 1997). Similar ionophore-mediated increases in $[Ca^{2+}]_i$ are non-toxic to primary cortical neurons, despite the fact that they induce Ca^{2+} entry to levels highly comparable to that initiated by glutamate receptor agonists (Durkin et al., 1996). Yet, in brain only levels of excitotoxins that are lethal to neurons induced calpain substrate hydrolysis, suggesting that calpain activation is linked with a death response (Siman et al., 1989). The question arises as to whether calpain activation is specifically linked to Ca^{2+} fluxes induced by glutamate receptor activation in neurons, or whether generalized increases in Ca^{2+} are sufficient to activate the protease. The former would suggest a specialized role for calpain in Ca^{2+}-mediated cell signaling events mediated by glutamate, while the latter would suggest a generalized role,

indiscriminant of signaling mechanisms. This is an important issue addressed in this chapter.

In order to understand the mechanisms of calpain activation in primary cortical neurons, we have characterized the response of calpain to lethal versus non-lethal increases in $[Ca^{2+}]_i$, using NMDA, glutamate, AMPA, ionomycin, KCl and *in vitro* hypoxia. In the previous chapter it was also established that calpain-mediated spectrin hydrolysis occurred in primary cortical neurons in response to NMDA, and that the proteolytic product was identical to that induced by *in vivo* global forebrain ischemia. However, in order to address the relevance of calpain activation to NMDA-induced cell death, a more detailed assessment of the protease's response relative to cell mortality was needed. We therefore examined the ability of specific glutamate receptor antagonists and calpain inhibitors to attenuate both calpain activation and neuronal cell death.

2.2 METHODS

2.2.1 Treatment Conditions

Primary cortical cell cultures were prepared in 35 mm dishes as described in the method section of Chapter 1 (section 1.2.2.1). For NMDA application, unless otherwise indicated, 14-18 DIV cells were used. The conditioned media from each plate was removed, and a portion was combined with NMDA to the indicated final concentration of the excitotoxin (0 – 100 μM). Cells were treated in a 1 ml volume of media + excitotoxin for 5 minutes at room temperature, the media was removed, and the cells were washed once in 1 ml of CSS (containing 120 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 15 mM

glucose, buffered with 25 mM Tris, pH 7.4). One ml of the original untreated conditioned culture media was re-applied to each plate, and the cells were incubated at 37°C until harvesting and cell lysis (see section 1.2.2.2) at the times indicated for Western blotting (as described in section 1.2.4) or cell viability determination (see below, section 2.2.3).

In some experiments, the calpain inhibitors calpeptin, aurintricarboxylic acid (ATA), AK275, AK295, calpain inhibitor I, and EST were added to the cultures at the indicated concentrations one hour prior to NMDA exposure, and remained present until the cells were harvested for Western blotting (at two hours) or cell viability determination (at 24 hours).

In experiments examining the effects of AMPA, 21-24 DIV cortical cells were used for all treatment conditions, since unlike NMDA, AMPA is relatively non-toxic to younger cultures (Durkin et al., 1997). Cells were treated exactly as described above for NMDA, except that exposure time was increased to 15 minutes. Furthermore, the concentration of NMDA required to induce toxicity in older cultures with this treatment duration was substantially less and therefore, the concentration of the excitotoxin was reduced as indicated. Otherwise, treatment conditions were as described above.

In experiments in which cells were exposed to high KCl or ionomycin, and for ion dependency experiments, cells were treated in appropriately modified CSS for the times indicated. The high KCl medium was an isotonic solution containing 75 mM NaCl, 50 mM KCl, 1.8 mM $CaCl_2$, 15 mM glucose, buffered with 25 mM Tris, pH 7.4. Ionomycin was added to normal CSS. For "zero" Na^+ conditions, the 120 mM NaCl in CSS was replaced with an equivalent amount of choline chloride (ChCl). For zero Ca^{2+} conditions,

2mM EGTA was added to CSS, which effectively chelated all free Ca^{2+} from the treatment solution, as determined by the software program EQCAL (as described in Chapter 1). In order to properly compare the effects of excitotoxins to these conditions, excitotoxins were added to normal CSS instead of conditioned media, as indicated in the legends of the relevant figures. Cell lysis (section 1.2.2.2), Western blotting (section 1.2.4) and cell viability (see below, section 2.2.3) were as described.

For all experiments, excitotoxins and MK-801 were dissolved in CSS. Other drugs were dissolved in DMSO such that the final concentration of DMSO did not exceed 0.1%.

2.2.2 Hypoxia and Preconditioning

Cortical cell cultures were prepared in 35 mm dishes as described in the Methods of Chapter 1 (section 1.2.2.1). Oxygen-glucose deprivation was achieved by placing culture dishes in a 37°C incubator housed in an anaerobic glovebox (Forma Scientific, Marjetta, OH). The culture media from 15-18 DIV cultures was removed and retained, the cells were washed twice in a glucose-free, HEPES buffered, balanced salt solution (BSS; containing 140 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 10 mM HEPES, 0.03 mM glycine, pH 7.4), and were subjected to the anaerobic environment of 95% N_2/5% CO_2 in 1 ml of glucose-free BSS. O_2 partial pressure under anaerobic conditions was equal to 10-15 torr, as measured with an oxygen microelectrode (Microelectrodes, Londonberry, NH). The duration of the hypoxic insult ranged from 180 - 240 minutes. Oxygen deprivation was terminated by removing the cultures from the incubator, returning the

retained culture medium to the plates, and incubating them at 37°C under normal atmospheric O_2 and 5% CO_2. Control cultures were incubated at 37°C under normal atmospheric conditions for the same duration in BSS containing 3 mM D-glucose. The glutamate receptor antagonists, MK-801 and CNQX were added to the media as indicated in the figure legends of the specific experiments.

For hypoxic preconditioning experiments, four treatment groups were established to assess the effects of hypoxic preconditioning on cell death and calpain activation. Treatment volumes were 1 ml of the indicated solutions. **Control** (Ctrl) cultures were subjected to 90 minutes of normoxia (standard incubator conditions) in BSS + 3 mM glucose, followed by a 24 hour recovery in conditioned media, and then 180 – 240 minutes of normoxia in BSS + 3 mM glucose. **Preconditioned** (PC) cultures were exposed to "sublethal" oxygen/glucose deprivation in glucose-free BSS for 90 minutes, followed by 24 hours recovery in conditioned media, and then 180- 240 minutes of normoxia in BSS + 3 mM glucose. **Ischemic** (ISCH) cultures were subjected to 90 minutes of normoxia in BSS + 3 mM glucose, allowed to recover for 24 hours in conditioned media, and then subjected to oxygen/glucose deprivation for 180 – 240 minutes in glucose-free BSS. **Preconditioned ischemic** cultures (ISCH/PC) were exposed to oxygen glucose deprivation for 90 minutes in glucose-free BSS, and following a 24 hour recovery in conditioned media, were subjected to another 180 – 240 minutes oxygen/glucose deprivation in glucose-free BSS.

Calpain-mediated spectrin hydrolysis was assessed two hours after final treatment, with cells prepared as described (section 1.2.2.2) for Western blot analysis using the Cal-

SBDP antibody (1:1000) (section 1.2.4). Cell viability (see below, section 2.2.3) was determined 24 hours post-treatment.

For preconditioning induced by exposure to MK-801, 15-18 DIV cells were treated with MK-801 (1 μM) for 30 minutes in 1 ml of conditioned media, at 37°C. Following this exposure, culture media was removed, the cells were washed twice with 1ml of CSS, and 1 ml of conditioned media was re-introduced to each plate. Forty-eight hours following transient MK-801, cells were treated with 50 μM NMDA for 5 minutes in conditioned media, washed with CSS, returned to normal conditioned media (1 ml), and harvested for Western blot analysis (as described in sections 1.2.2.2 and 1.2.4) or cell viability (see below, section 2.2.3).

2.2.3 Propidium Iodide Cell Viability Assay

A 10 μl volume of propidium iodide (PI, 15 μM final concentration) was added to the medium of each culture dish and incubated at 37°C for a minimum of 10 minutes. Cell viability was determined by counting phase-bright cells (live neurons) under phase-contrast microscopy, and the number of propidium iodide (PI)-labeled nuclei (dead neurons) under fluorescence microscopy. Cell death was expressed as the percentage of PI stained to total cells (i.e., PI stained/ [phase-bright + PI stained]).

2.2.4 Calcium Entry and $[Ca^{2+}]_i$ Detection

$[Ca^{2+}]_i$ was determined by using the Ca^{2+}-sensitive indicator Fura-2 (Molecular Probes Inc., Eugene, OR) in cultures plated on glass coverslips (plating procedures otherwise as described in section 1.2.2.1). Neurons were pre-loaded with Fura-2 by incubating them for 30 minutes at 37°C in a Mg^{2+}-free CSS salt solution (120 mM NaCl, 5.4 mM KCl, 0.8 mM $CaCl_2$, 15 mM glucose, buffered with 25 mM Tris, pH 7.4), containing 2.5 μM Fura-2/AM. After incubation, cells were washed three times and experiments conducted at room temperature, on groups of 5-15 cells per field. $[Ca^{2+}]_i$ was assessed by determining the ratio of the fluorescence intensities of Fura-2 emission as described previously (Black et al., 1995). Fluorescence recordings were obtained every 10 seconds for three minutes before the application of NMDA (50 μM, n = 3 experiments), KCl (50 mM, n = 2 experiments), and ionomycin (2.5 μM, n = 3 experiments). Following these additions, $[Ca^{2+}]_i$ was determined every 10 seconds for 15 minutes.

2.3 RESULTS

2.3.1 The early Ca^{2+}-dependent activation of calpain by NMDA correlates with excitotoxic cell death.

An NMDA-mediated increase in calpain activity was dependent upon the concentration of NMDA used, being evident only in those cultures treated with lethal concentrations of NMDA. Calpain-mediated spectrin hydrolysis was observed in cortical neurons as early as 30-60 minutes following transient exposure to 50 μM NMDA (Figure 2.1A), was strongly evident at two hours, and was sustained for up to 24 hours post-

treatment (see Figure 2.9A). This increase in calpain-mediated spectrin breakdown products correlated with the lethality of the insult. Concentrations of NMDA that caused early spectrin hydrolysis (in Figure 2.1A) resulted in cell death (Figure 2.1B). Furthermore, the increase in spectrin proteolysis at two hours preceded and was predictive of the loss in neuronal viability seen 24 hours following transient NMDA exposure. While no significant difference in neuronal viability was found between any of the treatment groups three hours following NMDA application ($p > 0.05$), cultures receiving transient, 50 μM NMDA exposure showed significant levels of cell death ($p < 0.01$) by 24 hours (Figure 2.1B). By contrast, 20 μM NMDA, which failed to stimulate spectrin hydrolysis, did not demonstrate any toxicity in neuronal cells when assayed with propidium iodide (PI) 24 hours post-insult ($p > 0.05$) (Figure 2.1B). Thus, a direct correlation between an early and persistent increase in calpain activity and the lethal activation of the NMDA receptor was established.

The association between calpain activation and NMDA toxicity could be directly linked to the influx of extracellular Ca^{2+} through the NMDA receptor. When Ca^{2+} was removed from the treatment solution by EGTA chelation, both calpain-mediated spectrin hydrolysis (Figure 2.2A) and NMDA toxicity (Figure 2.2B) were markedly attenuated. The replacement of Na^{+} in the medium by choline chloride had no effect on the ability of NMDA to either stimulate calpain (Figure 2.2A) or effect cell death (Figure 2.2B). This finding confirmed that both calpain activation and neuronal death were dependent on Ca^{2+} influx from the extracellular milieu.

2.3.2 Glutamate-induced calpain activation is specifically mediated by NMDA receptor activation.

Excitotoxicity in response to glutamate is regarded as a complex interaction of pathological events that involve both NMDA and AMPA receptor over-activation, since antagonism of either receptor subtype confers neuroprotection against *in vivo* ischemia (Gill and Woodruff, 1990; Ibarrola et al., 1998; Li and Buchan, 1993; Sheardown et al., 1993). In order to address whether both of these receptor subtypes could induce calpain activity, cells 21-24 DIV were treated with lethal levels of glutamate, NMDA, and AMPA. Each of these receptor agonists induced calpain-mediated spectrin hydrolysis in cortical cultures when assessed two hours following transient excitotoxin application (Figure 2.3A), suggesting that activation of either the AMPA or NMDA receptor was sufficient to activate calpain. To determine the relative contribution of NMDA and AMPA receptors during glutamate application, the selective NMDA receptor antagonist, MK-801 (20 μM) was used. It was predicted that with NMDA receptors blocked, the AMPA-mediated component of calpain activation would be evident. However, results showed that the application of MK-801 during glutamate exposure completely attenuated calpain-mediated hydrolysis (Figure 2.3B), suggesting that only NMDA receptor activation was crucial for calpain activity when all glutamate receptor subtypes were potentially activated.

To ensure that the effect of MK-801 on AMPA receptor-mediated calpain activation was not due to nonspecific effects of the drug at the concentration used, a more

detailed analysis of the AMPA response in the presence of NMDA receptor antagonists was carried out. Examination of the dose response of MK-801 (0 – 1 μM) on NMDA and AMPA-induced cell death and calpain activation revealed that the compound displayed highly similar effects for both excitotoxins (Figure 2.4A). Thus, the concentrations of MK-801 that were effective at inhibiting NMDA-induced cell death and calpain activation ($\geq$ 0.05 μM), were the same as those that attenuated the effects of AMPA. MK-801 reduced NMDA toxicity by 26, 64, and 78% at concentrations of 0.05, 0.1 and 1 μM, respectively. Similarly MK-801 reduced AMPA toxicity by 15, 35, and 70% at the corresponding doses. Like NMDA, this was reflected in the degree of spectrin hydrolysis observed. To ensure that these effects were not restricted to MK-801, but were characteristic of NMDA receptor antagonism, the NMDA receptor blocker memantine (0 – 100 μM) was also used (Figure 2.4B). Again, selective NMDA receptor blockade by memantine was effective at preventing both NMDA and AMPA-mediated calpain activation and cell death in the same dose range. Co-application of memantine at a concentration of 10 μM reduced NMDA and AMPA toxicity by 46 and 57%, respectively. These results strongly suggested that AMPA was acting indirectly to induce NMDA receptor activation in cortical neurons, since the concentrations of the inhibitors used were highly selective for the NMDA receptor complex.

Despite the fact that NMDA receptor antagonists could block the effects of AMPA in the cell cultures, the actions of AMPA were directly initiated by activation of its receptor subtype. The AMPA receptor antagonist CNQX attenuated AMPA-induced

calpain activation (Figure 2.5A) and cell death (Figure 2.5B) but, had no effect on NMDA-mediated responses (Figure 2.5C, 2.5D). Since AMPA receptors are believed to primarily form Na^+ permeable channels, the Na^+-dependency of the AMPA-mediated response was examined. When NaCl was replaced with ChCl, the calpain activation and cell death mediated by both AMPA (Figure 2.6A) and NMDA (Figure 2.6B) were unaffected. This suggested that AMPA-induced cell death in cortical neurons was independent of Na^+ influx, but dependent upon extracellular Ca^{2+}. Chelating Ca^{2+} from the extracellular medium would not differentiate between the need for Ca^{2+} through the AMPA receptor or the NMDA receptor since AMPA application was clearly activating both.

To further examine the mechanisms by which AMPA was able to activate NMDA receptors, a variety of compounds were employed that are thought to prevent either the spread of neuronal depolarization through voltage-sensitive Na^+ channels, or Ca^{2+}-dependent neurotransmitter release through synaptic vesicles. TTX (1 μM), riluzole (20 μM), and GVIA (1 μM) were co-applied with the receptor agonists, and compared to MK-801 (1 μM). All treatments, other than MK-801, were unable to attenuate the effects of AMPA (Figure 2.7A) or NMDA (Figure 2.7B) on neuronal viability or spectrin hydrolysis.

2.2.3 *In vitro* "ischemia" activates calpain selectively through the NMDA receptor.

An alternative method of inducing excitotoxicity in primary cultures involves the application of *in vitro* "ischemia" in which cells are placed under hypoxic conditions, depriving them of oxygen and glucose. The relative contribution of AMPA and NMDA receptors to this "ischemic" insult was determined by using the receptor antagonists CNQX and MK-801. Under these conditions, MK-801, but not CNQX was effective at attenuating calpain activity (Figure 2.8A) and cell death (Figure 2.8B). The combined application of the two antagonists did not produce a synergistic response. This suggests that under hypoxic conditions, the release of endogenous glutamate mediates toxicity and calpain activation selectively through the NMDA receptor subtype.

2.3.4 NMDA receptor activation is essential for calpain activity.

The above sets of experiments suggested a crucial role for Ca^{2+}-influx through the NMDA receptor complex for the activation of calpain. This raised the question as to whether or not calpain activation was selectively coupled to signaling through the NMDA receptor, or if other sources of Ca^{2+} entry could effect protease activity. To address this issue, a comparison between the effects of NMDA, Ca^{2+} influx through VSCCs mediated by KCl-induced depolarization, and non-specific Ca^{2+} entry produced by ionomycin was made. In contrast to NMDA, 50 mM KCl had either no effect (example 1) or only weakly resulted in calpain-mediated spectrin hydrolysis (example 2), when measured two and 24 hours after treatment (Figure 2.9A). In addition, while NMDA triggered a prolonged accumulation of spectrin breakdown products, the low level spectrin fragments produced by KCl at two hours (in example 2) was significantly attenuated by 24 hours post-

treatment. Moreover, the co-application of MK-801 (20 μM), completely prevented spectrin proteolysis caused by both NMDA and KCl depolarization (Figure 2.9A), suggesting that the effects of KCl on calpain, when present, were the indirect result of NMDA receptor activation. Ionomycin did not increase spectrin proteolysis above CSS control levels in any experiment (Figure 2.9A).

The inability of either KCl or ionomycin to effect sustainable calpain activation relative to NMDA correlated with their inability to cause cell death. As shown in Figure 2.9B, NMDA, but not KCl or ionomycin, was neurotoxic at concentrations used to assess their effect on calpain. Nevertheless, despite the fact that neither KCl nor ionomycin caused calpain activation or cell death, they were as potent as NMDA in causing significant and sustained increases in intracellular Ca^{2+} (Figure 2.9C). No statistical differences were found between the levels of $[Ca^{2+}]_i$ attained or the maintenance of these levels over the observation period ($p > 0.05$). Thus, relative to NMDA, other sources of Ca^{2+} entry were either unable to produce spectrin proteolysis, or produced only a transient, rather than sustained activation of the enzyme. The source of Ca^{2+} entry appeared to be crucially important for activating calpain in intact neurons.

2.3.5 The inhibition of calpain blocks NMDA-induced neuronal death.

The intimate association of calpain activity with NMDA receptor activation and cell death suggested that it was indeed part of the same signaling pathway that contributed to cellular demise. To determine whether calpain-mediated hydrolysis was playing a causal role in cell death, we examined the efficacy of numerous calpain

inhibitors in preventing calpain-mediated spectrin hydrolysis and cell death. The cell permeable calpain inhibitor calpeptin (0 – 5 μM) attenuated both spectrin hydrolysis (Figure 2.10A) and cell death (Figure 2.10B) in the same dose range (first evident at 0.5 μM calpeptin). Similarly, ATA (0 – 1000 μM) inhibited calpain activity (Figure 2.11A) and cell death (Figure 2.11B) at the same concentration (i.e., between 100 – 200 μM). The fact that calpeptin and ATA affected protease activation at the same concentrations at which they exhibited neuroprotection against NMDA, suggested a causal role for calpain in mediating NMDA-induced cell death.

Other putative calpain inhibitors tested were not effective at preventing calpain activation or cell death in primary neuronal cultures treated with NMDA. Calpain inhibitor I (CPI I, 0 – 135 μM) applied at similar concentrations previously shown to provide neuroprotection in primary cortical neurons against kainate (Cheng and Sun, 1994) or in hippocampal slice against hypoxia (Arai et al., 1991), was ineffective at preventing calpain-mediated spectrin hydrolysis (Figure 2.12A), and did not attenuate cell death (Figure 2.12B). The recently developed α-keto amide calpain inhibitors AK275 and AK295 (Li et al., 1996) were also unsuccessful *in vitro*. Although AK275 and AK295 have been shown to prevent ischemic cell death and calpain-mediated spectrin hydrolysis *in vivo* (Bartus et al., 1994a,b), neither compound was effective in preventing calpain activity (Figure 2.13A, 2.14A) or cell death (Figure 2.13B, 2.14B) in cells treated with NMDA. In fact, AK295 demonstrated mild toxicity at 100 μM, which was synergistic when co-applied with NMDA (Figure 2.14B).

The failure of CPI I, AK275 and AK295 as *in situ* calpain inhibitors and neuroprotectants contrasts with that reported in other cell culture models (Rami and Krieglstein, 1993; Cheng and Sun, 1994; Wang et al., 1996a; Rami et al., 1997) and in *in vivo* ischemia (Bartus et al., 1994a,b; Rami and Krieglstein, 1993; Lee et al., 1991). Since membrane permeability could not readily explain the observed differences between these results and ours, the effects of varying the severity of the insult on the efficacy of a calpain inhibitor was examined. For this purpose the effects of EST (50 μM), a cell permeable derivative of E64, on calpain inhibition and cell death was determined at increasing concentrations of NMDA (i.e., 50, 75 and 100 μM). Figure 2.15 illustrates that at the lowest concentration of NMDA used (50 μM), EST (50 μM) could partially attenuate calpain-mediated spectrin hydrolysis, and cell death. However, the inhibitory capabilities of EST on calpain activation and cell death were lost as the concentration of NMDA increased to 75 and 100 μM (Figure 2.15). This suggested that the efficacy of at least some calpain inhibitors was directly related to the severity of the insult.

2.3.6 Neuroprotection afforded by "preconditioning" is coupled to calpain.

Collectively, the above data suggest that *when* a calpain inhibitor is effective at attenuating calpain-mediated spectrin hydrolysis under conditions of NMDA-mediated toxicity, neuronal cells are protected from injury. As was the case with *in vivo* intra-ischemic hypothermia (see Figure 1.7), this suggested calpain inhibition may be a common characteristic, and perhaps a necessary feature, of a cell-mediated

neuroprotective response. To further address this generality, we examined the effects of preconditioning on calpain activation and cell death following an hypoxic insult. Preconditioning, induced by "sub-lethal" exposure to oxygen/glucose deprivation, confers significant neuroprotection *in vitro* (Gage and Stanton, 1996; Perez-Pinzon et al., 1996; Badar-Goffer et al., 1993; Schurr et al., 1986; Bossenmeyer-Pourie and Daval, 1998; Khaspekov et al., 1998; Bruer et al., 1997; Ying et al., 1997 Sakaki et al., 1995). Similar neuroprotective effects of transient, sub-lethal ischemia have also consistently been observed *in vivo* (Liu et al., 1993; Kirino et al., 1991; Kitagawa et al., 1990). Figure 2.16A demonstrates that 90 minutes of sublethal hypoxic/hypoglycemic preconditioning itself induced spectrin hydrolysis. However, during the subsequent ischemic insult 24 hours later, those cultures that had undergone preconditioning did not show a subsequent increase in spectrin hydrolysis in response to the insult, and had significantly less calpain-mediated spectrin breakdown compared to those cultures receiving "lethal" oxygen/glucose deprivation alone (Figure 2.16A). Figure 2.16B demonstrates that this attenuation of calpain activity correlated with the ability of preconditioning to reduce cell death during the subsequent insult. Since hypoxic cell damage and calpain activation are mediated through NMDA receptor activation (see Figure 2.6), these results suggest preconditioning acts to uncouple this response.

Preconditioning can also be induced in primary neuronal cultures by a 30 minute exposure to MK-801. The neuroprotection induced by this MK-801 pretreatment unlikely involves protracted NMDA receptor antagonism after the channel blocker is removed since, research conducted by others in this laboratory has demonstrated that it is also

effective at preventing non-glutamate-mediated forms of cell death, and that neuroprotection requires a delay (Tremblay and Durkin, manuscript in preparation). Figure 2.17 demonstrates that when cells were treated with MK-801 (1 μM) for 30 minutes and then exposed to transient NMDA (50 μM for 5 minutes in culture media) 48 hours later, both calpain-mediated spectrin hydrolysis (Figure 2.17A) and NMDA-mediated cell death (Figure 2.17B) were attenuated. These data support the notion that sustained calpain activation directly results in cell injury, and that inhibition of the protease is associated with neuroprotection against an excitotoxic insult.

DISCUSSION

It has been demonstrated that calpain activation in primary cortical neurons is associated with lethal increases in Ca^{2+} entry, specifically associated with NMDA receptor activation, and that the characteristics of calpain activation in this model system have distinct similarities to that reported *in vivo* following excitotoxin application or cerebral ischemia.

The similarities between *in vivo* ischemia and primary cell cultures treated with excitotoxins were especially apparent in terms of the *timing* and *persistence* of calpain-mediated spectrin hydrolysis in response to lethal NMDA receptor activation. Roberts-Lewis et al. (1994) demonstrated that calpain-mediated spectrin hydrolysis occurs as an early event post-ischemically in a number of brain areas. However, the persistent detection of spectrin fragments was confined to cells destined to die, suggesting that the

persistent activation of calpain was a feature of ischemically vulnerable neurons (Roberts-Lewis et al., 1994). Similarly, in primary cortical neurons exposed to NMDA, we have shown calpain activation to be an early, pre-death event, initiated hours prior to a loss in membrane integrity. Furthermore, only experimental treatments that resulted in cell death caused persistent spectrin hydrolysis. Thus, as seen in *in vivo* ischemia, the persistence of spectrin breakdown products appears characteristic of neuronal populations destined to die. At the very least, these data indicate calpain activation to be a reliable marker for impending cell death. However, the inhibitor data presented using calpeptin, ATA, and EST support the role of calpain as a mediator of excitotoxic cell death rather than merely an early marker of the cells' demise.

Strikingly, calpain activation in primary cortical neurons was exclusively associated with lethal NMDA receptor stimulation. The data from NMDA, AMPA, glutamate, and hypoxic insults consistently showed that early calpain activation and cell injury specifically arise from NMDA receptor overstimulation. Furthermore, although the entry of extracellular Ca^{2+} appeared necessary for calpain activation, the source of that Ca^{2+} was inherently important for proteolysis. Despite the fact that NMDA, KCl and ionomycin raised Ca^{2+} concentrations to comparable levels, only NMDA induced calpain activity. This suggests that calpain is part of a specific cell signaling pathway emanating from the NMDA receptor complex, and is a mediator of Ca^{2+} signals involved in the cell death cascade.

Although AMPA receptor stimulation could also induce calpain-mediated spectrin hydrolysis and cell death, NMDA receptor antagonism by MK-801 or memantine

completely prevented these AMPA-mediated effects. This suggested that AMPA-induced depolarization was activating NMDA receptors through the initiation of endogenous glutamate release. However, blocking voltage-dependent Na^+ channels with TTX, a marine guanidinium toxin that selectively blocks voltage-gated Na^+ channels, did not affect AMPA-induced calpain activity or cell death, suggesting that the release of glutamate was not mediated by Na^+-dependent depolarization through this ion channel class. Similarly, riluzole, a compound known to block presynaptic glutamate release (Kretschmer et al., 1998) and voltage-dependent Na^+ and Ca^{2+} channels (Yokoo et al., 1998), also did not prevent AMPA from activating calpain or inducing cell death. Blockade of N-type Ca^{2+} channels with omega-conotoxin GVIA also had no effect, however transmitter release can be mediated through other subtypes of Ca^{2+} channels, including the P/Q-type (Bergquist et al., 1998; Okuma et al., 1998; Reid et al., 1998; Vazquez and Sanchez-Prieto, 1997), making it difficult to ascertain if AMPA was initiating release via Ca^{2+}-influx through VDCCs. We suggest it is most likely that AMPA receptor activation under these experimental conditions induced a Ca^{2+}-dependent depolarization that was sufficient to initiate release. Ca^{2+} permeable AMPA receptors have been reported *in vitro* (Jensen et al., 1998) and *in vivo* (Mahanty and Sah, 1998), and the application of AMPA in rat spinal cord slices has been shown to activate receptors residing at the terminal region to induce transmitter release (Sundstrom et al., 1998). It is possible that a similar mechanism is operating under our experimental conditions in primary cortical cultures. AMPA-mediated Ca^{2+} influx as a mode of transmitter release is also supported by the observation that AMPA effects were blocked by CNQX, but were

not dependent upon the presence of extracellular Na^+. However, there is one caveat when interpreting these ion replacement results since substituting NaCl with ChCl may interfere with glutamate transporters present on neurons and glia, since their operation relies on the presence of extracellular Na^+ (Madl and Burgesser, 1993). These transporters are necessary for terminating the effects of synaptically released glutamate, and for maintaining levels of glutamate below toxic levels. The transporters carry three Na^+, one H^+ and one glutamate inward, while extruding one K^+ (Levy et al., 1998; Zerangue and Kavanaugh, 1996; Madl and Burgesser, 1993). Failure or reversal of the pump can occur when there is a decrease in the Na^+ and K^+ plasma membrane gradients, decreasing the driving force for glutamate uptake (Madl and Burgesser, 1993). Nonetheless, AMPA-mediated calpain activation and cell death were prevented by NMDA receptor antagonists at concentrations selective for the NMDA receptor, and thus the effects of AMPA must act to increase extracellular glutamate which then is capable of acting on NMDA receptors.

Despite the fact that calpain activation did not occur if extracellular Ca^{2+} was removed by chelating agents, the source of Ca^{2+}-influx was extremely important for activation of the protease. Ca^{2+} entry effected by sub-lethal NMDA, ionomycin, and, for the most part, KCl, was ineffective for inducing calpain activity. Depolarization-induced Ca^{2+} entry by KCl was observed, at times, to increase spectrin hydrolysis, but unlike NMDA, KCl caused only a weak and transient appearance of a spectrin breakdown product that had dissipated by 24 hours post-insult. The persistent activation of calpain has been described as a definitive marker of cell injury, capable of differentiating

between vulnerable and resistant neurons following *in vivo* ischemia (Roberts-Lewis et al., 1994). The KCl data are consistent with that of Roberts-Lewis et al. (1994) discussed above, who demonstrated that spectrin hydrolysis increased following global ischemia in the gerbil in a variety of brain areas, but in resistant areas the detection of hydrolytic products of spectrin was transient. Vulnerable brain areas showed persistent levels of calpain-mediated spectrin breakdown products. The present observation that transient KCl-induced spectrin hydrolysis in cortical cells was not associated with a cell death response, while a persistent presence of spectrin breakdown products following lethal NMDA was, is fully consistent with these previous *in vivo* studies.

KCl induction of spectrin hydrolysis was also dependent on NMDA receptor activation. The increase in spectrin hydrolysis initiated by KCl was completely prevented when MK-801 was present during the application. As argued above for AMPA, this observation suggests that KCl induces endogenous glutamate release which acts as an NMDA receptor agonist. This finding is consistent with previous studies demonstrating that the effects of KCl could be prevented by NMDA receptor antagonism (Bonthius and Steward, 1993; Schurr et al., 1995).

Examination of calpain activation under hypoxic conditions further supported the conclusion that calpain activation was selectively coupled to NMDA receptor activation, and that calpain was significantly contributing to neuronal decline. Under these *in vitro*, ischemic-like conditions, both calpain-mediated hydrolysis and cell damage were attenuated by NMDA receptor blockade with MK-801. Conversely, CNQX was not effective in preventing calpain activity or cell death, and no synergistic effect was

observed when MK-801 and CNQX were combined under hypoxic conditions. Thus, during ischemia, calpain activation appears selectively coupled to NMDA receptor activation, and part of the excitotoxic death process.

Ischemic preconditioning, which is neuroprotective in several *in vivo* models of cerebral ischemia (Liu et al., 1993; Kirino et al., 1991; Kitagawa et al., 1990), attenuated subsequent calpain activation triggered by lethal hypoxia. The mechanisms by which mild ischemic incursions provide subsequent neuroprotection against greater insults remain essentially unknown, despite intense investigation. A delay of at least 24 hours between preconditioning and the potentially lethal ischemic insult has been reported to be necessary for effective neuroprotection (Kato et al., 1991; Kirino et al., 1991; Kitagawa et al., 1990). This time delay suggests that the cell undergoes molecular adaption in response to cell signals initiated by preconditioning. The identity of these preconditioning signals is essentially unknown. The present study demonstrates that while calpain-mediated spectrin hydrolysis is associated with preconditioning, subsequent calpain hydrolysis is blocked during the succeeding insult. Since a selective link between Ca^{2+} entry through the NMDA receptor and calpain activation has been established, a change in Ca^{2+} permeability following preconditioning may be the most parsimonious explanation for the observed effects. However, calpain activation itself may potentially mediate the preconditioning effect. Calpain activation has been purported to induce a cleavage of both NMDA and AMPA receptors at their C-terminal domains (Bi et al., 1998a,b; Gellerman et al., 1997; Bi et al., 1997a,b; Bi et al., 1996; Bi et al., 1994). The physiological or functional significance of these alterations is unknown. However, at least

in terms of the AMPA receptor, the functional interaction between the C-terminal domain and Giα (Wang et al., 1998) would be lost since this region would be part of the cleavage product (Bi et al., 1996). At the same time, NMDA-mediated activation of calpain results in an increase in both low and high affinity [^{3}H]-AMPA binding sites in organotypic hippocampal slices (Gellerman et al., 1997). Since NMDA receptor activation and calpain-mediated proteolysis have been strongly implicated in synaptic plasticity and LTP, these NMDA-mediated alterations have been speculated to underlie long term functional changes that mediate the cells' subsequent response repertoire (Gellerman et al., 1997). Thus, calpain-mediated changes during preconditioning may alter the cells' response to the subsequent insult. Whether calpain activation is actually necessary for the induction of preconditioning remains to be determined, but whatever the underlying mechanism, neuroprotection by preconditioning involves the attenuation of calpain activation during a subsequent severe hypoxic insult.

The inhibition of calpain using calpeptin, ATA, and EST demonstrated that the attenuation of calpain-mediated spectrin hydrolysis was directly related to the preservation of neuronal viability. Calpeptin and ATA were neuroprotective at the same concentrations that evoked calpain inhibition, strongly supporting the conclusion that calpain activation directly contributes to neuronal injury and cell death. In contrast, CPI I, AK275, and AK295 were unable to prevent calpain activation and cell injury in response to NMDA. These results were perplexing since the compounds have reported efficacy *in vivo* (Bartus et al., 1994a,b) *in vitro* (Rami and Krieglstein, 1993; Cheng and Sun, 1994; Wang et al., 1996a; Rami et al., 1997), or both (Rami and Krieglstein, 1993; Lee et al.,

1991). However, the potency of an inhibitor in mediating its effects may be related to the severity of the insult faced, since the calpain inhibitor EST progressively lost its ability to attenuate calpain-mediated spectrin hydrolysis and cell death with increased NMDA concentrations. This suggests that calpain activation grew in degree with NMDA concentration, and that the concentration of EST was not saturated. All of the calpain inhibitors used in these experiments were di- or tri-peptide active-site-directed inhibitors (Sorimachi et al., 1997a) that compete with substrates for the active site of the protease. Newer calpain inhibitors with novel mechanisms of action are now becoming available. PD150606 has a high specificity for calpain, and interacts with its Ca^{2+}-binding domains, preventing Ca^{2+}-induced activation (Wang et al., 1996a). In combination with active-site-directed inhibitors, compounds such as this may offer potent and selective inhibition of the protease.

Under the above experimental conditions, we did not observe an incidence where a calpain inhibitor was efficacious at preventing protease activation, but failed to rescue cells from NMDA-mediated death. If an inhibitor was effective at attenuating the NMDA-induced increase in calpain activation, it also effectively reduced NMDA-mediated cell death. This is in contrast with the findings of Manev et al. (1991) and Brana et al (1998) who reported that neuronal cell death in response to excitotoxicity was independent of calpain inhibition. In both instances, calpain inhibitors were able to attenuate spectrin hydrolysis, but cell death still occurred. Nonetheless, our results are highly consistent with the majority of reports that demonstrate calpain inhibition is an

effective neuroprotectant both in *in vivo* and *in vitro* models of excitotoxicity and stroke (see *Calpain and Cell Death* in the General Introduction).

Collectively, the above results provide convincing evidence that calpain activation is selectively coupled to a Ca^{2+}-dependent signaling pathway emanating from the NMDA receptor, and that the attenuation of calpain activity results in neuroprotection from excitotoxicity. Understanding the regulatory features of the calpain proteolytic system in intact cells, and its potential interaction with other signaling events resulting from glutamate receptor activation will provide valuable insight into the determinants of cell death in instances of excitotoxicity and ischemia. It is this realization that promoted the study described in Chapter 3, to add clarity to the mechanisms responsible for NMDA-receptor-coupled calpain activation in neuronal cells.

Figure 2.1 Time-course of calpain activation and cell death in response to increasing doses of NMDA. Primary cortical cultures were exposed to NMDA (0, 20, or 50 μM) for 5 min in conditioned media, as described in Methods (section 2.2.1), and were **A:** harvested (as described in section 1.2.2.2) 15 min, 30 min, 1 hour, and 2 hours later, and subjected to Western blot analysis using the Cal-SBDP antibody (section 1.2.4) or; **B:** assessed 3 hours or 24 hours later for cell viability (as described in section 2.1.3). Bars represent the mean of three separate experiments (n = 3 per experiment) $\pm$ standard deviation (* significantly different from all other groups, $p < 0.01$).

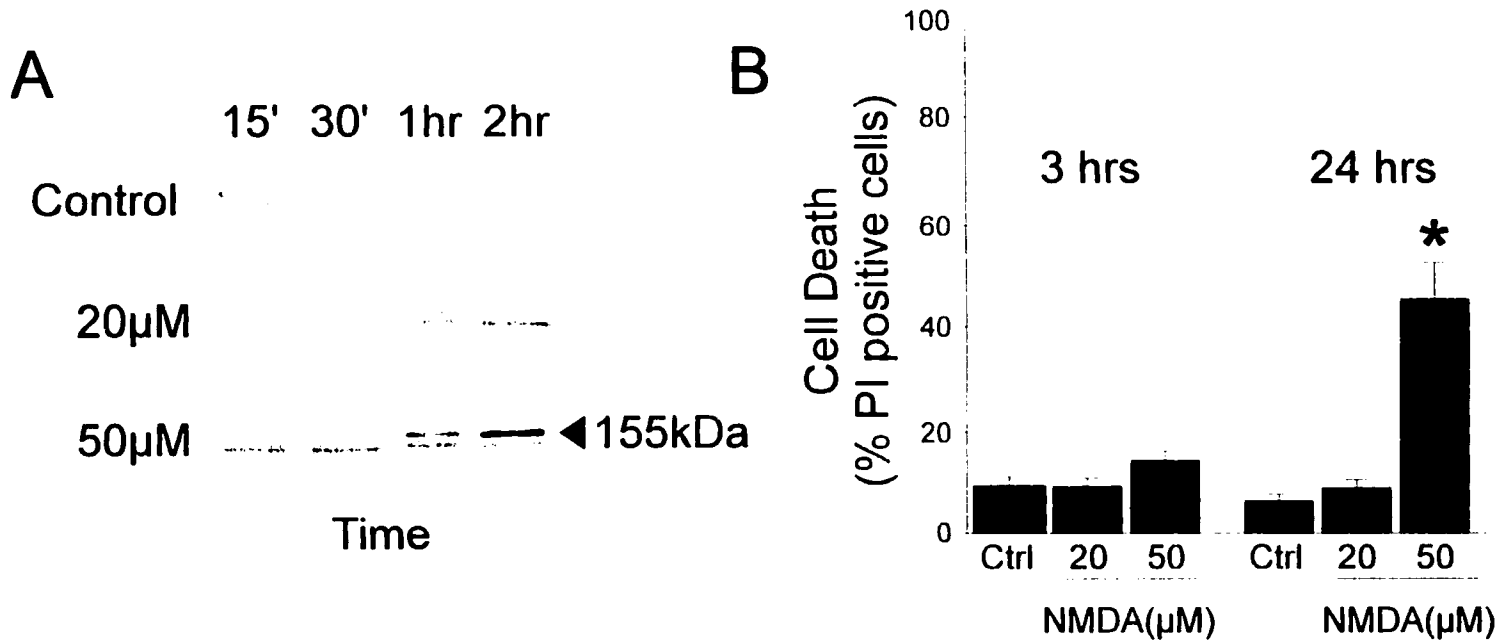
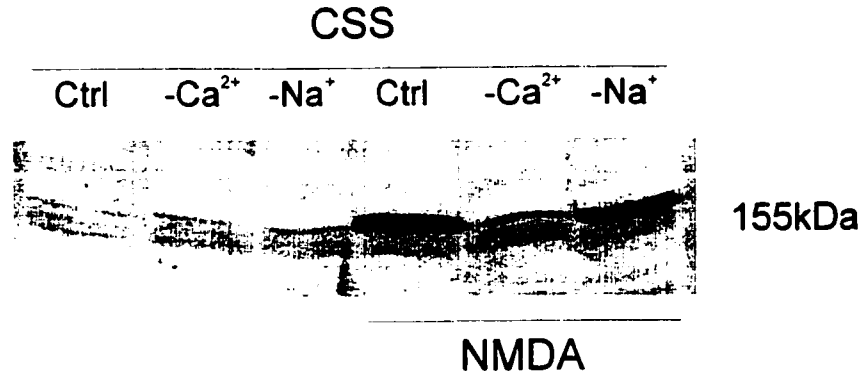

A
15' 30' 1hr 2hr
Control
20μM
50μM
155kDa
Time
B
Cell Death
(% PI positive cells)
100
80
60
40
20
0
3 hrs
24 hrs
*
Ctrl 20 50
Ctrl 20 50
NMDA(μM)
NMDA(μM)

Figure 2.2 The effect of extracellular Ca^{2+} or Na^{+} ions on NMDA-mediated calpain activation and cell death. **A:** Western blot analysis of the effects of extracellular Ca^{2+} and Na^{+} on calpain-mediated spectrin hydrolysis two hours following transient NMDA exposure. **B:** Corresponding viability of neuronal cells treated as in **A,** 24 hours after NMDA exposure. Cells were exposed for 5 minutes to 50 μM NMDA in normal CSS, in CSS containing 2 mM EGTA, or in CSS in which NaCl was replaced with ChCl. After treatment the cells were washed with normal CSS, and returned to conditioned media until being harvested (as described in section 1.2.2.2) for Western blot analysis (described in section 1.2.4) or cell viability (as described in Methods section 2.1.3). Bars represent the mean of three experiments (n = 3 per experiment) $\pm$ standard deviation. (* significantly different from all other NMDA treated groups, $p < 0.05$).

A


CSS
Ctrl
-Ca2+
-Na+
Ctrl
-Ca2+
-Na+
155kDa
NMDA

B

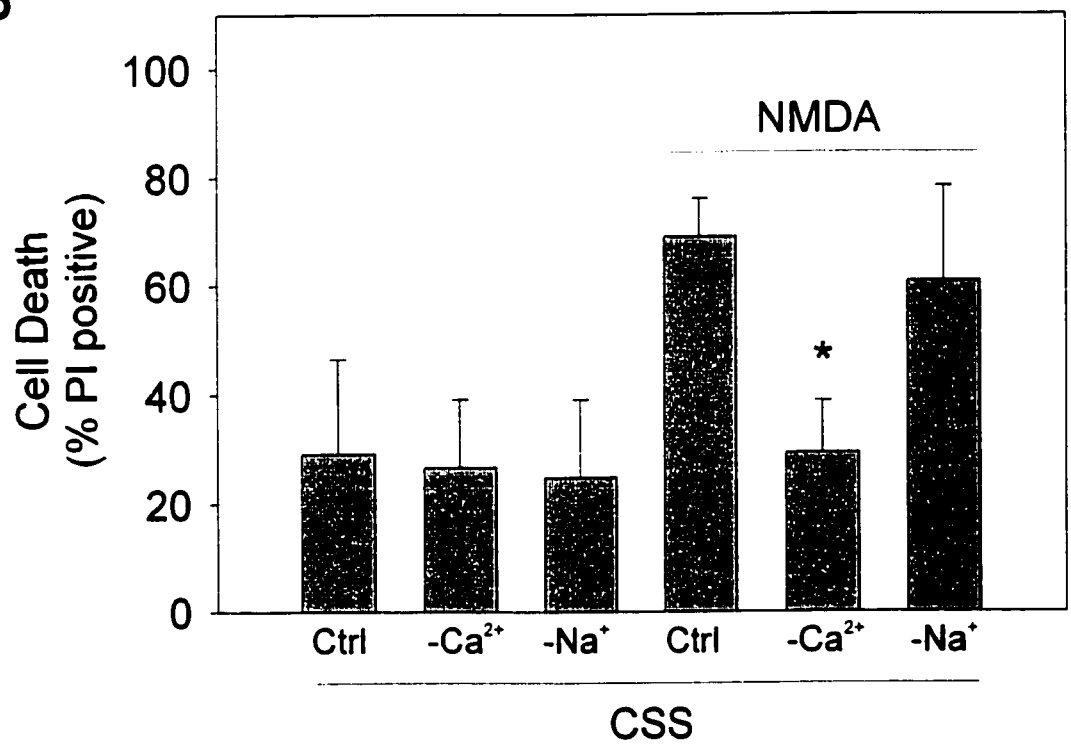

Cell Death
(% PI positive)
100
80
60
40
20
0
NMDA
*
Ctrl
-Ca2+
-Na+
Ctrl
-Ca2+
-Na+
CSS

Figure 2.3 The ability of glutamate, NMDA and AMPA to induce calpain activation in 22 DIV primary cortical neurons. **A:** The effect of transient (15 min) application of NMDA (50 μM), glutamate (50 μM), or AMPA (200 μM) on calpain-mediated spectrin hydrolysis measured two hours post-treatment by Western blot analysis, using the Cal-SBDP antibody. Methods for cell lysis and Western blotting were as described in Figure 2.1 **B:** The effect of NMDA receptor blockade with MK-801 (20 μM) on calpain activation induced by transient application of glutamate (50 μM), as in A. Blots are representative of 3 separate experiments.

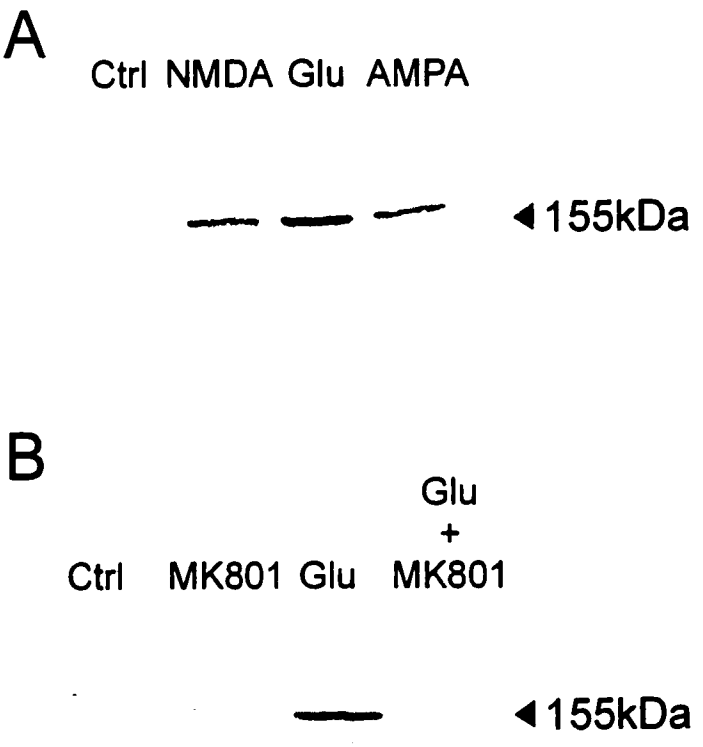

A
Ctrl NMDA Glu AMPA
155kDa
B
Glu
+
Ctrl MK801 Glu MK801
155kDa

Figure 2.4 The effect of NMDA receptor blockade on NMDA and AMPA-induced activation of calpain and cell death. **A:** The ability of the NMDA receptor blocker, MK-801 (in CSS), to attenuate spectrin hydrolysis and cell death in DIV 21 neuronal cultures treated with NMDA (25 μM) and AMPA (200 μM). The indicated concentrations of MK-801 were co-applied with the receptor agonists to the culture media of cortical neurons. Following a 15 minute application, cells were washed and returned to conditioned media for 2 hours before being lysed for Western blot analysis or for 24 hours for cell viability determination. Methods for cell lysis, Western blotting, and cell viability were as described in Figure 2.1 **B:** The effect of the NMDA receptor blocker memantine on NMDA and AMPA-mediated calpain activation and cell death. Memantine was co-applied with the receptor agonists at the indicated concentrations. Otherwise, cells were treated identically as in A. Bars represent the mean of three separate experiments (n = 3 per experiment) $\pm$ standard deviation (significantly different from respective agonist (NMDA or AMPA, as indicated) alone, * $p < 0.05$; ** $p < 0.01$).

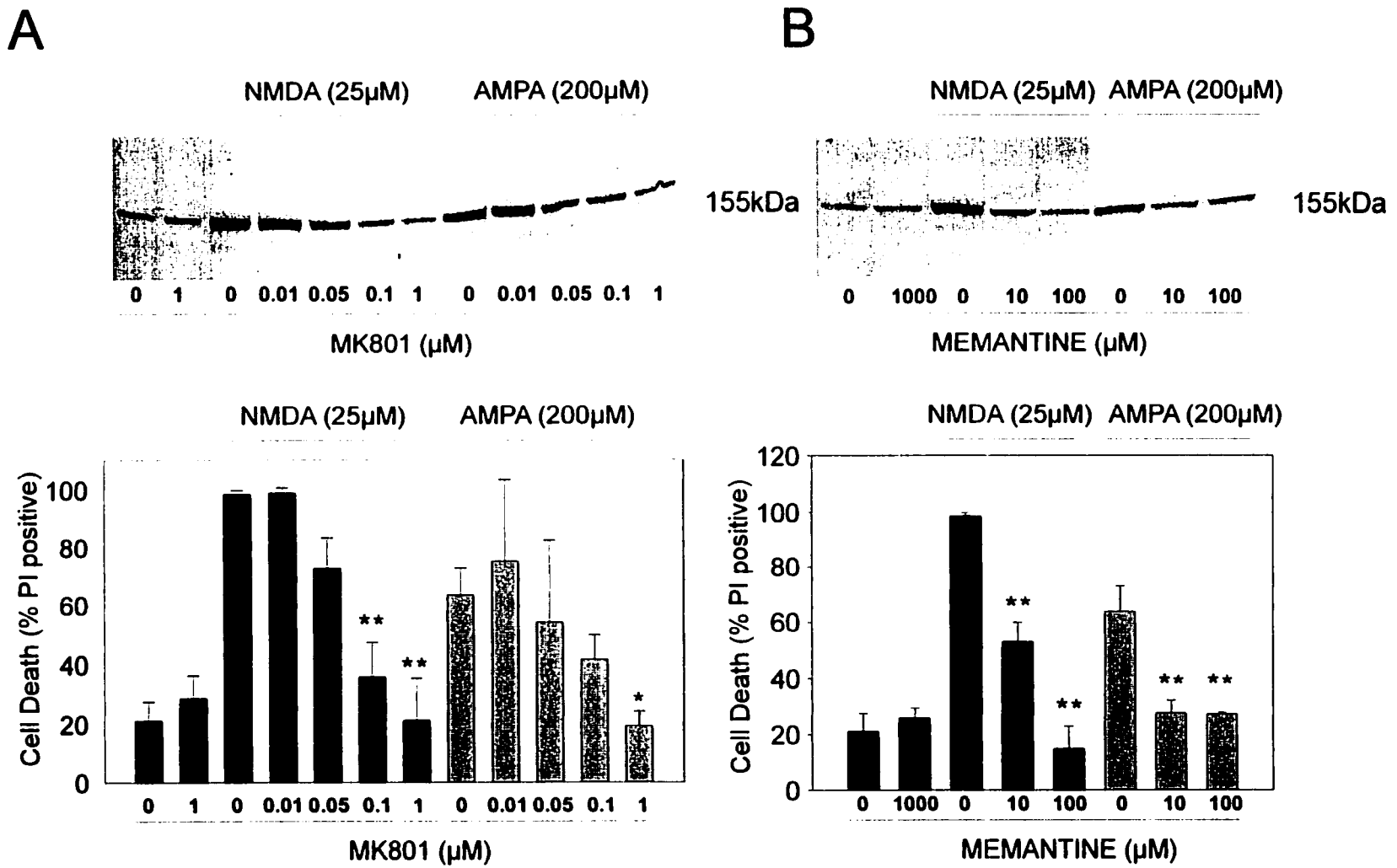
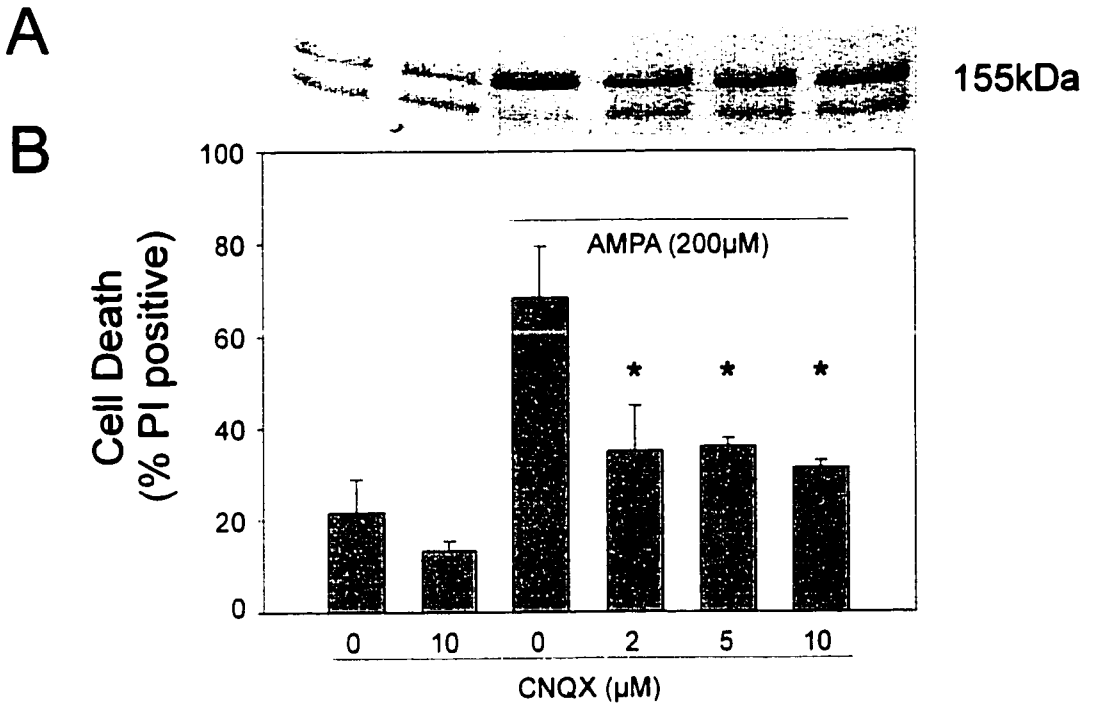

A
NMDA (25μM)
AMPA (200μM)
0 1 0 0.01 0.05 0.1 1 0 0.01 0.05 0.1 1
MK801 (μM)
B
NMDA (25μM) AMPA (200μM)
155kDa
155kDa
0 1000 0 10 100 0 10 100
MEMANTINE (μM)
NMDA (25μM)
AMPA (200μM)
Cell Death (% PI positive)
0 20 40 60 80 100
0 1 0 0.01 0.05 0.1 1 0 0.01 0.05 0.1 1
MK801 (μM)
NMDA (25μM) AMPA (200μM)
Cell Death (% PI positive)
0 20 40 60 80 100 120
0 1000 0 10 100 0 10 100
MEMANTINE (μM)

Figure 2.5 The effect of AMPA receptor antagonism by CNQX on **A:** AMPA and **B:** NMDA-mediated increases in calpain activity and cell death. The indicated concentrations of CNQX were co-applied to DIV 21 neuronal cultures with the receptor agonists for 15 minutes in culture media. Cell harvesting, Western blot analysis (2 hours post-treatment) and cell viability determination (24 hours post-treatment) were as described in Figure 2.1. Bars represent the mean of two separate experiments (n = 4 per experiment) $\pm$ standard deviation (* in A, significantly different from all other groups, $p < 0.01$; * in B, significantly different from non-NMDA treated groups, $p < 0.01$).


A
155kDa
B
Cell Death
(% PI positive)
100
80
60
40
20
0
AMPA (200μM)
*
*
*
0
10
0
2
5
10
CNQX (μM)

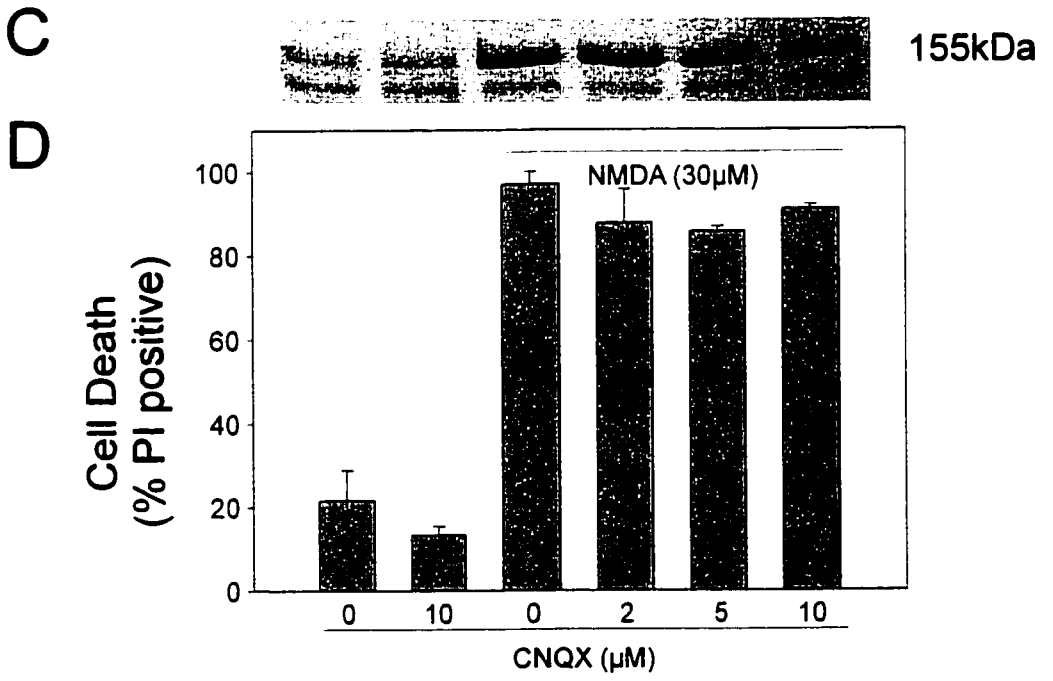

C
155kDa
D
Cell Death
(% PI positive)
100
80
60
40
20
0
NMDA (30μM)
0
10
0
2
5
10
CNQX (μM)

Figure 2.6 Effect of extracellular Na^+ on NMDA and AMPA-induced **A:** calpain activation, and **B:** cell death. DIV 21 cells were exposed for 15 minutes to NMDA (25 μM) or AMPA (200 μM) in normal CSS, or in Na^+-free CSS containing ChCl. Cells were then washed with normal CSS, and returned to conditioned media until being harvested for Western blotting (2 hours post-treatment) or cell viability determination (24 hours post-treatment), as described in Figure 2.1. Bars represent the mean of two separate experiments (n = 4 per experiment) $\pm$ standard deviation.

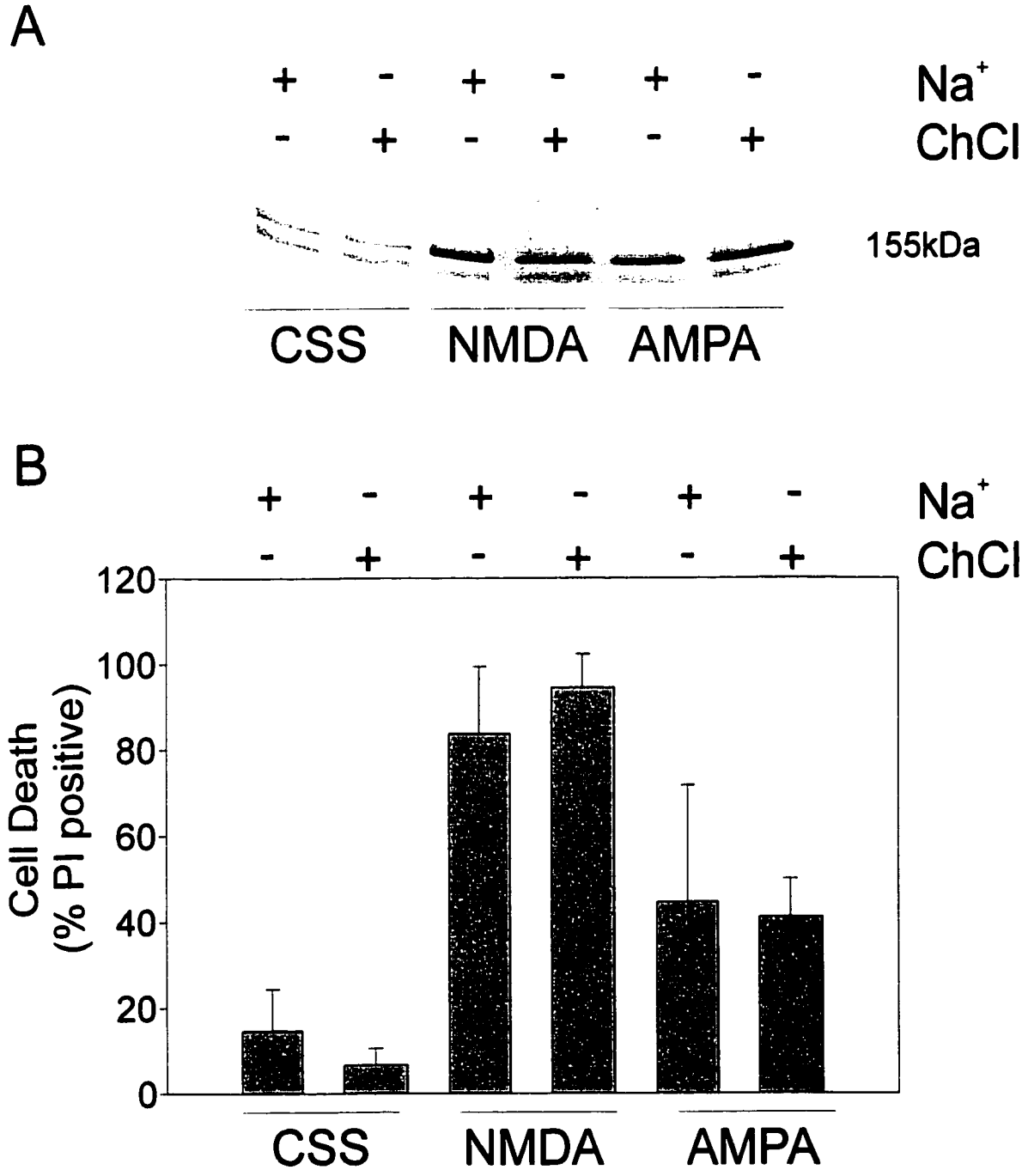
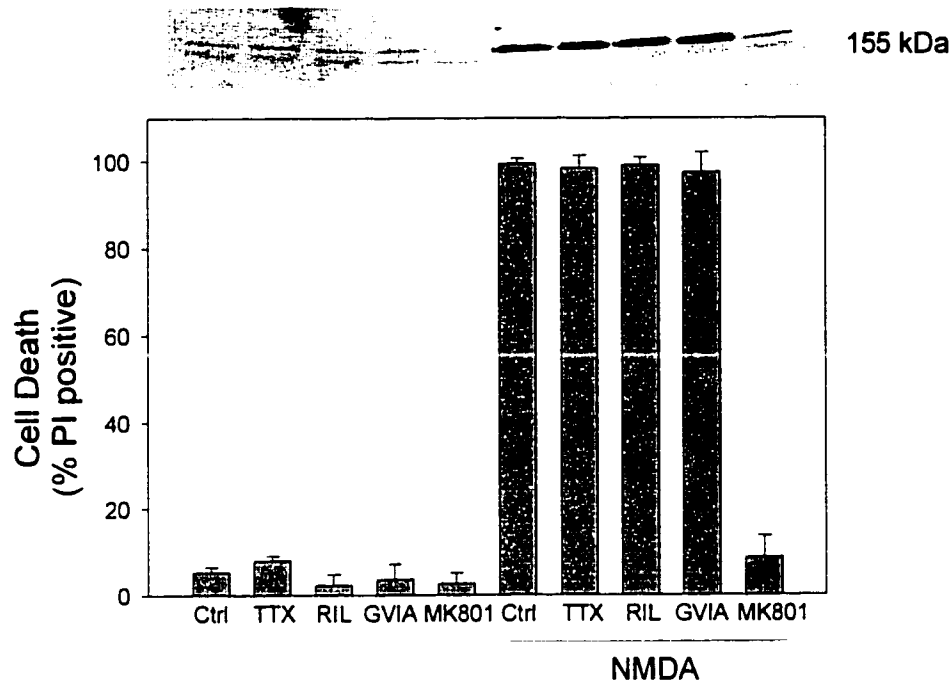

A
+ - + - + -
Na+
- + - + - +
ChCl
155kDa
CSS
NMDA
AMPA
B
+ - + - + -
Na+
- + - + - +
ChCl
120
100
80
60
40
20
0
Cell Death
(% PI positive)
CSS
NMDA
AMPA

Figure 2.7 The effect of TTX (1 μM), riluzole (20 μM), the ω-conotoxin GVIA (1 μM), and MK-801 (1 μM) on **A:** NMDA and **B:** AMPA-induced increases in calpain-mediated spectrin hydrolysis and cell death. Compounds were co-applied at the indicated concentrations with NMDA (25 μM) or AMPA (200 μM) to DIV 21 cultures in media for 15 minutes. Cell harvesting, Western blot analysis, and cell viability determinations were as described for Figure 2.1. Bars represent the mean of three separate experiemnts (n =3 per experiment) $\pm$ standard deviation.

A


155 kDa
Cell Death
(% PI positive)
100
80
60
40
20
0
Ctrl
TTX
RIL
GVIA
MK801
Ctrl
TTX
RIL
GVIA
MK801
NMDA

B

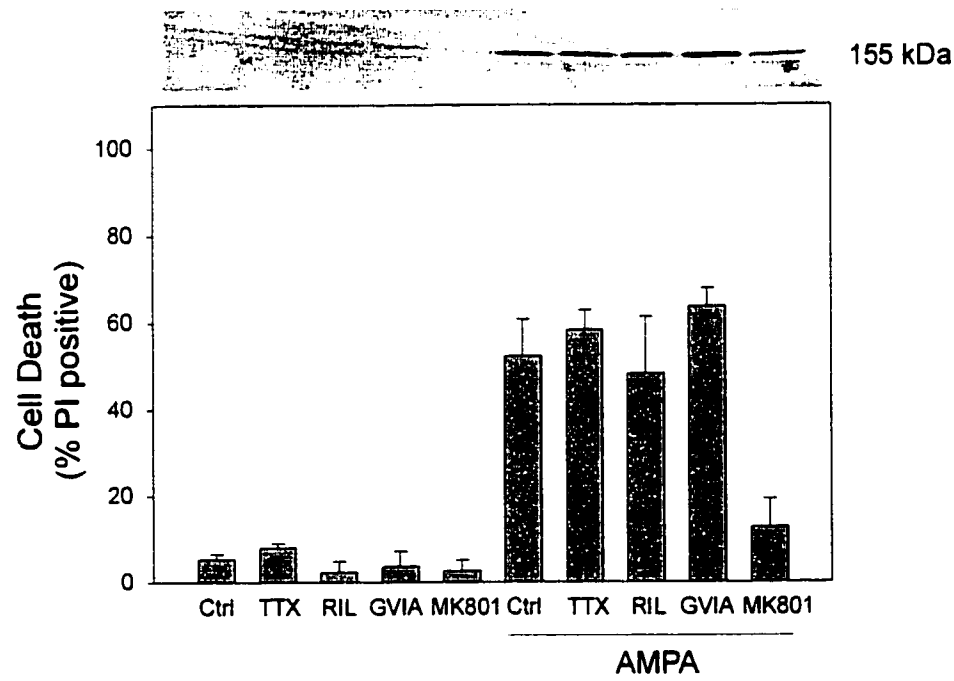
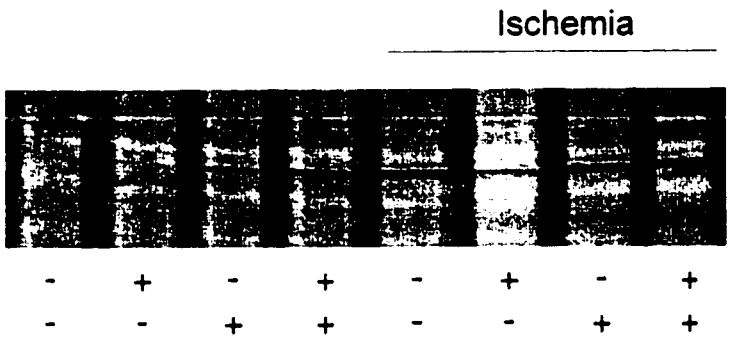

155 kDa
Cell Death
(% PI positive)
100
80
60
40
20
0
Ctrl
TTX
RIL
GVIA
MK801
Ctrl
TTX
RIL
GVIA
MK801
AMPA

Figure 2.8 The contribution of NMDA and AMPA receptors to *in vitro* "ischemia" (oxygen/glucose deprivation)-induced **A:** calpain activation and **B:** cell death. DIV 15 primary cortical cultures were subjected to oxygen/glucose deprivation for 180 minutes as described in Methods (section 2.2.2). MK-801 (5 μM) and CNQX (20 μM) were present during the insult, as indicated. Calpain-mediated spectrin hydrolysis and cell viability were determined 2 and 24 hours, respectively, following the termination of ischemic conditions (methods as described for Figure 2.1). Bars represent the mean of five separate experiments (n = 3 per experiment) $\pm$ standard deviation (* significantly different from unmarked groups, $p < 0.01$).

A


Ischemia
155 kDa
- + - + - + - +
- - + + - - + +
MK-801
CNQX

B

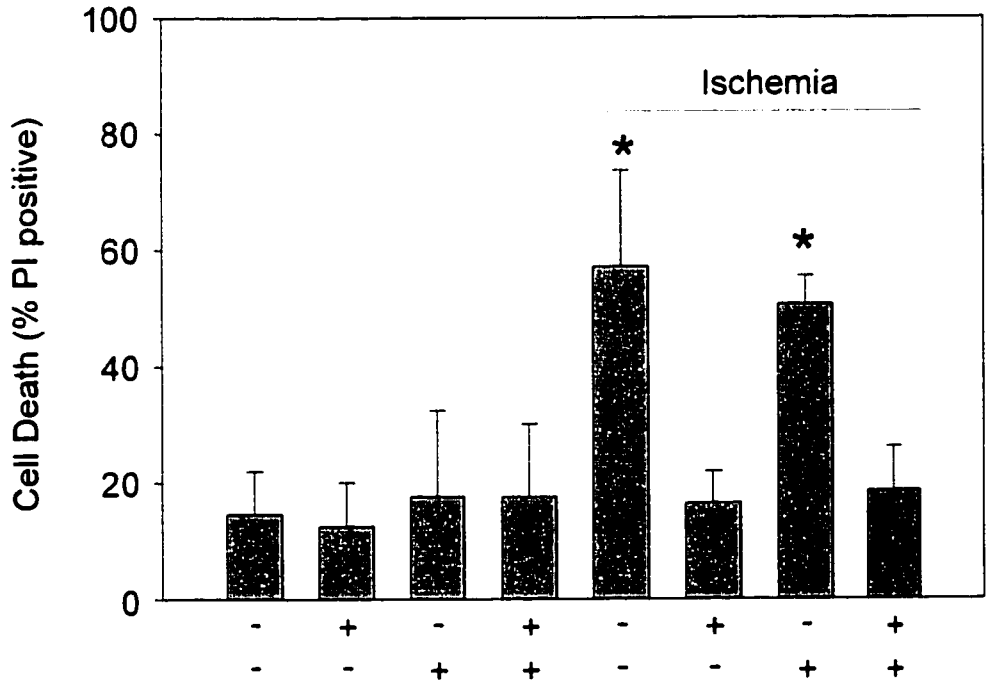
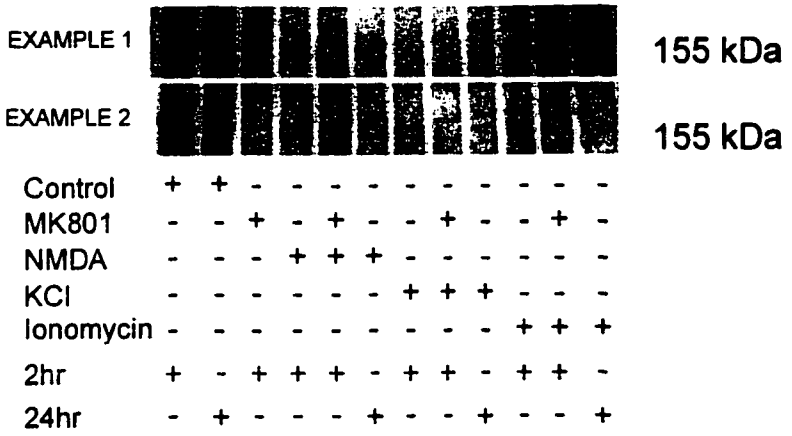
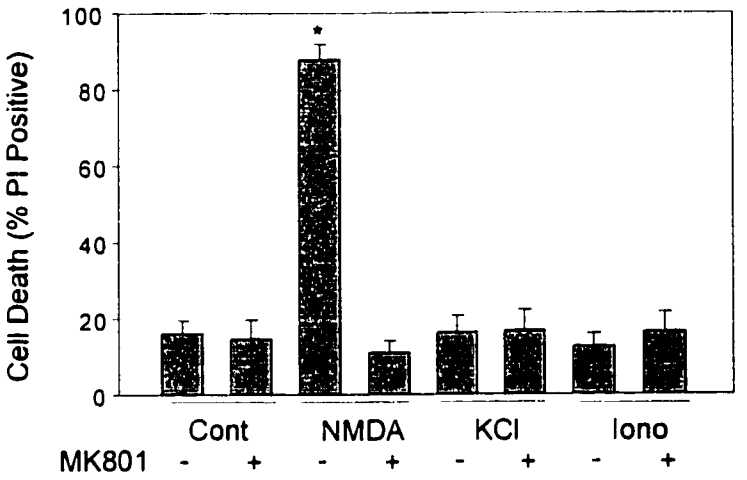

Cell Death (% PI positive)
100
80
60
40
20
0
Ischemia
*
*
- + - + - + - +
- - + + - - + +
MK-801
CNQX

Figure 2.9 The effects of a 15 min exposure to NMDA (50 μM; n = 3 experiments), ionomycin (2.5 μM; n = 2 experiments) or KCl (50mM; n = 3 experiments) ± MK801 (20 μM) on **A:** spectrin proteolysis measured by Western blot analysis 2 and 24 hours later using the Cal-SBDP antibody (section 1.2.4); **B:** cell viability measured at 24 hours (section 2.1.3), and **C:** changes in $[Ca^{2+}]_i$ determined as described in Methods (section 2.2.4). All treatments were carried out in Mg^{2+}-free CSS salt solution (120 mM NaCl, 5.4 mM KCl, 0.8 mM $CaCl_2$, 15 mM glucose, and 25 mM Tris, pH 7.4), with cells returned to their conditioned media prior to spectrin breakdown determination and cell viability. Control cultures were incubated for 15 min in CSS alone. Bars represent the overall means from the indicated number of experiments ± standard deviation (* significantly different from all other groups; $p < 0.01$).

A


EXAMPLE 1
155 kDa
EXAMPLE 2
155 kDa
Control + + - - - - - - - - - -
MK801 - - + - + - - + - - + -
NMDA - - - + + + - - - - - -
KCl - - - - - - + + + - - -
Ionomycin - - - - - - - - - + + +
2hr + - + + + - + + - + + -
24hr - + - - - + - - + - - +

B


Cell Death (% PI Positive)
100
80
60
40
20
0
*
Cont
NMDA
KCl
Iono
MK801 - + - + - + - +

C

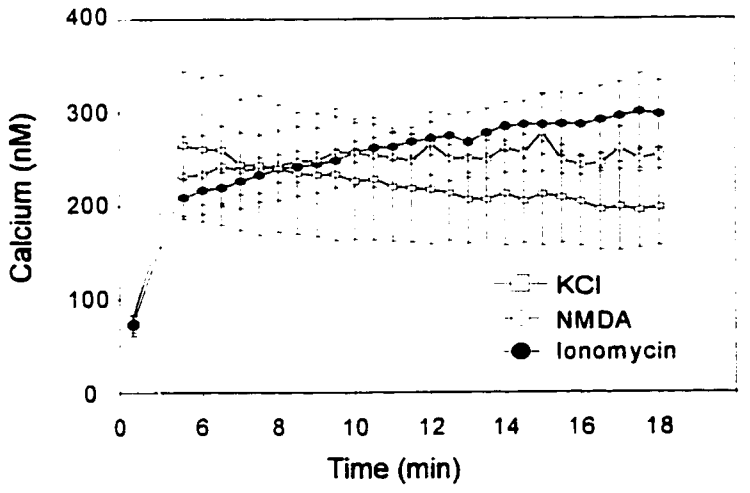

Calcium (nM)
400
300
200
100
0
KCl
NMDA
Ionomycin
0 6 8 10 12 14 16 18
Time (min)

Figure 2.10 The ability of the calpain inhibitor, calpeptin, to attenuate spectrin hydrolysis and cell death in neurons treated with NMDA. **A:** Representative Western blot of samples treated with NMDA (50 μM) and the indicated concentrations of calpeptin, probed with the Cal-SBDP antibody, as indicated in Methods (section 1.2.4). **B:** Cell viability determination (as described in section 2.1.3) of cultures (n = 9, 3 separate experiments) treated with NMDA and calpeptin as in A. Calpeptin was applied one hour prior to NMDA application, and remained in the culture media until time of harvesting for Western blot analysis (2 hours post-NMDA treatment) and cell viability determination (24 hours post-NMDA treatment). Bars represent the mean $\pm$ standard deviation (* significantly different from NMDA alone, $p < 0.05$).

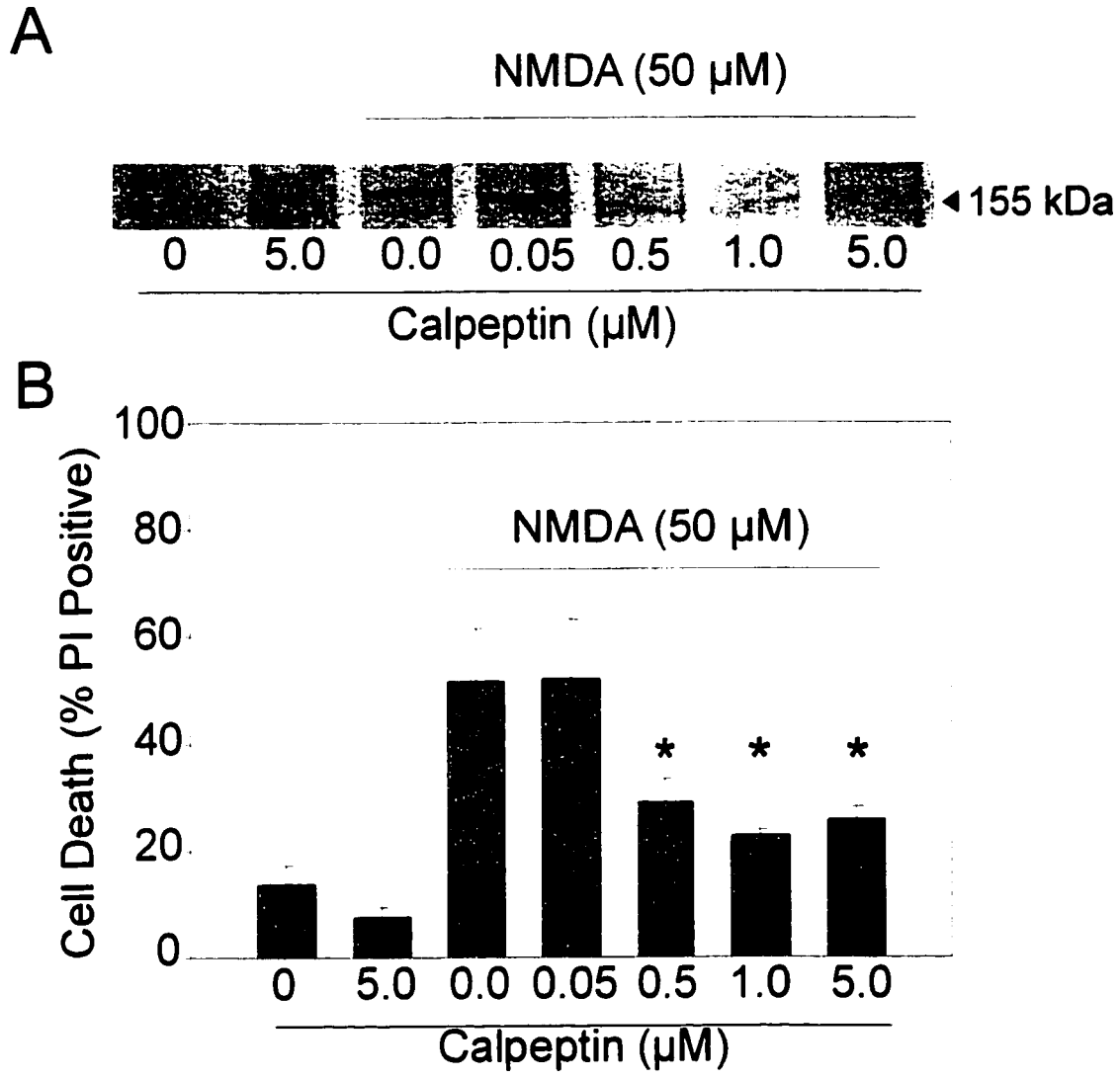
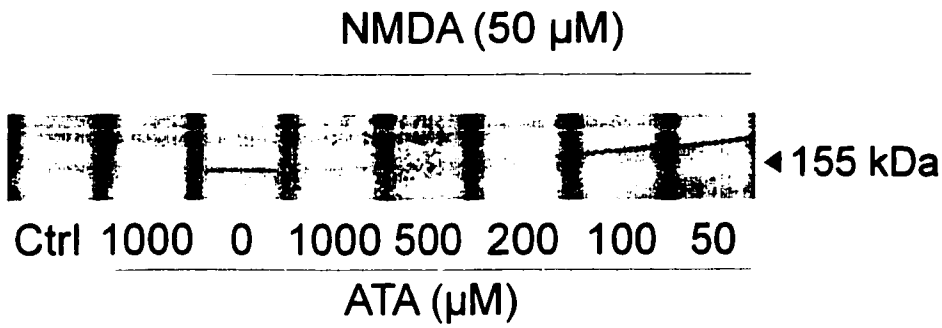

A
NMDA (50 μM)
155 kDa
0
5.0
0.0
0.05
0.5
1.0
5.0
Calpeptin (μM)
B
Cell Death (% PI Positive)
100
80
60
40
20
0
NMDA (50 μM)
*
*
*
0
5.0
0.0
0.05
0.5
1.0
5.0
Calpeptin (μM)

Figure 2.11 The ability of the calpain inhibitor, ATA, to attenuate spectrin hydrolysis and cell death in neurons treated with NMDA. **A:** Representative Western blot of samples treated with NMDA (50 μM) and the indicated concentrations of ATA, probed with the Cal-SBDP antibody, as indicated in Methods (section 1.2.4). **B:** Cell viability determination (see section 2.1.3) of cultures (n = 4 separate experiments, 3 plates per treatment) treated with NMDA and ATA as in A. As with all calpain inhibitor experiments, ATA was applied one hour prior to NMDA application, and remained in the culture media until time for harvesting for Western blot analysis (2 hours post-NMDA treatment) and cell viability determination (24 hours post-NMDA). Bars represent the mean $\pm$ standard deviation (* significantly different from NMDA alone, $p < 0.05$).

A


NMDA (50 μM)
155 kDa
Ctrl 1000 0 1000 500 200 100 50
ATA (μM)

B

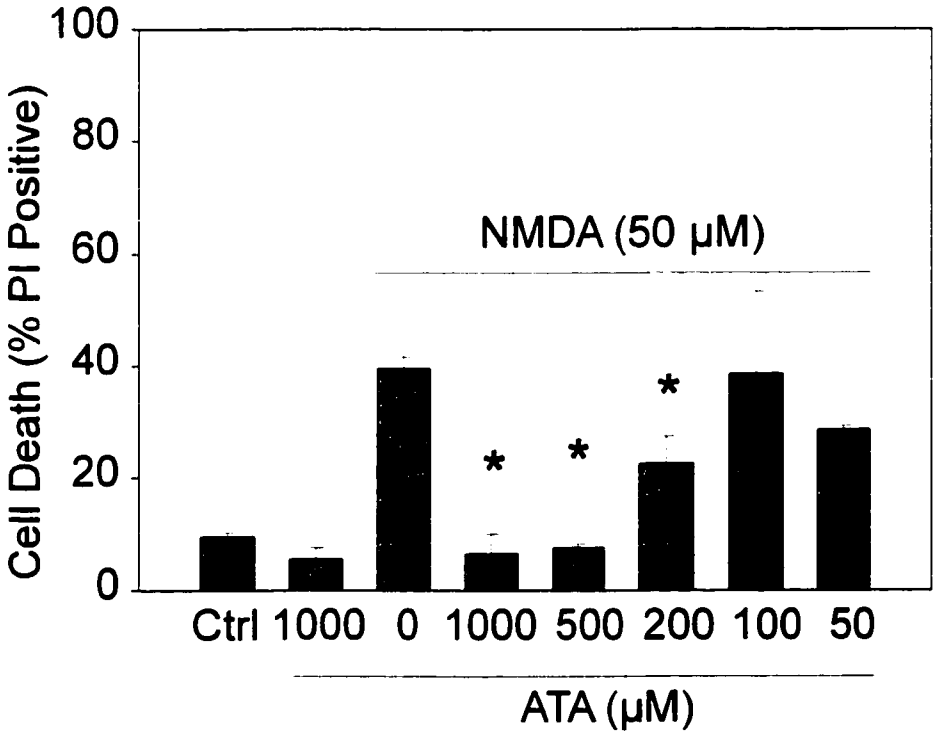
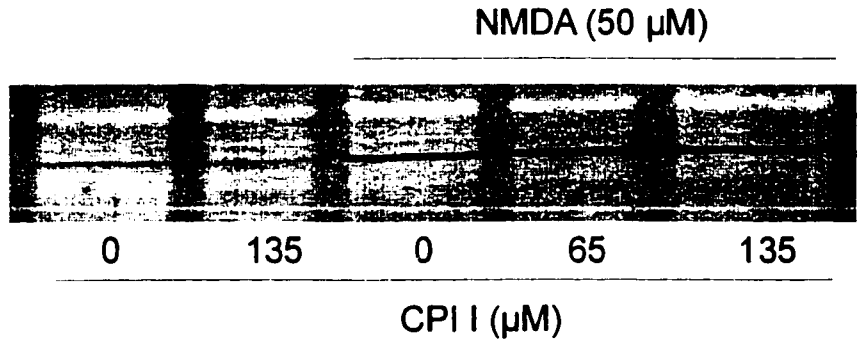

Cell Death (% PI Positive)
100
80
60
40
20
0
NMDA (50 μM)
*
*
*
Ctrl 1000 0 1000 500 200 100 50
ATA (μM)

Figure 2.12 The ability of the calpain inhibitor, calpain inhibitor I (CPI I), to attenuate spectrin hydrolysis and cell death in neurons treated with NMDA. **A:** Representative Western blot of samples treated with NMDA (50 μM) and the indicated concentrations of CPI I, probed with the Cal-SBDP antibody, as indicated in Methods (section 1.2.4). **B:** Cell viability determination (see section 2.1.3) of cultures (3 separate experiments, n = 9) treated with NMDA and CPI I as in A. CPI I was applied one hour prior to NMDA application, and remained in the culture media until time of harvesting for Western blot analysis (2 hours post-NMDA treatment) and cell viability determination (24 hours post-NMDA treatment). Bars represent the mean $\pm$ standard deviation.

A


NMDA (50 μM)
155 kDa
0
135
0
65
135
CPI I (μM)

B

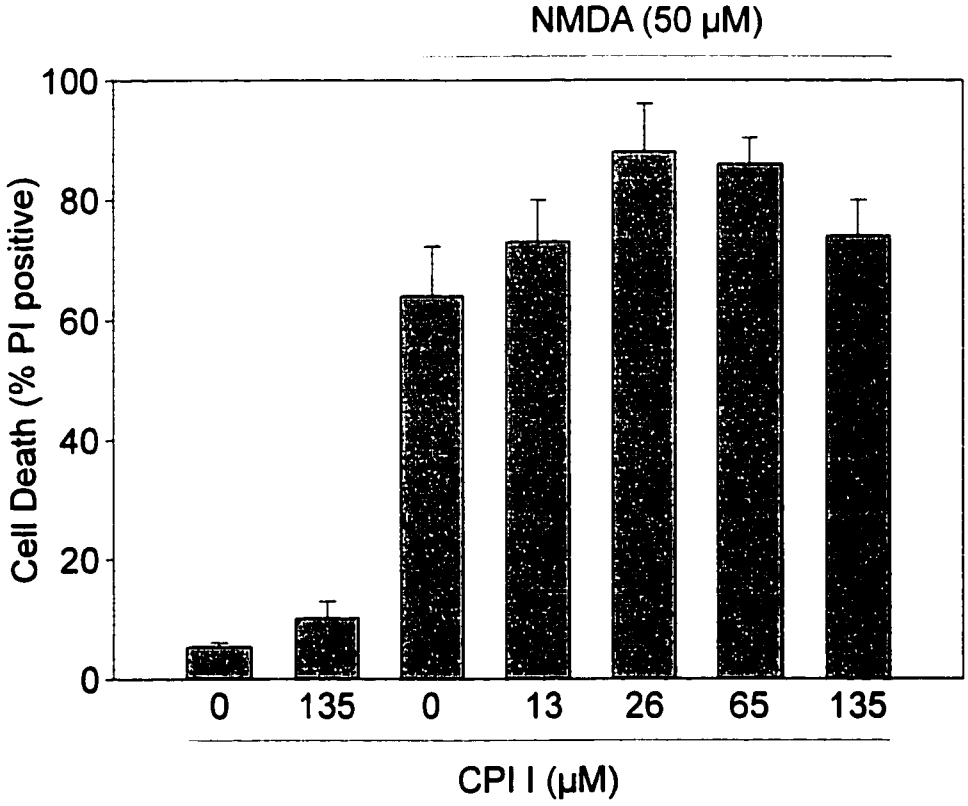

NMDA (50 μM)
Cell Death (% PI positive)
100
80
60
40
20
0
0
135
0
13
26
65
135
CPI I (μM)

Figure 2.13 The ability of the α-keto amide calpain inhibitor, AK275, to attenuate spectrin hydrolysis and cell death in neurons treated with NMDA. **A:** Representative Western blot of samples treated with NMDA (50 μM) and the indicated concentrations of AK275, probed with the Cal-SBDP antibody, as indicated in Methods (section 1.2.4). **B:** Cell viability determination (see section 2.1.3) of cultures (3 separate experiments, n = 9) treated with NMDA and AK275 as in A. AK275 was applied one hour prior to NMDA application, and remained in the culture media until time for harvesting for Western blot analysis (2 hours post-NMDA treatment) and cell viability determination (24 hours post-NMDA treatment). Bars represent the mean ± standard deviation.

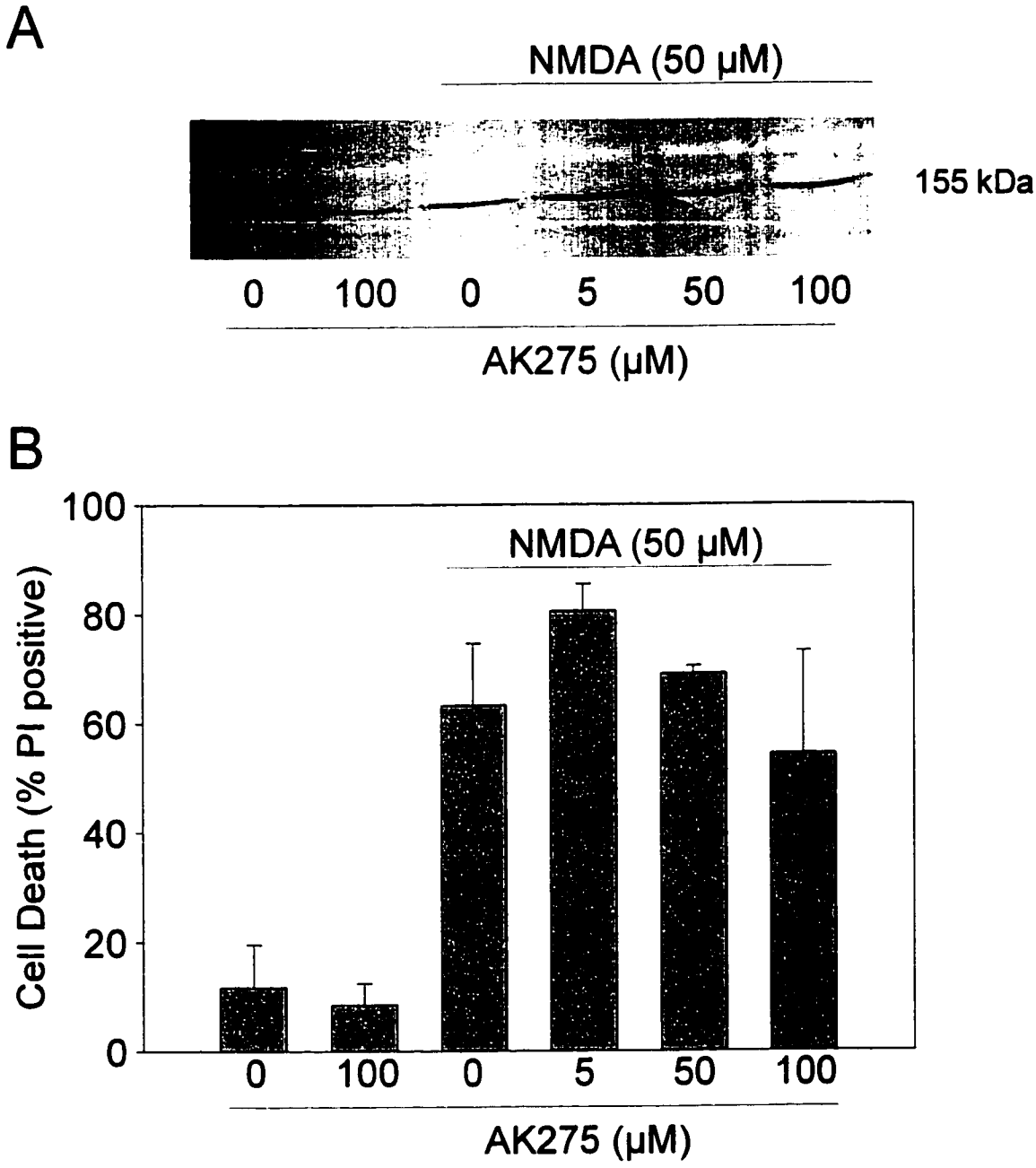

A
NMDA (50 μM)
155 kDa
0
100
0
5
50
100
AK275 (μM)
B
Cell Death (% PI positive)
100
80
60
40
20
0
NMDA (50 μM)
0
100
0
5
50
100
AK275 (μM)

Figure 2.14 The ability of the α-keto amide calpain inhibitor, AK295, to attenuate spectrin hydrolysis and cell death in neurons treated with NMDA. **A:** Representative Western blot of samples treated with NMDA (50 μM) and the indicated concentrations of AK295, probed with the Cal-SBDP antibody, as indicated in Methods (section 1.2.4). **B:** Cell viability determination (as described in section 2.1.3) of cultures (2 separate experiments, n = 8) treated with NMDA and AK295 as in A. AK295 was applied one hour prior to NMDA application, and remained in the culture media until time for harvesting for Western blot analysis (2 hours post-NMDA treatment) and cell viability determination (24 hours post-NMDA treatment). Bars represent the mean ± standard deviation.

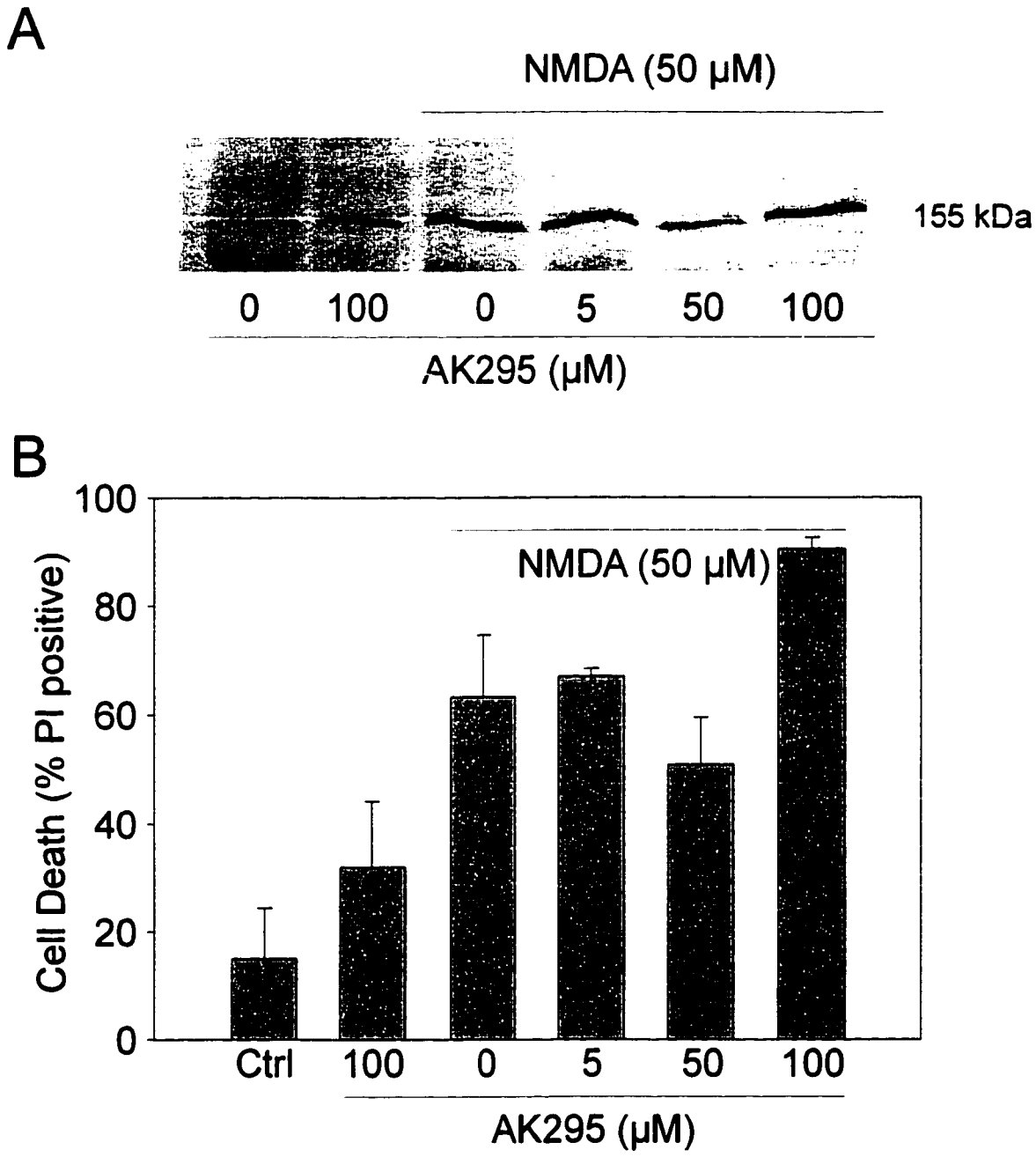

A
NMDA (50 μM)
155 kDa
0
100
0
5
50
100
AK295 (μM)
B
Cell Death (% PI positive)
100
80
60
40
20
0
NMDA (50 μM)
Ctrl
100
0
5
50
100
AK295 (μM)

Figure 2.15 The effect of increasing concentrations of NMDA on the ability of EST to attenuate calpain activation (2 hours post-NMDA treatment) and cell death (24 hours post-NMDA treatment). **A:** Representative Western blots probed with the Cal-SBDP antibody demonstrating the effect of EST (50 μM) on calpain-mediated spectrin hydrolysis produced by 50, 75, and 100 μM NMDA. Cells were treated in culture media, and contained EST and NMDA as indicated. As with other calpain inhibitors, EST was pre-applied for one hour prior to NMDA application, and was present for the entire duration of the experiments. Cell lysis and Western blot analysis were as described in sections 1.2.2.2 and 1.2.4 of Methods, respectively. **B:** Cell viability determination (as described in section 2.1.3) in cultures treated as in A. Bars represent the means of two separate experiments (n = 8) $\pm$ standard deviation. (* significantly different from NMDA-treated counterpart, $p < 0.01$).

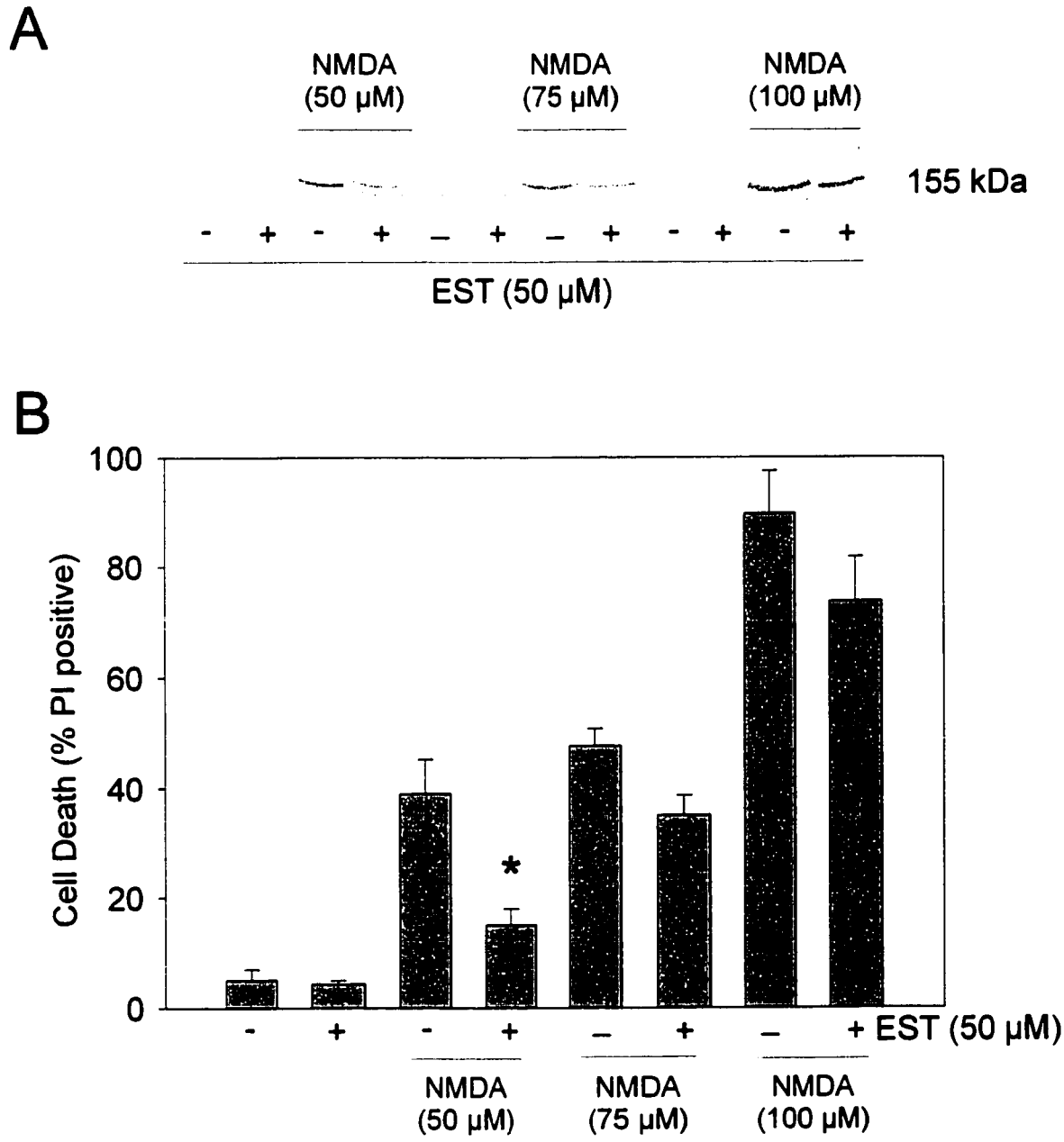
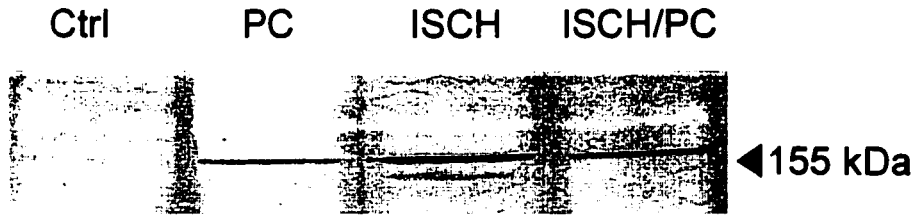

A
NMDA
(50 μM)
NMDA
(75 μM)
NMDA
(100 μM)
155 kDa
- + - + – + – + - + - +
EST (50 μM)
B
Cell Death (% PI positive)
100
80
60
40
20
0
*
- + - + – + – + EST (50 μM)
NMDA
(50 μM)
NMDA
(75 μM)
NMDA
(100 μM)

Figure 2.16 The effect of ischemic preconditioning (PC) on **A:** calpain activation (measured 2 hours post-oxygen/glucose deprivation) and **B:** cell death (24 hours post) induced by *in vitro* oxygen/glucose deprivation (ISCH). Oxygen/glucose deprivation and ischemic preconditioning were carried out as described in Methods (section 2.2.2). Cell harvesting, Western blot analysis, and cell viability determination were also as described in Figure 2.1 Bars represent the mean of 5 separate experiments (n = 3 per experiment) $\pm$ standard deviation.

A


Ctrl
PC
ISCH
ISCH/PC
155 kDa

B

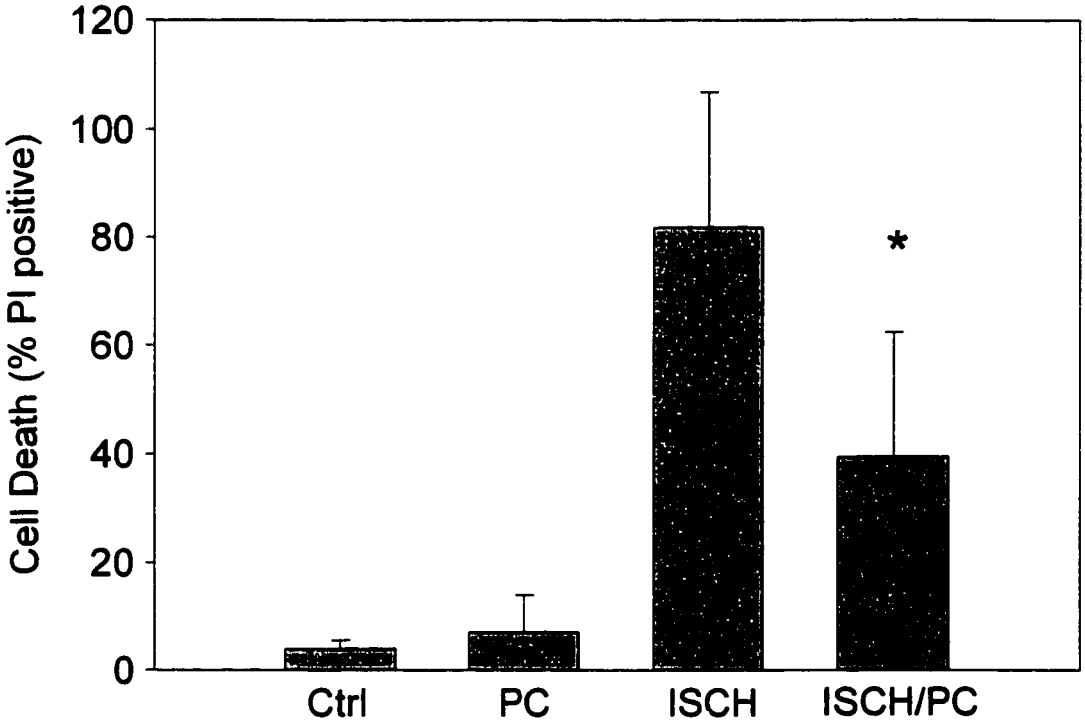
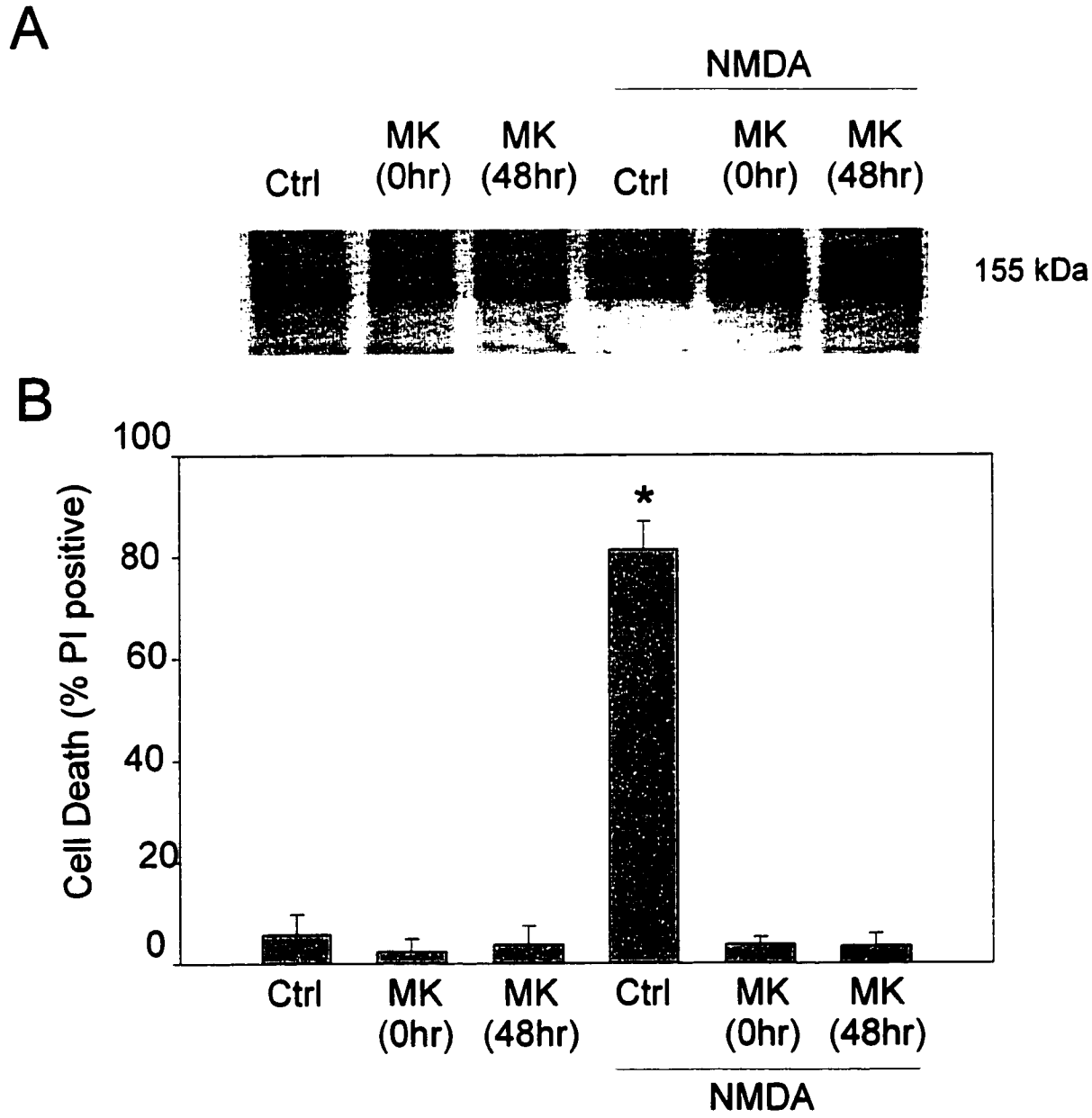

Cell Death (% PI positive)
120
100
80
60
40
20
0
*
Ctrl
PC
ISCH
ISCH/PC

Figure 2.17 The effect of preconditioning mediated by a 30 minute MK-801 pretreatment 48 hours prior to lethal NMDA exposure on **A:** calpain activation and **B:** cell death. Forty-eight hours after a 30 minute pretreatment with 1 μM MK-801 (MK 48hr), cells were treated with 50 μM NMDA for 5 minutes in conditioned media. Following treatment, cells were washed with CSS, and returned to normal conditioned media until cell harvesting and Western blot analysis 2 hours post-NMDA (as described in Methods sections 1.2.2.2 and 1.2.4), or cell viability determination 24 hours post-NMDA treatment (section 2.1.3). The effects of the same concentration of MK-801 co-applied with NMDA (MK 0hr) were also examined for comparison purposes. Results in A are representative of four separate experiments. Bars in B represent the mean cell viability of four separate experiments (n = 3 per experiment) $\pm$ standard deviation. (* $p < 0.01$, significantly different from all other groups).


A
NMDA
Ctrl
MK (0hr)
MK (48hr)
Ctrl
MK (0hr)
MK (48hr)
155 kDa
B
Cell Death (% PI positive)
100
80
60
40
20
0
*
Ctrl
MK (0hr)
MK (48hr)
Ctrl
MK (0hr)
MK (48hr)
NMDA

Chapter 3. Regulation of Calpain by Interactions with PKC

3.1 Background

Understanding the essential biochemical processes responsible for neuronal cell death following glutamate-receptor hyperactivation has become the quintessential goal of much research striving to advance pharmaceutical therapies for brain damage due to stroke or related illnesses. As outlined in the General Introduction, it is generally acknowledged that a complex interplay of signaling effectors ensues following toxic events such as cerebral ischemia. By selectively intervening in essential upstream biochemical events or their downstream targets, it is believed therapies can be developed to ameliorate presently irreversible brain damage. In order to meet these goals, a clearer understanding of the critical cell signaling events mediating cell death and survival pathways is required.

One essential signaling step in the biochemical cascade leading to neuronal cell death following glutamate-mediated toxicity appears to involve the loss of PKC activity. The inactivation of PKC following excitotoxic or ischemic insults has been extensively characterized. This loss of kinase activity appears to be a pre-death event (Busto et al., 1994; Murphy et al., 1994; Cardell and Wieloch, 1993; Yamaoka et al. 1993; Domanska-Janik and Zalewska, 1992; Cardell et al., 1990, 1991; Louis et al., 1991; Wieloch et al., 1991; Crumrine et al., 1990; Zivin et al., 1990; Louis et al., 1988); one that is a necessary step in the excitotoxic death process (Durkin et al., 1996, 1997). The functional significance of this loss in kinase activity will be expressed by its downstream targets,

and therefore defining the kinase substrates that are aberrantly affected by a loss of PKC activity will advance the understanding of toxic signaling events.

Previous examination of the loss of PKC activity following glutamate or glutamate-analogue toxicity in primary neuronal cultures showed that the attenuation did not result from hydrolysis of the kinase, despite the fact that calpain is activated following excitotoxic insults (Wang et al., 1996a; Bednarski et al., 1995; del Cerro et al., 1994; Caner et al., 1993; Roberts-Lewis and Siman, 1993; Siman et al., 1989; Siman and Noszek, 1988), and that PKC is a potential substrate of the protease (Tanabe et al., 1998; Dwyer-Nield et al., 1996; Shea et al., 1995, 1996; Hong et al., 1995; Patel et al., 1994; Al and Cohen, 1993; Savart et al., 1992; Croall and Demartino, 1991; Adachi et al., 1990; Young et al., 1987; Kajikawa et al., 1983; Kishimoto et al., 1983). Rather than undergoing calpain-mediated hydrolysis, the loss in kinase activity in primary cortical cells and neuronal tissue is reversible and may involve the actions of an intrinsic inhibitory factor. Recently, a membrane-bound PKC supressor protein has been identified in N1E-115 neuroblastoma cells which appears necessary for PKC suppression and the induction of differentiation (Chakravarthy et al., 1995). Whether the actions of this or a related suppressor protein is involved in glutamate-receptor-induced inactivation of PKC remains to be determined. Nonetheless, suppression of PKC is a reversible event (Durkin et al., 1996), suggesting that irreversible hydrolysis is not responsible for the observed changes in PKC.

The loss of PKC under these toxic conditions is however, specifically mediated through Ca^{2+} entry coupled to glutamate receptor activation, and equivalent increases in

$[Ca^{2+}]_i$ induced by ionomycin or KCl-depolarization are not sufficient to induce a loss of PKC activity or cell death (Durkin et al., 1996). This Ca^{2+} specificity of PKC inactivation is remarkably similar to that described for calpain activation (see Chapter 2), and suggests that both calpain activation and the loss of PKC activity are coupled to the same Ca^{2+}-signaling pathway responsible for the death response of primary cortical neurons following NMDA treatment. The question as to whether these two cellular events occur independently, or whether they are sequentially linked, has not been previously addressed under conditions of excitotoxicity.

The results of the previous chapter also suggest that calpain activation requires additional modulatory factors associated with NMDA receptor activation since generalized increases in Ca^{2+} (via ionomycin or KCl) were unable to activate the protease. This Ca^{2+} selectivity of calpain in neurons contrasts sharply with that reported for the protease in non-neuronal cells. Typically, non-specific increases in Ca^{2+} mediated by ionophore application are sufficient to induce protease activation, as measured by translocation and autolysis, susceptibility to calpain inhibitors, and/or substrate hydrolysis (Chan et al., 1998; Falet et al., 1998; Guttmann and Johnson, 1998; Pasquet et al., 1998; Stewart et al., 1998; Walowitz et al., 1998; DePetrillo, 1997; Fujitani et al., 1997; Fukiage et al., 1997; Rock et al., 1997; Shea et al., 1997; Michetti et al., 1996; Sanderson et al., 1996; Wang et al., 1996a; Molinari et al., 1994), and in some cases cell death (Schnellmann and Williams, 1998; Edelstein et al., 1997; Waters et al., 1997). These differences indicate that calpain activation is a more regulated and complex process in neuronal cells. It further suggests that calpain is directly involved in cell

signaling processes emanating from NMDA receptor activation, implying a functional role for the protease during NMDA-mediated signaling events under both physiological and pathological conditions.

In this chapter we address the translocation and autolytic profile of calpain in neuronal and non-neuronal cells (erythrocytes) in response to ionophores, as well as the effects of NMDA on these parameters in neurons. Furthermore, we have examined the interaction between NMDA receptor mediated increases in $[Ca^{2+}]_i$, the loss of PKC activity, and calpain activation. We have shown that calpain possesses unique localization and regulatory properties not present in non-neuronal cells. In addition, we have demonstrated an essential link between the NMDA-mediated loss of PKC activity and calpain activation, suggesting that calpain activity is modulated by a complex interaction of events stemming from NMDA receptor activity.

3.2 METHODS

3.2.1 Determination of Calpain Localization and Autolysis

The localization and autolysis of calpain was compared in rat erythrocytes and primary neuronal cultures using a modification of the method of Molinari et al. (1994). Briefly, rat erythrocytes from female Sprague-Dawley rats (Charles River) were obtained from freshly collected blood by centrifugation. Cells were washed twice with 10 mM HEPES buffer containing 140 mM NaCl, 5 mM KCl, 2 mM EDTA, pH 7.3 (Buffer A), and three times with the same buffer without EDTA (Buffer B). The pelleted cells were

resuspended in 10 mM HEPES, 140 mM NaCl, 5 mM KCl-buffer containing 3 mM $MgCl_2$ (Buffer C). Primary neurons were also washed with Buffer B, as above. Both erythrocytes and cortical cells were treated for 15 and 60 minutes with 2.5 μM ionomycin and 100 μM $CaCl_2$, at 37°C, or maintained in Buffer C alone. Following the incubation, cells were lysed in a hypotonic lysis buffer (10 mM HEPES, pH 7.3) and membrane and cytosolic fractions separated by centrifugation. The resulting fractions were subjected to 8% SDS-PAGE, transblotted, and probed with a monoclonal antibody against the ubiquitous form of μ-calpain (supplied by Dr. John Elce, Queen's University, Kingston, Ontario). In addition, primary neurons were also transiently exposed (5 min) to NMDA (50 μM) in conditioned media, washed, and subjected to hypotonic lysis as above.

3.2.2 BDNF Neuroprotection Assays

Primary neuronal cultures were prepared as described (section 1.2.2.1). All treatments were carried out in conditioned media. Neurons (15-18 DIV) were pretreated with 50 ng BDNF (Alomone Labs, Jerusalem, Israel) overnight in 1 ml of conditioned media prior to a 5 min exposure to NMDA (50 μM). Following NMDA treatment, the culture media was removed, and prior to a return to normal conditioned media, the cells were washed with 1 ml CSS. Two hours post-NMDA treatment cells were harvested for Western blot analysis as described (section 1.2.4). Twenty-four hours post-NMDA treatment, sister cultures were used for viability assessment using PI exclusion (method as described in section 2.1.3). In some instances, the PKC inhibitors staurosporine (stauro, 50 nM), GF-109203X (GF-X, 100 nM), or calphostin C (Cal-C, 250 nM) were

co-applied with NMDA where indicated.

3.2.3 Calcium Determination with Fura-2

$[Ca^{2+}]_i$ was determined by using the Ca^{2+}-sensitive indicator Fura-2 (Molecular Probes Inc., Eugene, OR) in cultures plated on glass coverslips (otherwise cell preparation was as described in section 1.2.2.1). Neurons were pre-loaded with Fura-2 by incubating them for 30 minutes at 37°C in a Mg^{2+}-free CSS salt solution (120 mM NaCl, 5.4 mM KCl, 0.8 mM $CaCl_2$, 15 mM glucose, buffered with 25 mM Tris, pH 7.4), containing 2.5 μM Fura-2/AM. After incubation, cells were washed three times and experiments conducted at room temperature, on groups of 5-15 cells per field. $[Ca^{2+}]_i$ was assessed by determining the ratio of the fluorescence intensities of Fura-2 emission as described previously (Black et al., 1995). Fluorescence recordings were obtained every 10 seconds for approximately three minutes before the application of NMDA (50 μM) in cells with (n = 3) or without (n = 3) BDNF pretreatment. Following NMDA application, $[Ca^{2+}]_i$ was determined every 10 seconds for 10 minutes.

3.2.4 *In vitro* PKC-Dependent Phosphorylation Assay

This assay was conducted using a purified rat brain PKC extract (supplied by Dr. B. Chakravarthy, Institute for Biological Sciences, NRC) to examine the ability of PKC to phosphorylate spectrin. PKC activity was measured as described by Chakravarthy et al. (1991) in a reaction mixture (100μl) containing 10 μl of purified PKC (1.3 μg of protein),

50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 2 mM $CaCl_2$, 10 μg phosphatidylserine, 1 μM PMA, 100 μM sodium vanadate, 100 μM sodium pyrophosphate, 1 mM sodium fluoride, 100 μM PMSF (phenylmethylsulfonyl fluoride), 50 μM [^{32}P]ATP (220 cpm/pmol) and either 75μM PKC-selective peptide substrate (MARCKS peptide) (Fujise et al., 1994) or 5 μg purified erythrocyte spectrin (Sigma Chemical Co.) as a substrate for PKC. After incubation for 10 or 30 minutes at 25°C, the reaction was stopped by adding 10 μl of 5% acetic acid, and the samples were clarified by centrifugation at 16,000 g for 5 minutes in a microfuge. A 45 μl sample of each supernatant was applied to P81 Whatman paper (1cm^2) and the paper was washed twice for 10 minutes in 5% acetic acid (10ml/cm^2 of paper). The radioactivity bound to the paper was then determined by liquid scintillation counting. All reactions were carried out in triplicate.

3.2.5 Immunoblotting

Western blot analysis was carried out as described in Methods (section 1.2.4). For spectrin hydrolysis the Cal-SBDP antibody was used at a concentration of 1:1000. For calpastatin, a monoclonal antibody (Affinity Bioreagents, Inc., Golden, CO) was used, which purportedly recognizes four immunologically reactive species of calpastatin in immunoblots with apparent molecular weights of 150, 125, 90 and 70 kDa. Analysis of the calpastatin protein was carried out with 12% SDS-PAGE, using an alkaline phosphastase-conjugated anti-mouse IgG secondary (Sigma Chemical Co.). Standard prestained molecular weight markers were used to determine apparent molecular weights

of immunoreactive proteins.

3.3 RESULTS

3.3.1 Calpain translocation and autolysis

The characteristics of μ-calpain activation in primary cortical neurons were compared to that occurring in rat erythrocytes under identical treatment conditions. The effects of ionomycin on calpain localization, translocation and autolysis were examined under conditions previously shown to cause definitive translocation and autolysis of the protease in erythrocytes (Molinari et al., 1994). As shown in Figure 3.1A, calpain was localized mainly in the cytosol of rat erythrocytes, where it underwent translocation to membranes and autolysis to a 76 kDa fragment in the presence of 2.5 μM ionomycin and 100 μM Ca^{2+}. By contrast, the majority of calpain in primary cortical neurons was detected in the membrane fraction under non-stimulating conditions, and ionomycin was unable to induce translocation or detectable autolysis of the protease in cortical cells (Figure 3.1A). Moreover, the transient application of 50 μM NMDA was also unable to induce detectable translocation or autolysis of ubiquitous μ-calpain in cortical cells (Figure 3.1A).

When samples exposed to the same experimental conditions used in Figure 3.1A were probed with the Cal-SBDP antibody (neurons) or a commercial spectrin antibody (240/235E; Chemicon) (erythrocytes), calpain-mediated spectrin hydrolysis was evident in both cell types, dependent on the treatment conditions. As shown in Figure 3.1B, and

consistent with that previously reported by Croall et al. (1986), ionomycin substantially increased spectrin proteolysis in erythrocytes resulting in the production of several lower molecular weight fragments ranging from 210 kDa to 43 kDa daltons, including a 155 kDa product. This complex degradation of erythrocyte spectrin was initiated by calpain activation since it was largely reversed in the presence of the selective calpain inhibitor, calpeptin (0.5 μM). In contrast, ionomycin did not induce detectable spectrin hydrolysis in cortical neurons, a finding consistent with the results in Figure 3.1A using the calpain antibody. However, despite the fact that 50 μM NMDA did not induce an apparent increase in calpain translocation or autolysis in cortical neurons, it did cause a substantial increase in spectrin breakdown as measured by the appearance of the calpain-specific 155 kDa band. Relative to the effects of ionomycin on spectrin in erythrocytes, NMDA-mediated fragmentation of spectrin was largely restricted. This limited proteolysis was evident in experiments in which immunoblots were probed with the same 240/235E antibody used in Figure 3.1B for erythrocytes (see Figure 1.5). These results demonstrate that even though ionomycin caused a substantial increase in $[Ca^{2+}]_i$ in cortical cells (see Figure 2.9C), this increase was unable to induce calpain translocation, autolysis or activation as it did in erythrocytes. This again suggested that calpain's regulation in neuronal cells was strictly regulated and more complex than that observed in non-neuronal cells.

3.3.2 Effect of PKC activity on calpain and neuroprotection

Recently, the neurotrophin BDNF has been shown to prevent excitotoxic neuronal

cell death *in vitro* (Tremblay et al., 1999; Cheng and Mattson, 1994; Lindholm et al., 1993; Shimohama et al., 1993), and ischemic damage *in vivo* (Beck et al., 1994; Yamashita et al., 1997). We have demonstrated that BDNF neuroprotection of primary cortical neurons is maximal when the neurotrophin is applied 4 and 8 hours before a transient and normally lethal NMDA or glutamate application (Tremblay et al., 1999). This neuroprotective effect of BDNF against NMDA was afforded without any apparent change in NMDA receptor subunit distribution or the levels of $[Ca^{2+}]_i$ triggered by NMDA receptor activation. This suggested that the neurotrophin was targeting some critical cell signaling event downstream of lethal Ca^{2+} influx. We have also demonstrated that several PKC inhibitors (i.e., staurosporine, GF-109203X, and calphostin C) were able to reverse the neuroprotective effects of BDNF when added to cells along with transient NMDA application (Tremblay et al., 1999). These observations indicate that the critical cellular effect of BDNF is to block a crucial step in the mechanism by which lethal Ca^{2+} influx via the NMDA receptor causes PKC inactivation in primary cortical cells.

To determine whether calpain activation was affected by BDNF in the face of unaltered Ca^{2+} flow through the NMDA receptor, we examined the effects of an overnight pre-treatment of BDNF on NMDA-induced calpain activity and cell death. Figure 3.2A demonstrates the effect of BDNF on NMDA-induced activation of calpain. In the presence of BDNF calpain-mediated spectrin hydrolysis was partly attenuated. This coincided with BDNF's ability to provide neuroprotection against NMDA (Figure 3.2B). When cells were pretreated with BDNF, NMDA-mediated cell death was significantly

reduced by more than half, from 90% to 40% ($p < 0.01$). Consistent with our previous results (Tremblay et al., 1999), BDNF effects were mediated without affecting Ca^{2+} entry in response to NMDA (Figure 3.2C). These results were the first to suggest that NMDA-induced calpain activation could be modulated by some additional factor, independent of Ca^{2+} flow through the NMDA receptor.

As stated above, the neuroprotective effects of BDNF are lost if PKC inhibitors are present during NMDA application (Tremblay et al., 1999). To address the potential for interaction between PKC inactivation and calpain activity, we tested the effect of PKC inhibitors on the BDNF-induced attenuation of spectrin hydrolysis following NMDA treatment. Figure 3.3A demonstrates that the ability of BDNF pretreatment to attenuate calpain activity in response to NMDA was reversed in the presence of 50 nM staurosporine, a concentration believed to be selective for PKC and not other kinases (Gordge and Ryves, 1994). The ability of a PKC inhibitor to induce calpain activation under these experimental conditions suggested that the activation of calpain by NMDA was downstream of PKC inhibition.

The ability of staurosporine to reverse BDNF's attenuation of calpain activity was also associated with its ability to affect cell survival. NMDA application resulted in 74% cell death, however an overnight pretreatment with BDNF reduced this to 37%, such that this group was not statistically different from control groups ($p > 0.05$) (Figure 3.3B). When staurosporine was present during NMDA application, BDNF could no longer confer neuroprotection to cortical neurons, and cell death was nearly double that observed in the absence of PKC inhibition (37% cell death increasing to 63%). Collectively, the

data suggest that PKC inhibition modulated an increase in calpain activity that was responsible for reversing the protective effects of BDNF, and for promoting cell death.

To further address the relationship between NMDA receptor activation, the loss of PKC, and calpain activation, cortical cells were treated with staurosporine (50 nM) and/or, a sublethal concentration of NMDA (20 μM). It has been previously shown that only lethal concentrations of glutamate, AMPA, or NMDA cause an early PKC inactivation (Durkin et al., 1996), and as shown in Figure 2.1, calpain also requires lethal levels of NMDA to exact spectrin hydrolysis. Neither the inhibition of PKC alone, nor the sublethal activation of NMDA receptors stimulated spectrin hydrolysis (Figure 3.4A). However, when sublethal NMDA receptor activation was combined with the inhibition of PKC, the signature 155 kDa calpain-mediated spectrin fragment was detectable (Figure 3.4A). This result was not restricted to staurosporine. Both the PKC inhibitor GF-109203X (100 nM) and calphostin C (250 nM) co-applied with sublethal NMDA (10 μM) promoted calpain-mediated spectrin breakdown (Figure 3.4B), while having no effects on their own (data not shown). These results further suggested that the selective activation of calpain by NMDA-induced Ca^{2+} influx was mediated by the inactivation of PKC coupled to that Ca^{2+} flow. However, the combination of PKC inhibition with sublethal NMDA receptor activation was not sufficient to induce cell death (Figure 3.4C), a circumstance analyzed in the Discussion section of this Chapter.

The effects of PKC inhibition were selectively coupled to NMDA receptor activation. If PKC activity was inhibited during KCl-induced depolarization or ionomycin

application, increased spectrin proteolysis was not observed. Thus, the co-application of GF-109203X (100 nM) (Figure 3.5) or staurosporine (50 nM) (Figure 3.6) was unable to cooperate with KCl (50 mM) or ionomycin (2.5 μM) to mediate an increase in spectrin hydrolysis (Figure 3.5A, Figure 3.6A), or to effect cell death (Figure 3.5B, 3.6B).

3.3.3 Search for potential substrates of PKC that mediate calpain activation

While PKC inhibition clearly caused an increase in calpain-mediated spectrin breakdown products in the above experiments, its substrate(s) under these circumstances was not clear. While the loss of PKC could be impacting calpain directly, there is little direct experimental evidence to suggest that calpain is phosphorylated *in vivo* (Adachi et al., 1986). We investigated the alternative possibility that a loss of PKC activity was impacting substrate hydrolysis by changing spectrin's phosphorylational state, and thereby altering its susceptibility to calpain. Indeed, changes in substrate phosphorylation have been shown to impact calpain's activity against other substrates such as MAP-2 (Alexa et al., 1996), tau (Shea, 1997), and connexin (Elvira et al., 1993). To address this issue, we first performed an *in vitro* kinase assay to determine whether PKC could potentially mediate the phosphorylation of spectrin.

Figure 3.7 shows that spectrin did not act as a substrate for partially purified rat brain PKC in the *in vitro* assay, making it unlikely that spectrin would be phosphorylated *in vivo* by the kinase. Consistent with these results, no detectable levels of spectrin phosphorylation resulting from incubation with active PKC and [32P]-ATP were observed in samples subjected to SDS-PAGE and autoradiography (results not shown).

The inability of spectrin to act as a substrate for PKC could not be attributed to a failure of the assay conditions to promote PKC-mediated phosphorylation since the selective PKC substrate peptide (MARCKS) was effectively phosphorylated under the same assay conditions (Figure 3.7).

We proposed that an alternative potential substrate impacted by the loss of PKC during NMDA toxicity might be the endogenous calpain inhibitor, calpastatin. As described in the General Introduction, calpastatin interacts with the calmodulin-like domains of calpain to prevent its activation. This interaction is dependent on Ca^{2+}, and is highly modulated by phosphorylation (Adachi et al., 1991; Pontremoli et al., 1992; Salamino et al., 1994a,b; Salamino et al., 1997). In some instances the post-translational modifications and efficacy of calpastatin has been directly linked to phosphorylation by PKC (Adachi et al., 1991), thus making calpastatin a potential target for the NMDA-mediated inactivation of PKC. Figure 3.8A shows that like calpain (see Figure 3.1), calpastatin was largely membrane associated in control cultures maintained in conditioned media. A monoclonal calpastatin antibody detected several protein bands corresponding to apparent molecular weight products of 94, 66 and 34 kDa. Following a transient (5 min) NMDA (50 μM) application, there was a marked decrease in the amount of the 94 and 34 kDa species of calpastatin protein detected in the membrane fraction two hours following treatment. Western blots were unable to resolve any detectable hydrolytic products of the protein, or a systematic shift from the membrane compartment to the cytosol. Nonetheless, a time course analysis of this NMDA-mediated effect on the 94 kDa species of calpastatin revealed that there was a detectable decrease from the

membrane 30 minutes post-treatment, which continued to decline over the time course of examination (between 30 and 120 min) (Figure 3.8B). This change in calpastatin closely coincided with the time course observed for calpain-mediated spectrin hydrolysis in response to NMDA (see Figure 2.1A).

Since the phosphorylational state of calpastatin has been shown to affect its localization in some cell types (Adachi et al., 1991), we postulated that an NMDA-induced PKC inactivation might mediate this loss of calpastatin from the membrane, and hence promote the activation of calpain by lethal NMDA. We therefore examined the effects of BDNF (thereby preventing the loss of PKC activity) on NMDA-induced changes in membrane-calpastatin, in the presence or absence of PKC inhibitors. Figure 3.9A illustrates that following NMDA treatment, the reduction in the amount of detectable membrane-associated 94 kDa calpastatin species was reversed when cells were pretreated with BDNF (50 ng/ml) (Figure 3.9A). However, when the PKC inhibitor staurosporine (50 nM) was co-applied with NMDA, the effect of BDNF on calpastatin was not affected (Figure 3.9A). Similar results were observed for the PKC inhibitors GF-109203X (100 nM) and calphostin C (250 nM) (results not shown). Despite the fact that the inhibition of PKC did not reverse BDNF's effect on calpastatin, its effect on cell viability was still reversed under these conditions (Figure 3.9B). Thus, the PKC-dependent effect on calpain activity did not appear to involve calpastatin, although BDNF was able to prevent calpain activity and the loss of its endogenous inhibitor from the membrane fraction. This again suggests that *at least* two coincident events emanating from NMDA receptor activation act in concert to affect calpain activity.

3.4 DISCUSSION

Conventional models of calpain activation are based on knowledge gained from non-neuronal cells, in which calpain exists as an inactive cytosolic enzyme requiring Ca^{2+}-induced translocation to membranes as a prerequisite for activation. The assumption that calpain displays a homogenous activation profile in all cell types has up until now resulted in the generalized application of this model to neuronal cells. Indeed, this was the approach initially taken in this research thesis in an attempt to examine calpain activation in response to NMDA or ionomycin using a calpain antibody to detect intact and autolytic forms in cytosolic and particulate cell fractions. However, experiments in which neurons and erythrocytes were directly compared under identical treatment conditions revealed that the protease behaved quite differently in neuronal cells. Calpain was found to be membrane associated under basal, non-stimulating conditions in primary cortical neurons, an observation seemingly at odds with previous reports indicating μ-calpain to be mainly cytosolic in brain tissue (Guroff, 1964; Murachi et al., 1980; Sandoval and Weber, 1978). However, the preparation of samples in those previous studies involved the use of EGTA and/or other chelating agents, which would effectively remove any calpain bound to membranes in a peripheral, largely electrostatic, manner. Since chelators were not used in the present study, our data are consistent with the idea that the majority of calpain in cortical cells (and perhaps in rat brain) is peripherally-bound to membranes. It may be argued that in the absence of chelators, cell lysis may artificially drive the protease to the membrane, however identical cell lysis procedures in erythrocytes did not result in membrane-association under basal conditions. This strongly supports our conclusion that

calpain is membrane-associated in neurons. Indeed, immuno-electron microscopic studies of calpain localization, in which chelating agents were not used, revealed that μ-calpain is primarily associated with cytoskeletal elements in neurons (Perlmutter et al., 1988), consistent with its localization to the insoluble particulate fraction. m-Calpain is also membrane-associated (Li and Banik, 1995), but Western blot analysis of purified μ- and m-calpain (both purchased from Calbiochem, La Jolla, CA) with the monoclonal calpain antibody used in this study confirmed that the antibody was specific for μ-calpain (data not shown), and that the low calcium-requiring isoform of the protease was indeed associated with the membrane or other insoluble particulate proteins in neuronal fractions.

Autolytic conversion of calpain during activation was also not detectable in primary cortical neurons. This finding could not be attributed to an inability of the anti-μ-calpain antibody to recognize the autolytic product of ubiquitous μ-calpain in the rat brain, since it effectively did so in the rat erythrocyte. In addition, there is no evidence for brain specific calpain isoforms, and the ubiquitous form of the μ-calpain (found in red blood cells, muscle, and other tissues) appears to be the predominant type in neurons (Sorimachi et al., 1994). The finding that the autolytic conversion of calpain is not necessary for the expression of protease activity in neurons is in agreement with previous observations in erythrocytes (Molinari et al., 1994). Alternatively, one can not rule out the possibility that membrane associated calpain is already the autolyzed form in unstimulated cultured neurons. Interaction of the protease with membrane phospholipids has been reported to enhance enzymatic activity (Arora et al., 1996; Melloni et al, 1996; Chakrabarti et al.,

1990), and the 78 kDa autolytic product of calpain has been shown to be the preferential form associated with membranes (Melloni et al., 1996). It is possible that the single band of neuronal calpain observed in neurons may represent an active, 78 kDa autolysed form of calpain. It is also not possible to completely rule out spectrin hydrolysis arising in cortical cells, in whole or in part, from NMDA-mediate changes in spectrin substrate availability, or susceptibility to proteolysis. We did however, rule out the likelihood that such a change could be mediated by NMDA-induced changes in PKC, and resultant changes in spectrin phosphorylation. Nonetheless, the results do support the notion of additional regulatory factors for calpain activity.

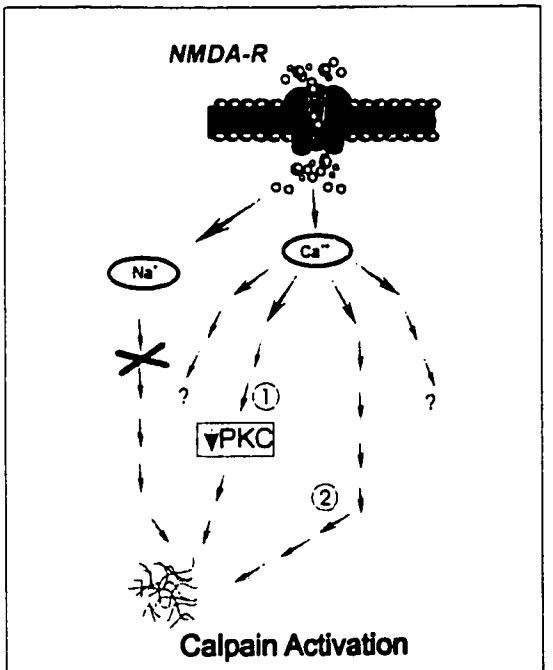
Several lines of evidence indicate μ-calpain, and not m-calpain, is responsible for the NMDA-induced hydrolysis of spectrin in neurons. First, the Ca^{2+} requirement for purified m-calpain is at least several orders of magnitude higher than that required to activate μ-calpain under *in vitro* assay conditions (Croall and DeMartino, 1991). Secondly, μ-calpain has been shown to be the predominant isoform of the protease in neurons, while m-calpain is highly expressed in glial cells (Hamakubo et al., 1986). Since it is generally accepted that rat primary glial cells do not possess functional NMDA receptors (Steinhauser and Gallo, 1996; Gallo and Russell, 1995; Gallo et al., 1994; McNaughton and Hunt, 1992; Glaum et al., 1990; Jensen and Chiu, 1990; Enkvist et al., 1989; Pearce et al., 1986), the NMDA-mediated effects observed are unlikely ascribed to glial m-calpain. Finally, using a monoclonal antibody specific for μ-calpain and a polyclonal antibody (generously supplied by Dr. Naren Banik, University of South Carolina, Charleston, South

Carolina) which recognizes both μ- and m-calpain isoforms, we have determined that μ-calpain is the predominant form of calpain in our cultures (data not shown). Furthermore, neither ionomycin or NMDA caused detectable translocation or autolysis of calpain on immunoblots probed with the polyclonal calpain antibody (data not shown), suggesting that even if m-calpain was activated, it too did not undergo translocation or autolysis in primary neuronal cultures.

Since calpain was largely membrane-associated in primary cortical neurons, it precluded the possibility that increases in $[Ca^{2+}]_i$ could induce a translocation-based activation of μ-calpain in these cells. This suggested other regulatory factors were at play in association with Ca^{2+} entry, since the presence of this cation was still an essential feature of calpain-mediated hydrolysis of spectrin. Moreover, the source of Ca^{2+} was strictly defined in terms of its ability to activate calpain. Non-specific Ca^{2+} entry induced by ionomycin was unable to activate μ-calpain in cortical neurons, but Ca^{2+} influx via the NMDA receptor was crucial for μ-calpain-mediated spectrin proteolysis. These data suggested that μ-calpain regulation was more complex in cortical cells than that documented for erythrocytes and other non-CNS cells, in that calpain was not promiscuously activated in response to just any source of $[Ca^{2+}]_i$. We postulated that the factors conferring such receptor and Ca^{2+} selectivity would most likely be coupled to NMDA receptor activation as well.

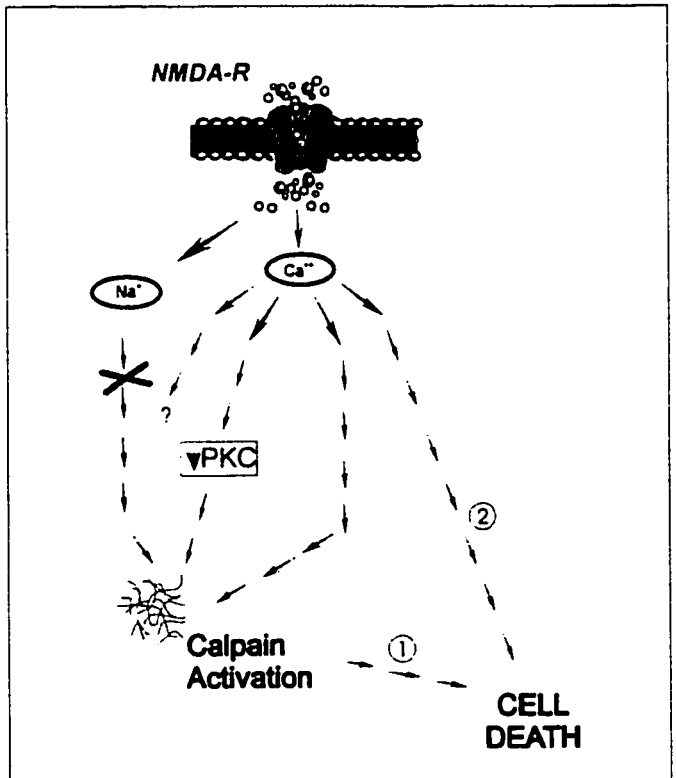
One such candidate was PKC. Previous results from this laboratory have shown that a loss of PKC activity is essential for NMDA-induced cell death in primary cortical

neurons, and that this loss was selectively linked to Ca^{2+} influx through glutamate receptors (Durkin et al., 1996; 1997). The neurotrophin BDNF prevents this loss of PKC in response to NMDA, and is neuroprotective (Tremblay et al., 1999). By examining the effects of BDNF on calpain activation, we have shown calpain activation to be downstream of PKC. An overnight pre-treatment with BDNF attenuated NMDA-induced calpain-mediated spectrin hydrolysis and cell death. This effect was not mediated through the attenuation of Ca^{2+} influx, since BDNF did not change the levels of free intracellular Ca^{2+} induced by NMDA. The co-application of PKC inhibitors with NMDA reversed the effects of BDNF on both cell viability and calpain activation. PKC inhibition was not sufficient to activate calpain alone, suggesting that this loss interacts with other regulatory features of calpain activity impacted by NMDA receptor activation. These other NMDA-related events directly or indirectly link to other Ca^{2+}-dependent changes effected by NMDA-receptor activation (see Scheme 5). The data clearly indicate that at least two independent events triggered by NMDA receptor overstimulation converge to affect the activation of calpain in primary cortical neurons. This is consistent with the widespread realization that excitotoxicity is



Scheme 5 - NMDA-induced calpain activation proceeds via a minimum of two independent steps.

not a simple process, but results from multiple cellular insults initiated by aberrant NMDA receptor activity. The necessity for the interaction of multiple regulatory components affecting protease activation and cell death was also supported by the results obtained following combined sublethal NMDA receptor activation and PKC inhibition. Neither activated calpain in isolation, but in combination were capable of inducing calpain-mediated spectrin hydrolysis. Thus, combined NMDA receptor activation and inhibition of PKC activity was sufficient to induce spectrin hydrolysis. It is unlikely a coincidence that this biochemical profile characterizes the events occurring following lethal NMDA application. These data again suggest that at least two coincident events resulting from NMDA receptor activation act together to induce calpain activity. In the case of sublethal NMDA, it is the PKC inactivating step that is limiting, and calpain activation is not observed unless PKC is inactivated pharmacologically by inhibitors. Under sublethal NMDA conditions then, other regulatory components of calpain activity are initiated, but without PKC inactivation the protease remains quiescent. Multiple activation requirements such as this can provide strict regulation for

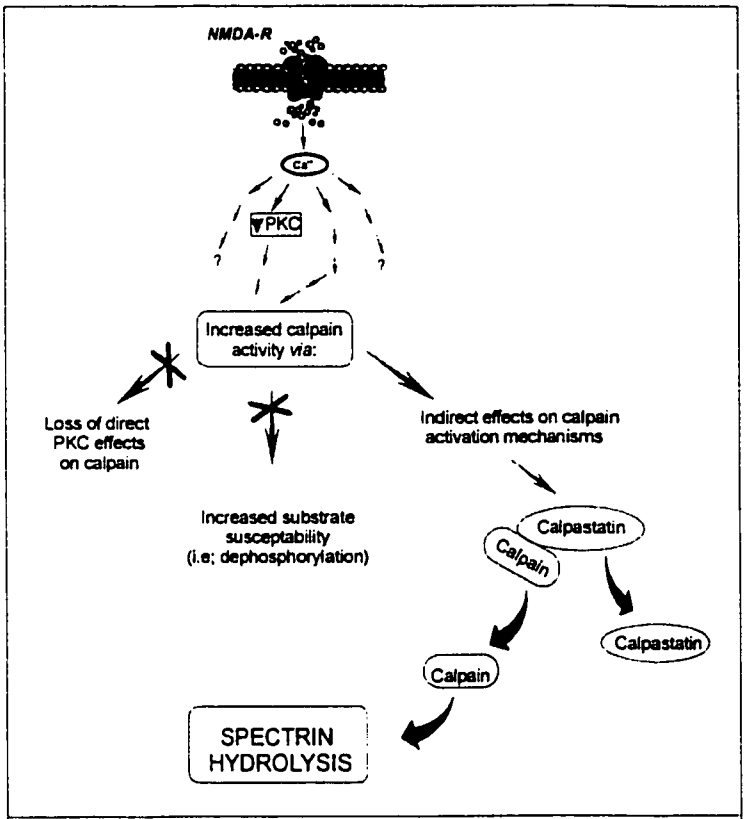


***Scheme* 6**- Relationship between calpain-mediated spectrin hydrolysis and NMDA-induced neuronal death

protease activity. It is important to note however, that sublethal NMDA combined with PKC inhibition was not sufficient to induce cell death. This suggests that other NMDA-mediated cellular events coalesce with calpain activation to promote cell injury (see Scheme 6 above). Alternatively, the extent to which calpain was activated under these conditions may have been insufficient to cause significant pathology. Indeed the amount of spectrin hydrolysis produced under these conditions was markedly less than that seen with lethal NMDA (data not shown). Furthermore, the persistence of these spectrin fragments was not determined, leaving the possibility that they were transient in cells treated with sublethal NMDA and PKC inhibitors. As we have demonstrated in Chapter 2, it is the persistent detection of calpain-mediated spectrin breakdown products that is a feature of cells destined to die.

The PKC cellular substrates affected by a loss of kinase activity were not obvious under conditions where PKC inhibition promoted calpain activity. Dephosphorylation of calpain as a mechanism of protease activation seemed unlikely since there was little evidence to suggest that μ-calpain was a substrate for PKC phosphorylation (Adachi et al., 1986). As an alternative, we examined the possibility that calpain was already active in untreated neurons and that PKC inactivation by NMDA could be affecting the susceptibility of spectrin to calpain hydrolysis. In this case, there is precedence suggesting that the phosphorylational state of calpain substrates could affect their vulnerability to hydrolysis (Shea, 1997; Alexa et al., 1996; Litersky and Johnson, 1995; Elvira et al., 1993). The hyperphosphorylation of tau is thought to prevent normal proteolytic

processing of the protein by calpain, which may contribute to the neuropathology of Alzheimer's Disease (Shea, 1997). Furthermore, Elvira et al. (1993) have shown that phosphorylation of connexin-32 by PKC prevents calpain-mediated proteolysis of the protein. Similar phosphorylation-dependent susceptibility has been reported for MAP2 (Alexa et al., 1996), and evidence exists that this phosphorylational state is modulated by glutamate receptor activation (Quinlan and Halpain, 1996). However, when we examined the ability of spectrin to serve as a PKC substrate, no detectable incorporation of labelled phosphate into spectrin under the *in vitro* assay conditions was observed. The inability of PKC to phosphorylate spectrin under these extremely permissive *in vitro* assay conditions made it unlikely that the kinase would act on spectrin *in vivo*. Since the observed effects of PKC inhibition on calpain were unlikely due to PKC action on either calpain itself or spectrin, we proposed that the loss of PKC activity could be directly impacting the activity of a calpain modulator (see Scheme 7). A potential candidate was the endogenous calpain inhibitor calpastatin, whose interaction with calpain



Scheme 7- Possible routes by which NMDA causes increased spectrin hydrolysis in cortical neurons

has previously been shown to be regulated by both Ca^{2+} and phosphorylation (Salamino et al., 1994b; Adachi et al., 1991; Maki et al., 1991). Examination of calpastatin in untreated primary cortical neurons revealed that, like calpain, calpastatin was membrane-associated, and therefore localized to an environment where it could suppress otherwise activated calpain. Consistent with this notion, NMDA caused a loss of immunoreactive calpastatin from the membrane fraction. This suggested that NMDA-mediated increases in Ca^{2+} acted to remove calpastatin's inhibitory regulation of the protease (see Scheme 7). Such regulatory processes responding to changes in specific, and perhaps localized, increases in $[Ca^{2+}]_i$ would give the cell significantly more versatility and control over calpain activity than would be possible were calpain activity regulated directly and simply by $[Ca^{2+}]_i$ fluctuations alone.

Despite its sensitivity to NMDA, calpastatin did not appear to be regulated by a loss of PKC activity. While the application of BDNF attenuated the loss of calpastatin from the membrane, PKC inhibition failed to reverse this effect. Since BDNF attenuated both calpain activity and the loss of calpastatin from the membrane perhaps calpastatin was acting as a substrate for calpain (Saido et al., 1997; Nagao et al., 1994; Melloni et al., 1992; Pontremoli et al., 1991). This has been reported to occur following global forebrain ischemia, where calpastatin immunoreactivity transiently increased (at 4 hours), and then subsequently declined below control levels over the next 1 – 7 days in vulnerable areas of the hippocampus (Saido et al., 1997). In this same experiment, areas resistant to ischemic damage such as CA2 showed continuously elevated levels of calpastatin reactivity. It was

proposed that calpastatin acted as a "bait" substrate, and its over-expression protected other preferred substrates from calpain-mediated hydrolysis (Saido et al., 1997). In human epidermoid carcinoma KB cells, activation of calpain resulted in the hydrolysis of membrane-associated calpastatin and release of the hydrolytic fragments into the cytosol (Nagao et al., 1994). However, it was recognized early on that fragmented calpastatin can fully retain its inhibitory capacity (DeMartino et al., 1988), making an interpretation of the effects of calpastatin hydrolysis on calpain activation somewhat difficult. In the current set of experiments, neither a shift of calpastatin to the cytosolic compartment, nor an increase in detectable hydrolytic products was observed following NMDA treatment in primary cortical cells. As a result we can not definitively account for the changes observed on Western blots for decreased immunoreactivity. It is unlikely that BDNF prevented calpain activation in these cells by causing the over-expression of calpastatin since BDNF pre-treatment alone did not result in increased immunoreactivity of the protein. Thus, inhibition of calpain by BDNF appeared to depend on an as of yet undefined PKC-mediated process, and the PKC-independent relocation or hydrolysis of calpastatin.

The above data can not determine what PKC substrate is responsible for mediating increases in calpain activity. Other modulators of calpain activity include facilitatory molecules, which have been deemed calpain activator proteins. In platelets an activator protein with a molecular weight of approximately 48 kDa has been identified (Shiba et al., 1992). It did not change the Ca^{2+} sensitivity of calpain, but increased activation by 2-fold (Shiba et al., 1992). Similarly, a membrane-associated 40 kDa protein in erythrocytes was

shown to selectively bind calpain, and promote protease activation, however association with the activator reduced the Ca^{2+} requirement of proteolytic activation to within physiological levels (Salamino et al., 1993). More recently, Melloni et al. (1998b) have demonstrated the existence of a calpain activator protein that is specific for μ-calpain in rat brain. This protein was shown to be about 30 kDa in size, and bore significant similarity to UK114, a goat liver protein. Melloni et al. (1998b) demonstrated that this protein binds to the 80 kDa subunit of μ-calpain, competes with calpastatin, and is localized to the inner surface of membranes. They proposed that this molecule defines the sites of calpain activation, allowing the protease to escape the negative control exerted by calpastatin (Melloni et al., 1998b). Whether the interaction of this molecule and calpain is modulated by phosphorylation is unknown, however given the necessity for an exchange from calpastatin-mediated inhibition to activator protein binding, a glutamate-mediated transition of the phosphorylation state of the activator may very well determine this interaction. Since PKC inhibition in combination with KCl-induced depolarization or ionomycin application did not induce calpain activity, the additional modulatory factors, appear coupled to and dependent upon NMDA receptor activated events initiated by Ca^{2+} influx. Future experiments addressing these issues may well determine the role of PKC in modulating calpain activity in neuronal cells.

Figure 3.1 A comparison of calpain translocation, autolysis, and calpain-mediated spectrin breakdown in primary cortical neurons and rat erythrocytes. **A:** The presence of intact (80 kDa) or autolytically cleaved (76-78 kDa) calpain in membrane and cytosolic fractions isolated from erythrocytes and cortical neurons exposed for 15 minutes to ionomycin (2.5 μM)/ Ca^{2+} (100 μM) (Iono), for 5 minutes to NMDA (50 μM; neurons only), and vehicle controls (Buffer C as described in Methods, section 3.2.1) (Cont), in Western blots probed with an anti-μ-calpain monoclonal antibody (1:10000 dilution; 10% SDS-polyacrylamide gel). The cells were treated, lysed (section 3.2.1 of Methods), and analysed by Western blot (section 1.2.4) as described. **B:** The presence of spectrin breakdown products in the membrane fraction of erythrocytes and cortical neurons isolated 1 hour after transient (5 min) NMDA treatment (50 μM; neurons only), 1 hour after exposure to ionomycin (Iono) (2.5 μM) and calcium (100 μM) $\pm$ calpeptin (0.5 μM), or vehicle controls (Cont; Buffer C as in A), in Western blots (prepared as in section 1.2.4) probed with anti-spectrin antibodies (1:1000 dilution; 8% SDS-polyacrylamide gels). The Cal-SBDP antibody was used in neurons, however since this antibody is specific for brain α-spectrin, a commercial antibody (240/235E) for spectrin was used in erythrocytes. Treatment conditions and cell lysis (section 3.2.1) were as described. Westerns are representative of 4 separate experiments.

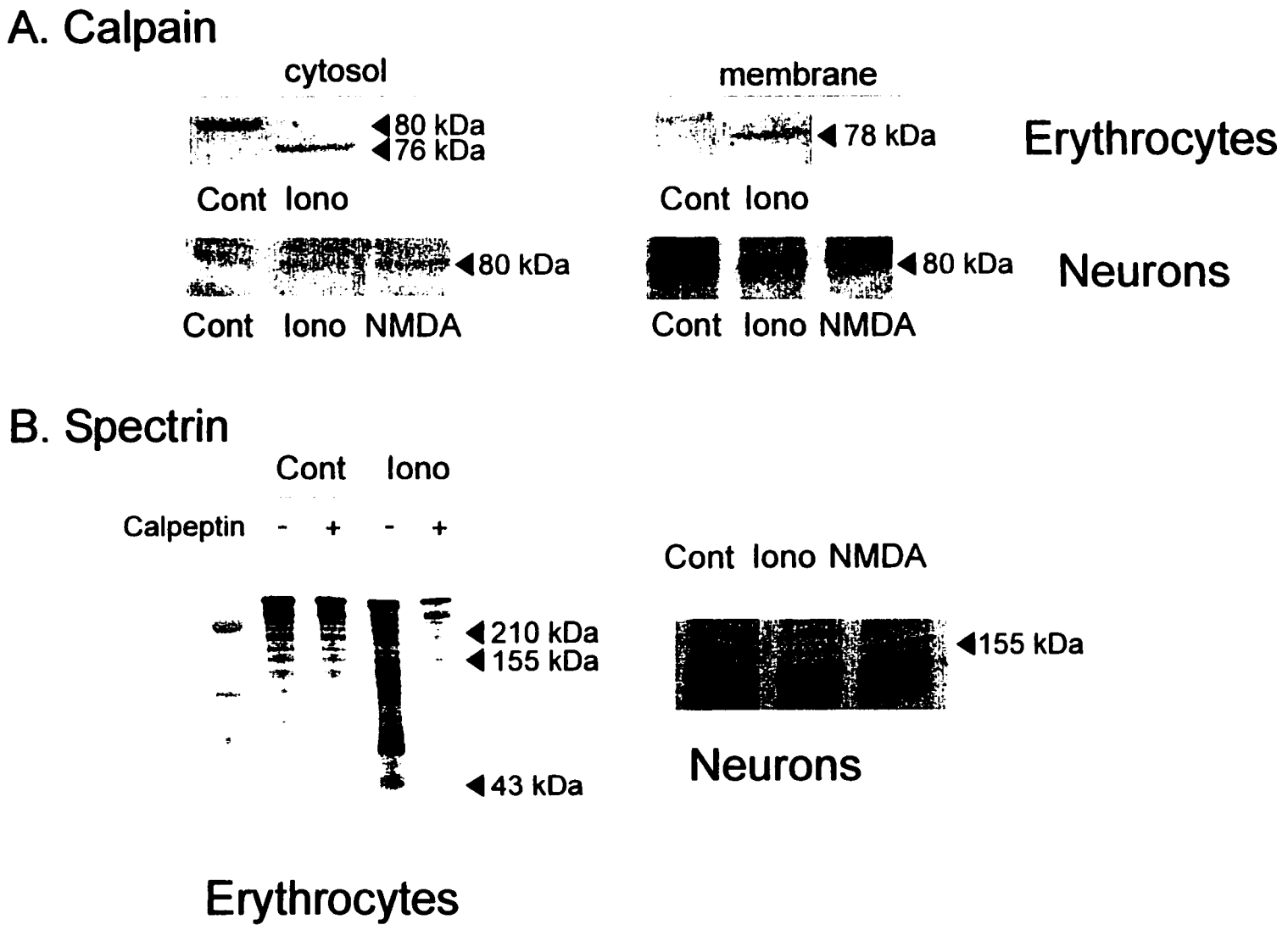

A. Calpain
cytosol
membrane
80 kDa
76 kDa
78 kDa
Erythrocytes
Cont Iono
Cont Iono
80 kDa
80 kDa
Neurons
Cont Iono NMDA
Cont Iono NMDA
B. Spectrin
Cont Iono
Calpeptin - + - +
Cont Iono NMDA
210 kDa
155 kDa
155 kDa
43 kDa
Neurons
Erythrocytes

Figure 3.2 The effect of BDNF on NMDA-induced calpain activity, neuronal cell death, and Ca^{2+} influx. **A:** Representative Western blot probed with the Cal-SBDP antibody showing calpain-mediated spectrin hydrolysis following a transient (5 min) NMDA (50 μM) application or vehicle (media) control (Cont), and the effect of an overnight BDNF (50 ng/ml) pretreatment. Treatment conditions were as described in Methods (section 3.2.2). Cells were lysed (as described in section 1.2.2.2) and prepared for Western blot analysis (antibody dilution 1:1000, 8% SDS-polyacrylamide gel) two hours post-NMDA application, as described (section 1.2.4). **B:** The effect of treatments as in A on neuronal viability. Bars represent the mean of 3 separate experiments (n = 3 per experiment) $\pm$ standard deviation (* $p < 0.01$). **C:** Representative traces demonstrating the effect of BDNF on NMDA-mediated increases in Ca^{2+} entry. Ca^{2+} was determined in fura-2 loaded neurons as described in Methods (section 3.2.3). BDNF (50 ng/ml) or vehicle (CSS) (Cont) was added to media overnight, prior to NMDA application (50 μM; arrowheads indicate the time of application). Traces are representative of at least six independent experiments.

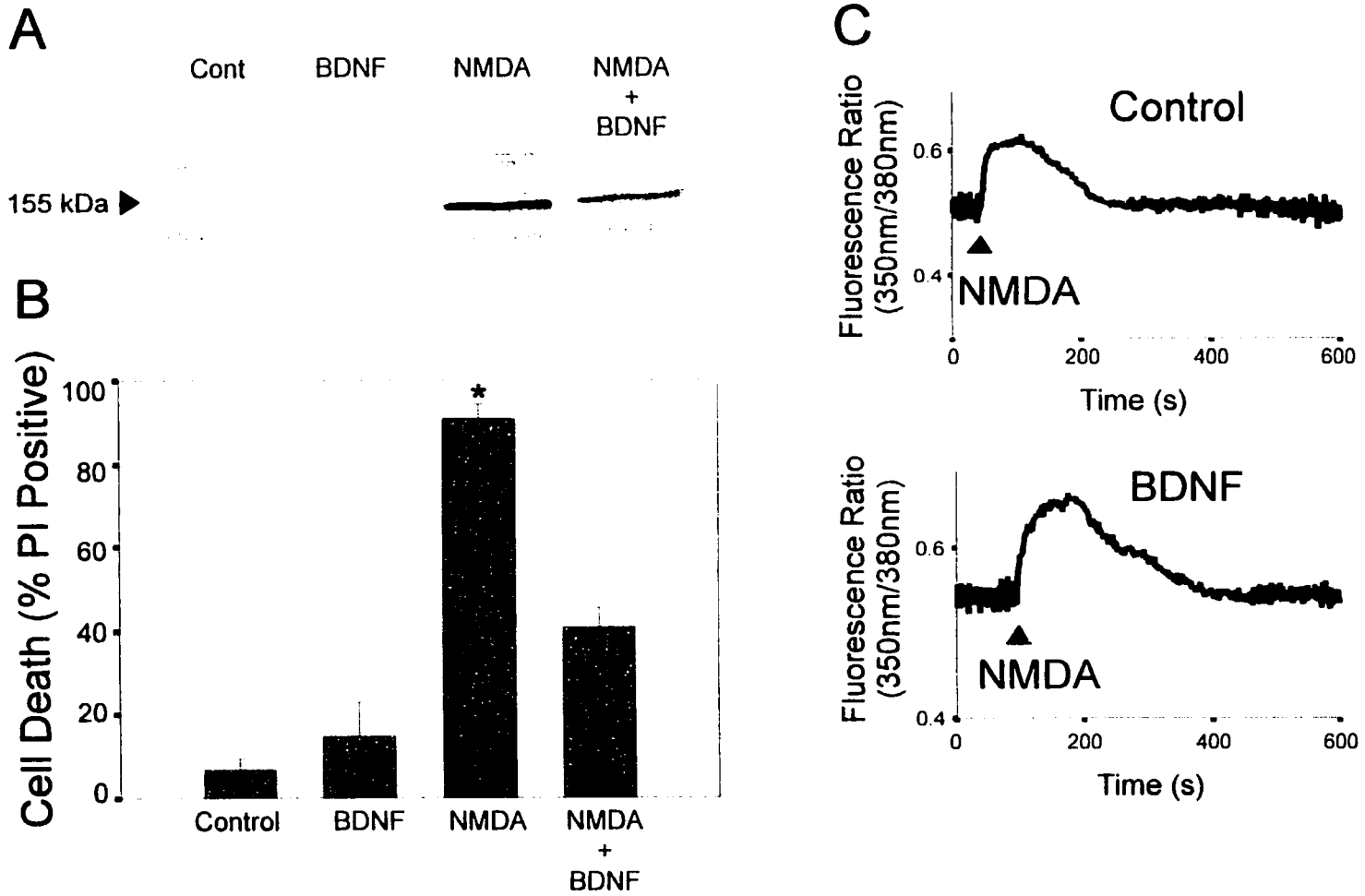

A
Cont
BDNF
NMDA
NMDA + BDNF
155 kDa
B
Cell Death (% PI Positive)
100
80
60
40
20
0
*
Control
BDNF
NMDA
NMDA + BDNF
C
Fluorescence Ratio (350nm/380nm)
Control
0.6
0.4
NMDA
0
200
400
600
Time (s)
Fluorescence Ratio (350nm/380nm)
BDNF
0.6
0.4
NMDA
0
200
400
600
Time (s)

Figure 3.3 PKC inhibition reverses the effect of BDNF on NMDA-mediated increases in calpain activity and cell death. **A:** Representative Western blot of calpain-mediated spectrin hydrolysis in response to transient (5 min) NMDA (50 μM) or vehicle (media) (Ctrl), attenuation by overnight BDNF (50 ng/ml) pretreatment, and reversal of this attenuation by the PKC inhibitor staurosporine (Stauro) (50 nM) added during the NMDA exposure period. Treatments were as described in method (section 3.2.2). Cell lysis (section 1.2.2.2) and Western blot analysis (section 1.2.4) were as described using the Cal-SBDP antibody (1:1000) and 10% SDS-polyacrylamide gels. **B:** The effect of treatments in A on neuronal viability, as assessed by PI staining (described in section 2.1.3 of Methods). Bars represent the mean of 5 separate experiments (n = 2 –3 per experiment) $\pm$ standard deviation. (* $p < 0.01$, statistically different from Ctrl, BDNF, and Stauro).

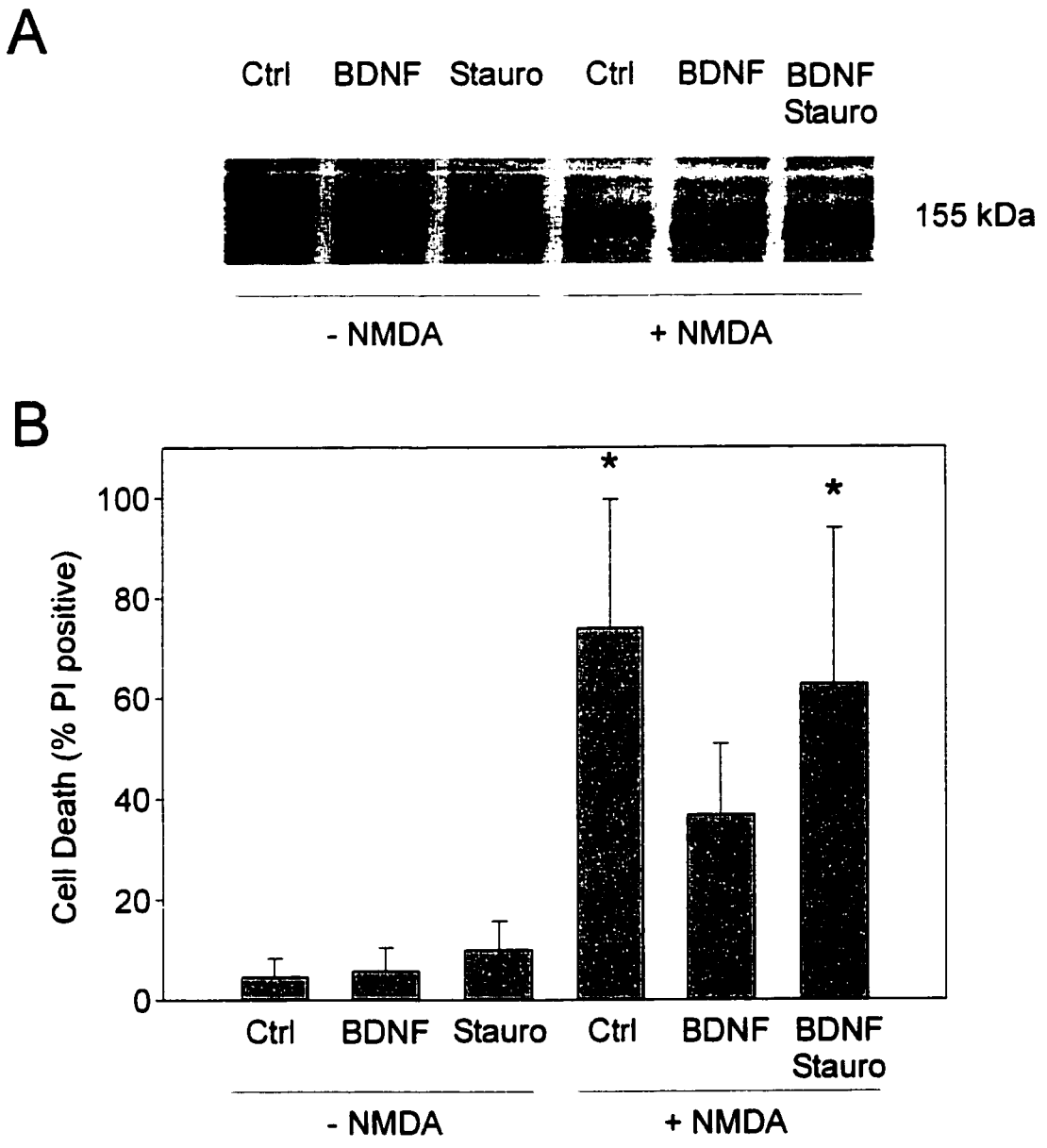

A
Ctrl
BDNF
Stauro
Ctrl
BDNF
BDNF
Stauro
155 kDa
- NMDA
+ NMDA
B
Cell Death (% PI positive)
100
80
60
40
20
0
*
*
Ctrl
BDNF
Stauro
Ctrl
BDNF
BDNF
Stauro
- NMDA
+ NMDA

Figure 3.4 The combined effects of sublethal NMDA receptor activation and PKC inhibition on calpain-mediated spectrin hydrolysis and cell viability. **A:** Representative Western blot of spectrin hydrolysis induced by sublethal NMDA (20 μM), vehicle (media) control (Ctrl), staurosporine (50 nM; Stauro), and the combination of NMDA and staurosporine applied for 5 minutes in conditioned media. Following treatment, cells were washed in 1 ml CSS, returned to 1 ml of conditioned media for 2 hours prior to cell lysis (section 1.2.2.2). For Western blot analysis (as described in section 1.2.4), samples were ran on 8% SDS-polyacrylamide gels and transblots were probed with the Cal-SBDP antibody (1:1000 dilution). **B:** The effect of the PKC inhibitors GF-109203X (GF-X, 100 nM) and calphostin C (Cal-C, 250 nM) on calpain-mediated spectrin hydrolysis when combined with sublethal (10 μM) transient (5 min) NMDA application. All treatment procedures and Western blot analysis were as described in A. **C:** The effect of treatments in (A) and (B) on cell viability, determined by PI staining (as described in section 2.1.3), 24 hours post-treatment. Bars represent the means of 2 – 4 separate experiments $\pm$ standard deviation (n = 3 per experiment). Because no statistical differences were found between the effects of 10 and 20 μM NMDA on spectrin hydrolysis or cell death, these values were combined for viability.

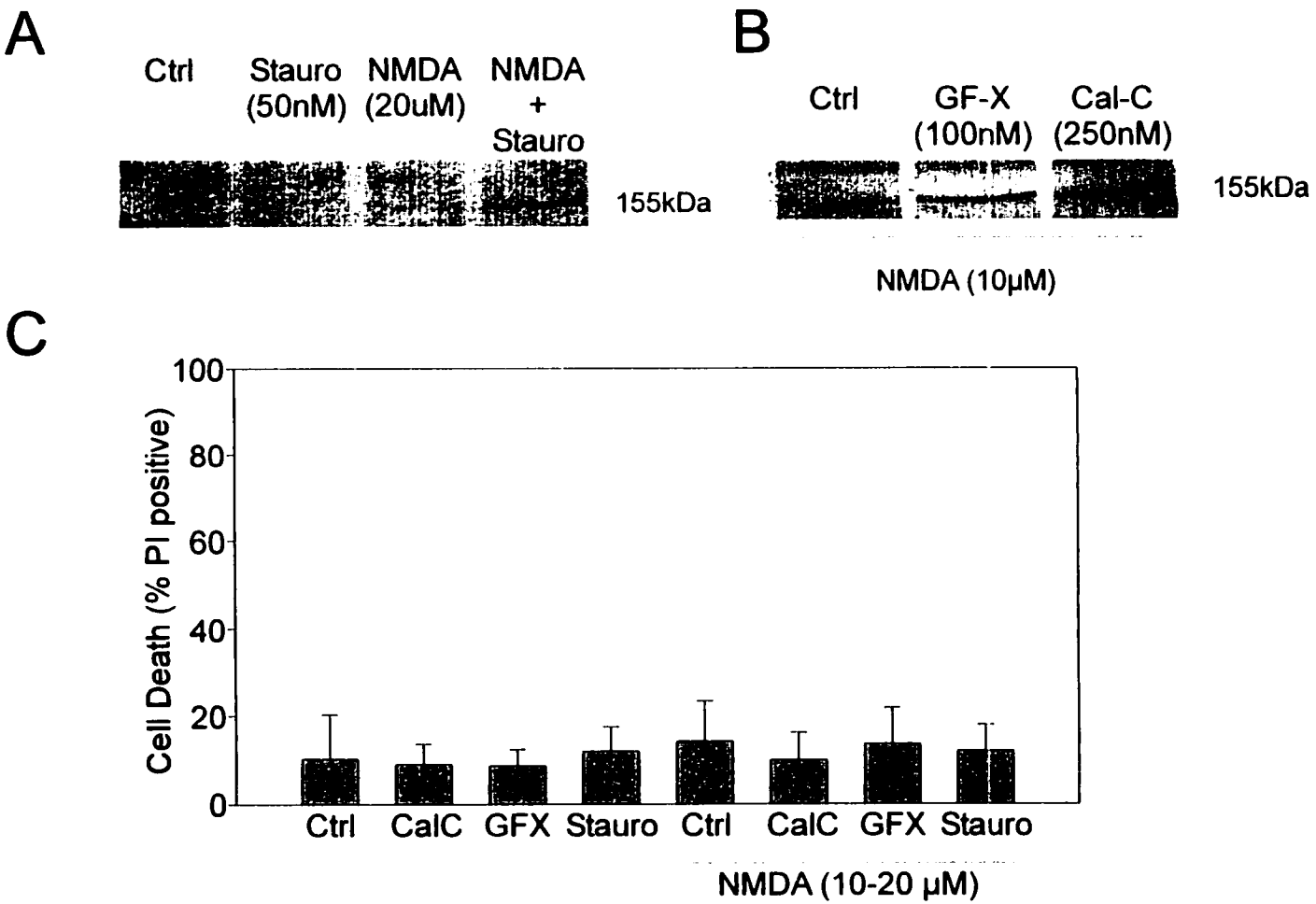
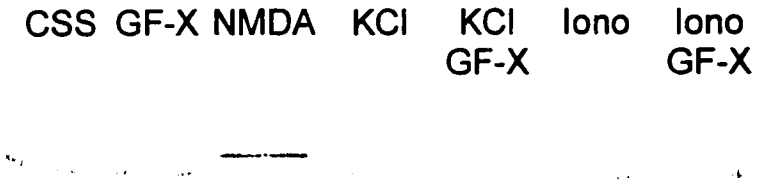

A
Ctrl
Stauro (50nM)
NMDA (20uM)
NMDA + Stauro
155kDa
B
Ctrl
GF-X (100nM)
Cal-C (250nM)
155kDa
NMDA (10μM)
C
Cell Death (% PI positive)
100
80
60
40
20
0
Ctrl
CalC
GFX
Stauro
Ctrl
CalC
GFX
Stauro
NMDA (10-20 μM)

Figure 3.5 The effect of PKC inhibition by GF-109203X on calpain activity and cell death when combined with non-specific Ca^{2+} entry through KCl-induced depolarization or ionomycin application. **A:** Primary cortical cells were treated with isotonic CSS or modified CSS containing 50 mM KCl or 2.5 μM ionomycin for 15 minutes in the presence or absence of GF-109203X (GF-X, 100 nM). For the purpose of comparison, cells were also treated with NMDA (50 μM) for 15 min in CSS. Following treatments, cells were washed with 1 ml of normal CSS, and returned to conditioned media for two hours before being lysed (as described in section 1.2.2.2). Western blot analysis was as described in Methods (section 1.2.4) using the Cal-SBDP antibody (1:1000 dilution, 8% SDS-polyacrylamide gels). **B:** Cell viability determination 24 hours post-treatment using PI staining (as described in section 2.1.3) in cortical cultures treated as described in A. Bars represent the mean of two separate experiments (n = 3-4 per experiment) $\pm$ standard deviation (* $p < 0.01$, different from all other groups).

A


CSS
GF-X
NMDA
KCl
KCl GF-X
Iono
Iono GF-X
155 kDa

B

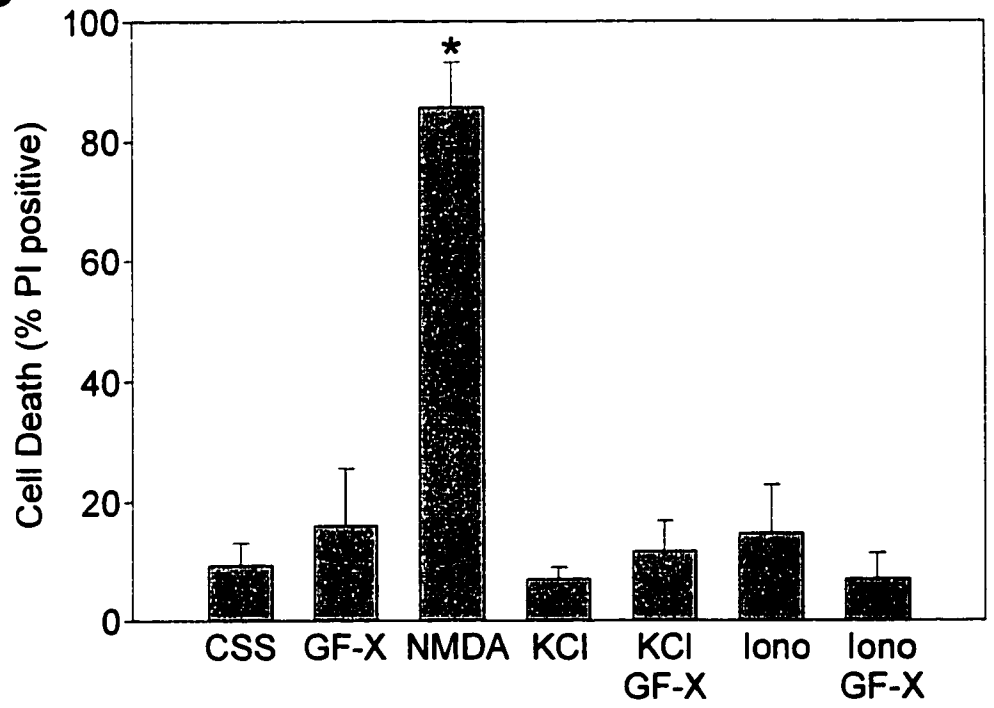

Cell Death (% PI positive)
100
80
60
40
20
0
*
CSS
GF-X
NMDA
KCl
KCl GF-X
Iono
Iono GF-X

Figure 3.6 The effect of PKC inhibition by staurosporine on calpain activity and cell death when combined with non-specific Ca^{2+} entry through KCl-induced depolarization or ionomycin application. **A:** Primary cortical cells were treated with isotonic CSS or modified CSS containing 50 mM KCl or 2.5 μM ionomycin (Iono) for 15 minutes in the presence or absence of staurosporine (Stauro, 50 nM). For comparison purposes, cells were also treated with NMDA (50 μM) for 15 min in CSS, or were left untreated (UT) to rule out any effects of CSS alone. Following treatments, cells were washed with 1 ml of normal CSS, and returned to conditioned media for two hours prior to cell lysis (as described in section 1.2.2.2). Western blot analysis was as described in Methods (section 1.2.4) using the Cal-SBDP antibody (1:1000 dilution, 8% SDS-polyacrylamide gels). **B:** Cell viability determination at 24 hours using PI staining (as described in section 2.1.3) in cortical cultures treated as described in A. Bars represent the mean of two separate experiments (n = 2-3 per experiment) $\pm$ standard deviation (* $p < 0.01$, different from all other groups).

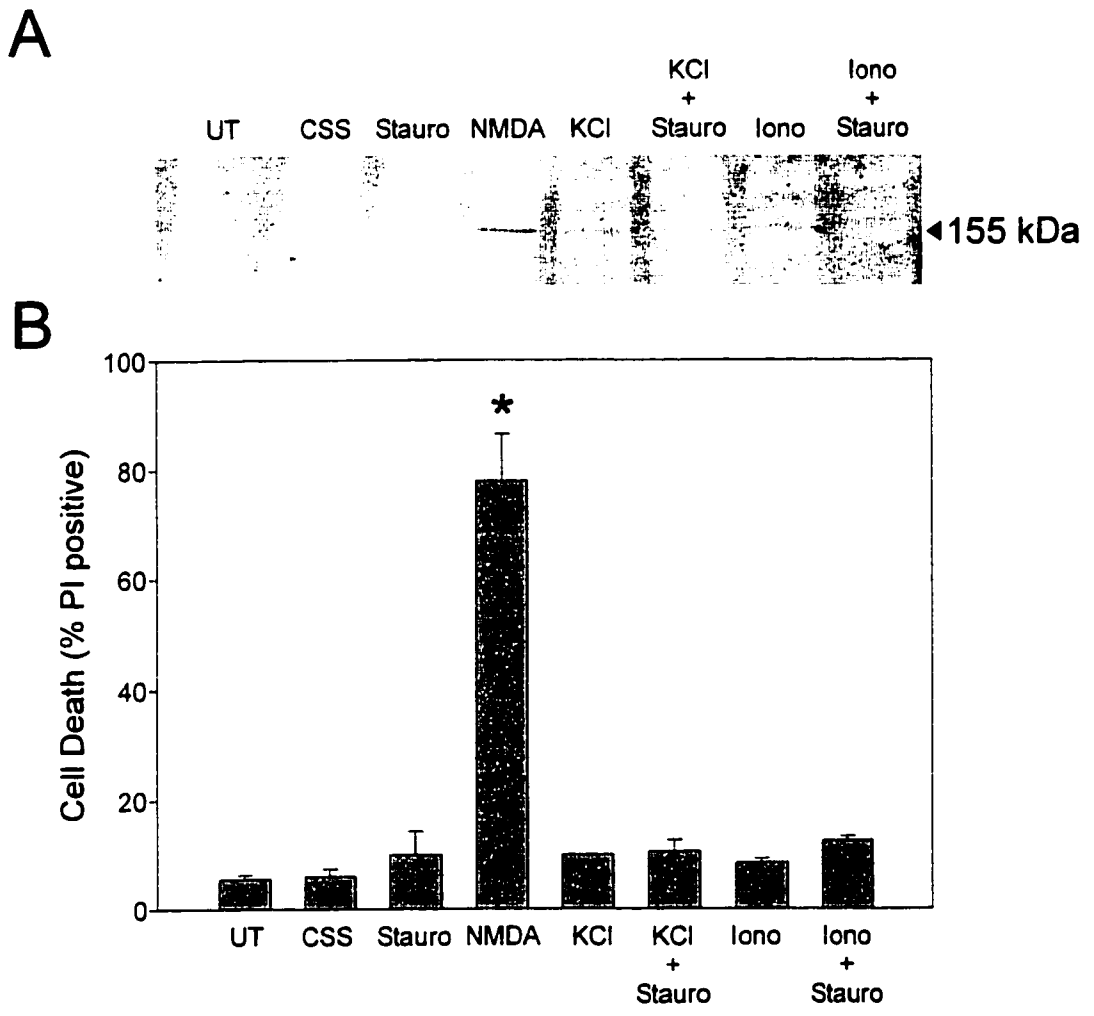

A
KCl + Stauro
Iono + Stauro
UT
CSS
Stauro
NMDA
KCl
Iono
155 kDa
B
Cell Death (% PI positive)
100
80
60
40
20
0
*
UT
CSS
Stauro
NMDA
KCl
KCl + Stauro
Iono
Iono + Stauro

Figure 3.7 The ability of partially purified rat brain PKC to phosphorylate spectrin *in vitro*. The PKC catalyzed incorporation of [32P] into spectrin (◕), or the selective PKC substrate MARCKS peptide (●) was compared to non-specific binding in the absence of substrate (▲) over a 30 minute time course. Assay conditions were exactly as described in Methods (section 3.2.5). Symbols in the plot represent the mean of 3 separate determinations ± standard deviation.

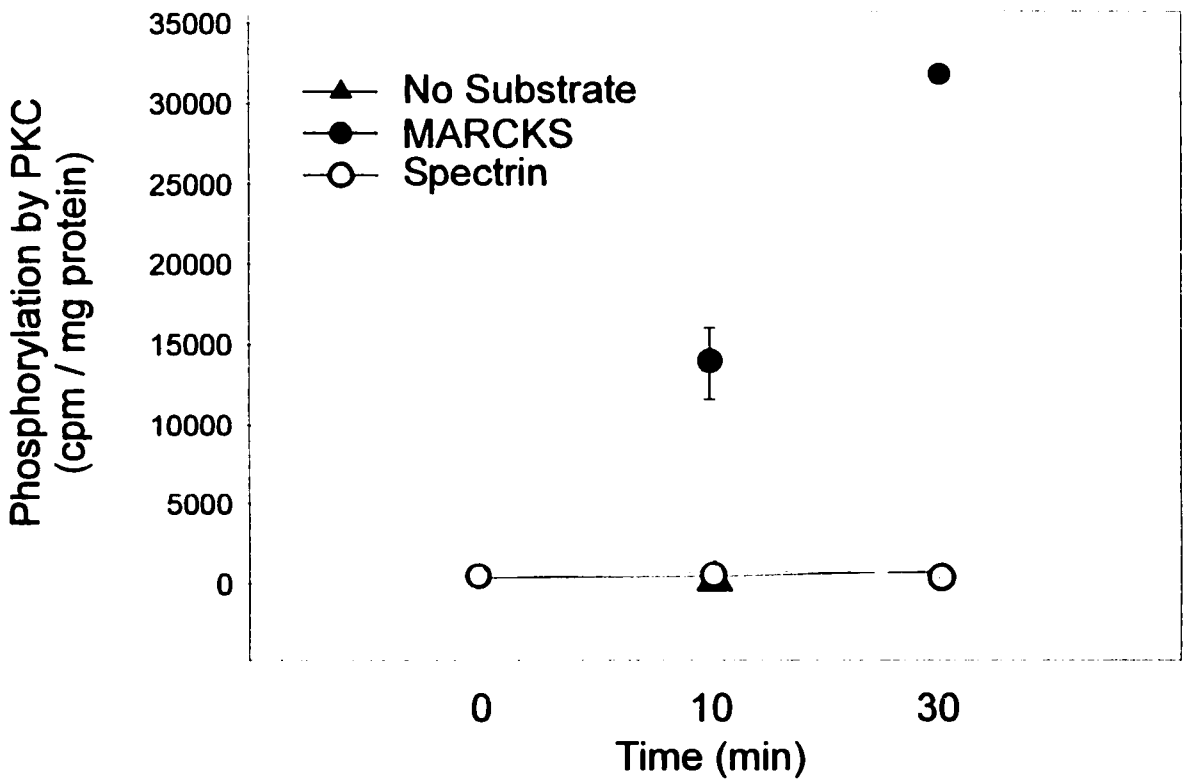

Phosphorylation by PKC
(cpm / mg protein)
35000
30000
25000
20000
15000
10000
5000
0
No Substrate
MARCKS
Spectrin
0
10
30
Time (min)

Figure 3.8 NMDA-mediated changes in the localization of the endogenous calpain inhibitor, calpastatin. **A:** Western blot analysis of calpastatin using a monoclonal antibody (1:10000) showing cellular localization under basal conditions (Ctrl), and following NMDA (50 μM) treatment for 5 minutes in conditioned media. Samples were lysed two hours post-treatment in a hypotonic buffer containing 50 mM Tris, pH 7.4. Cell fractionation was otherwise as described in Methods (section 1.2.2.2) to obtain cytosolic (soluble) (C) and membrane (particulate) (M) fractions. Western blot analysis was carried out as described (section 1.2.4) using 12% SDS-polyacrylamide gels with standard pre-stained molecular weight markers used to estimate molecular weight (open arrows). Closed arrows represent values in kDa of immunodectable bands. Results are representative of 6 out of 7 separate experiments. **B:** Representative Western blot showing the time course of change in the 94 kDa species of calpastatin in the membrane fraction following NMDA (50 μM) treatment for 5 min in conditioned media. Cells were lysed 30, 60 and 120 minutes following transient excitotoxin application and harvested for Western blot analysis as in A. Data are representative of 3 separate experiments.

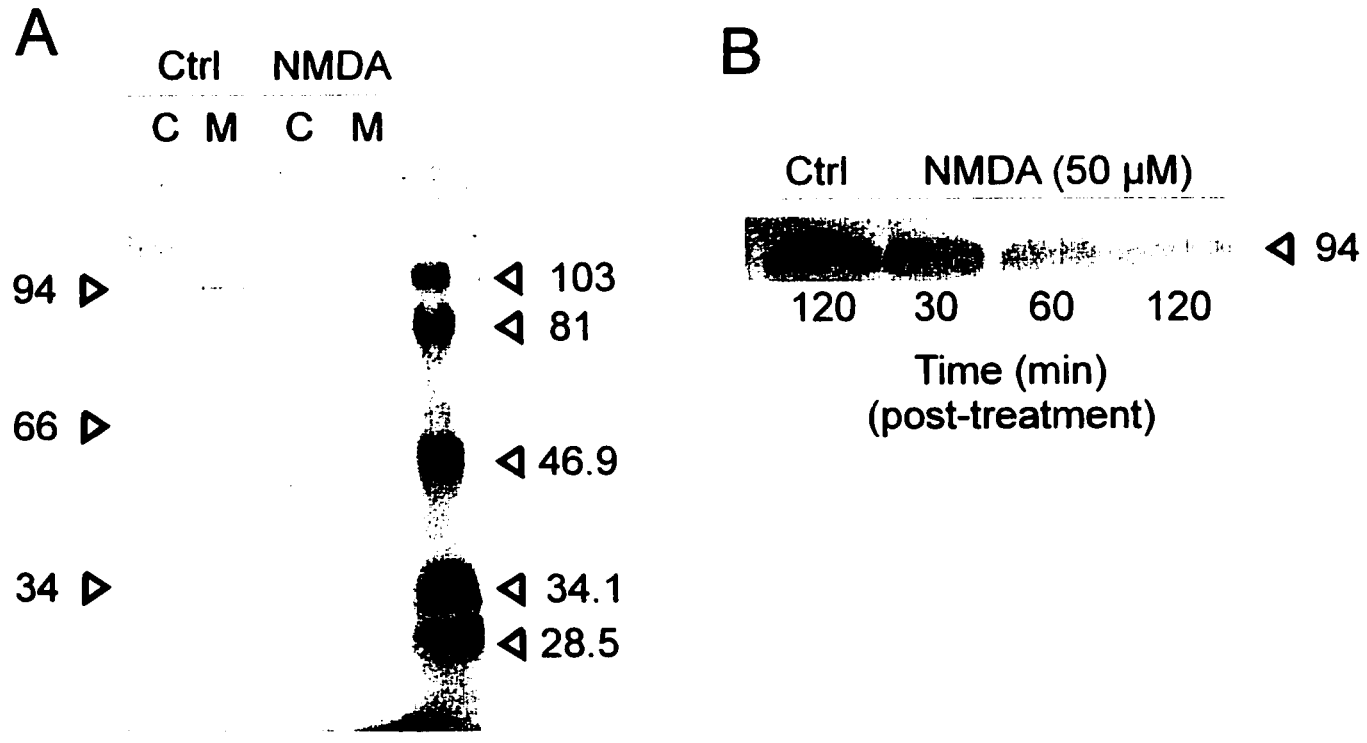
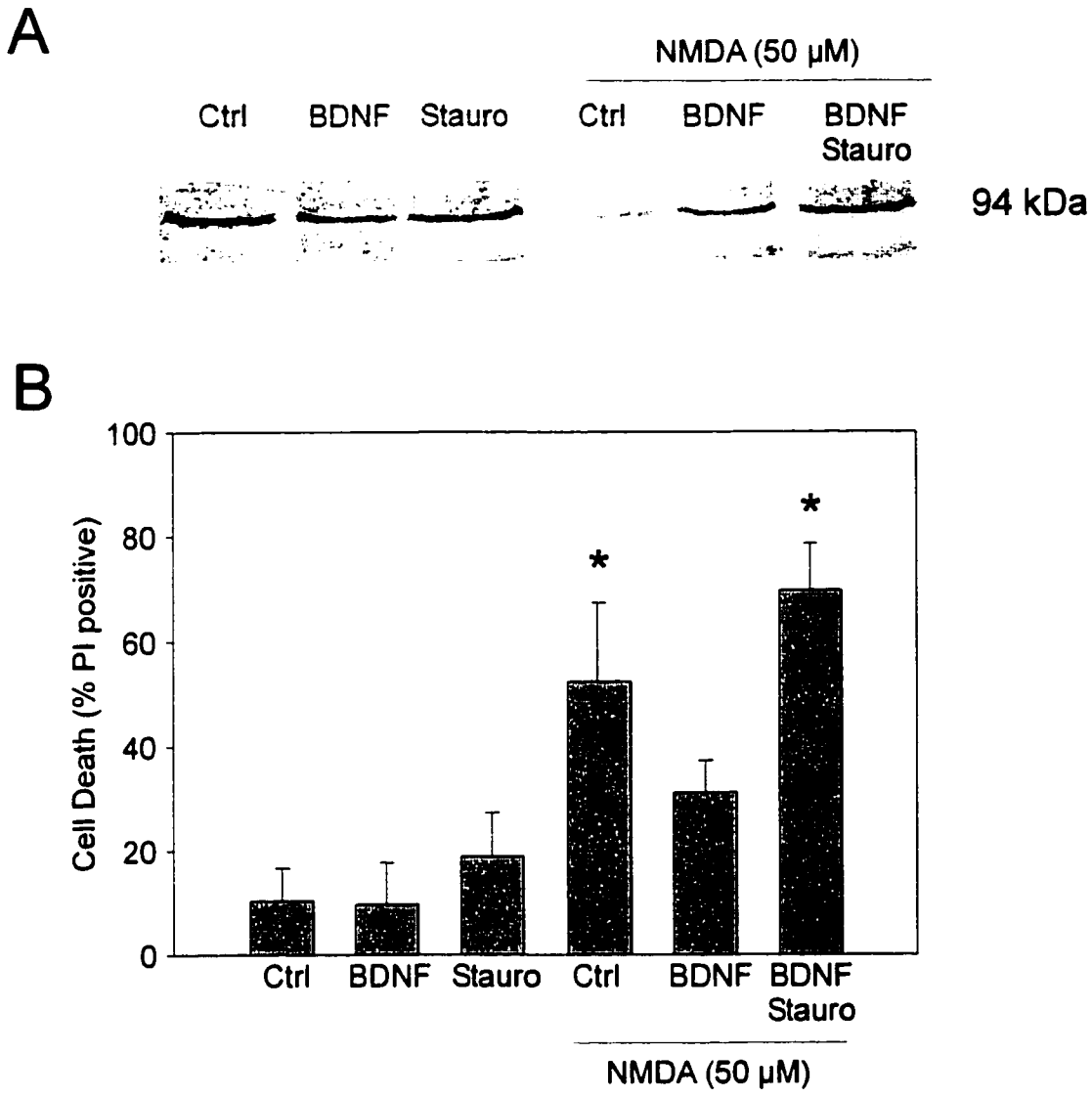

A
Ctrl
NMDA
C M C M
94
66
34
103
81
46.9
34.1
28.5
B
Ctrl
NMDA (50 μM)
94
120 30 60 120
Time (min)
(post-treatment)

Figure 3.9 The effect of BDNF on the loss of membrane 94 kDa form of calpastatin, and the effect of co-applied PKC inhibition. **A:** Control cultures maintained in conditioned media, or cultures treated overnight with BDNF (50 ng/ml) were subjected to NMDA or conditioned media alone (Ctrl) for 5 minutes in the presence or absence of staurosporine (Stauro, 50 nM). Following treatment cells were washed with 1 ml of CSS, and returned to conditioned media for two hours prior to being lysed and analysed by Western blot, using the anti-calpastatin antibody as described in Methods (section 3.2.5 and 1.2.4). **B:** Neuronal viability assessment of cells 24 hours after treatment as in A using PI staining (as described in section 2.1.3). Bars represent the mean of two separate experiments (n = 4 per experiment) $\pm$ standard deviation (* $p < 0.05$, significantly different from all other groups).


A
NMDA (50 μM)
Ctrl
BDNF
Stauro
Ctrl
BDNF
BDNF
Stauro
94 kDa
B
Cell Death (% PI positive)
100
80
60
40
20
0
*
*
Ctrl
BDNF
Stauro
Ctrl
BDNF
BDNF
Stauro
NMDA (50 μM)

GENERAL DISCUSSION

The actions, selectivity and regulation of the ubiquitous, Ca^{2+}-dependent, neutral protease calpain in neurons was the central focus of this thesis. It was demonstrated that calpain activation is selectively linked to the NMDA receptor, its activity contributes to cell injury resulting from toxic exposure to NMDA, and that inhibition of calpain rescues cells from NMDA-induced death. Furthermore, the selectivity of calpain activation with the NMDA receptor was observed in conjunction with unique activation properties and intracellular localization in neurons. Calpain was shown to be membrane associated in neurons, and it did not require autolysis for activation. This suggests that categorizing calpain as a "proenzyme" is inappropriate since the intact protease is clearly an active form. Based on the results of this thesis, it was shown that calpain activation in cultured neurons proceeds by a mechanism clearly distinct from this "traditional" route observed in erythrocytes and other non-CNS cells.

A central theme emerging from these data strongly suggests that intracellular signals coupled to the NMDA receptor are responsible for activating calpain already associated with cellular membranes in cortical neurons. This regulation was coupled to the NMDA-induced down regulation of PKC activity, however what mechanisms convey such specificity to NMDA's effects on both calpain and PKC are unknown. Recently, Sattler et al. (1999) reported that nitric oxide neurotoxicity was specifically coupled to NMDA receptor activation by PSD-95. As in this thesis, they demonstrated that equivalent increases in intracellular Ca^{2+} by non-NMDA means was non-toxic, suggesting the route of calcium in association with the degree of entry is the determining factor of

cell fate. Furthermore, it was demonstrated by the same authors that suppressing PSD-95 blocked Ca^{2+}-activated NO production by NMDA receptor activation, suggesting that PSD-95 imparts specificity to the excitotoxic Ca^{2+} signal. Given that the activation of calpain in cultured neurons is a tightly controlled event that appears to play a major role in excitotoxic processes, a similar association may exist and warrants direct investigation.

The selective regulation of calpain activity by the NMDA receptor is consistent with the proposed role of neuronal calpain activity under "physiological" conditions. Both the NMDA receptor and calpain have been highly implicated in synaptic plasticity and shaping of the nervous system. Under non-pathological conditions calpain is thought to mediate Ca^{2+}-dependent cell signaling events involved in synaptic plasticity and remodeling (Dosemeci and Reese, 1995; del Cerro et al., 1990; Denny et al., 1990; Lynch and Baudry, 1987). Indeed, calpain activation can significantly alter the morphology of postsynaptic densities by exacting limited and specific proteolysis of spectrin (Dosemeci and Reese, 1995). Aberrant activation of the protease can lead to hydrolysis of numerous substrates, but under limiting conditions calpain exacted significant morphological change by selectively cleaving spectrin, leaving other substrates such as CaMKII and tubulin intact (Dosemeci and Reese, 1995). These changes resulted in the widening and "loosening" of the PSD central lamina, and were thought to result in the exposure of previously occluded functional sites within this domain (Dosemeci and Reese, 1995). The role of calpain as a modulator of synaptic function is also supported by the observation that calpain activity results in increased receptor density at the postsynaptic site (Baudry et al., 1981), and may also functionally modulate glutamate-mediated alteration of

glutamate receptors by limited proteolysis (Bi et al., 1998a,b; Gellerman et al., 1997; Bi et al., 1997a,b; Bi et al., 1996; Bi et al., 1994). Under normal circumstances, it appears then that this protease is involved in redefining the signaling repertoire of specific neuronal areas in response to NMDA-mediated increases in Ca^{2+}. Under pathological conditions, when Ca^{2+} influx becomes excessive and prolonged, as opposed to brief and transient, it is easy to envision the destructive results of calpain activity on normal synaptic function. The mediators of plasticity become destructive, and mechanisms of synaptic rearrangement lead to derangement. Defining the modulators of calpain activation under both physiological and pathological conditions remains an important endeavour which may further our understanding of neuronal function. Fundamental to the impetus of this research, it is hoped that this knowledge could promote effective therapies for ameliorating neuronal destruction following injurious events such as stroke.

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