Immunolocalization of Calpain I-mediated Spectrin Degradation to Vulnerable Neurons in the Ischemic Gerbil Brain

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Transient ischemia-induced perturbations in calcium homeostasis have been proposed to lead to pathological activation of the cysteine protease calpain I and subsequent delayed neuronal death in the CA1 region of hippocampus. We report here on the design and characterization of antibodies selective for calpain-generated fragments of brain spectrin, and their use for immunoblot and immunohistochemical analyses of calpain activation following cerebral ischemia in the gerbil. Although spectrin was susceptible to degradation *in vitro* by many mammalian proteases, only calpain degraded spectrin to generate fragments immunoreactive with the antibodies. Following 5 min of global ischemia, immunoreactivity for calpain-degraded spectrin was rapidly (within 30 min) and markedly elevated in the perikarya and dendrites of several populations of forebrain neurons. The rapid calpain activation was completely prevented by the NMDA receptor antagonist MK-801. At later times postischemia, but prior to frank neuronal necrosis, calpain-degraded spectrin was restricted to hippocampal area CA1 pyramidal neurons. Silver impregnation histochemistry confirmed that neuronal damage was confined to area CA1. The results indicate that while nonpathological NMDA receptor stimulation can activate calpain, only those neurons showing sustained calpain activation are destined to die.

[Key words: calpain, ischemia, protein degradation, neuronal death, NMDA receptor, calcium overload]

Transient global cerebral ischemia can initiate a precipitous and sustained rise in intracellular calcium levels and subsequent delayed neuronal death in vulnerable regions of the CNS (Kirino, 1982; Pulsinelli et al., 1982; Siesjö, 1988). This ischemia-induced perturbation in calcium homeostasis could activate a number of calcium-dependent enzymes that may be involved in neuronal death, such as protein kinases, phospholipases, endonucleases, and proteases (Choi, 1988; Bazan, 1989; Manev et al., 1990; Seubert and Lynch, 1990; Farooqui and Horrocks, 1991). Although the relative contributions of various calcium-regulated processes to neuronal damage are not yet known, the calcium-activated cysteine protease calpain I, also known as μ -calpain, has several characteristics that suggest it may play a

role in the delayed neuronal death following transient global cerebral ischemia. First, calpain I is present in neurons, including those populations susceptible to ischemic cell death (Siman et al., 1985; Hamakubo et al., 1986; Perlmuter et al., 1988; Manev et al., 1991). Second, calpain I is activated by the low micromolar concentrations of free calcium that are believed to be reached intraneuronally under pathological conditions (Kudo and Ogura, 1986; Murphy et al., 1987). Third, there is considerable indirect evidence for calpain activation following ischemic or related toxic insults. Thus, insults that provoke neuronal degeneration also enhance the degradation of proteins that are preferred calpain substrates (Siman and Noszek, 1988; Seubert et al., 1989; Siman et al., 1989a; Lee et al., 1991; Manev et al., 1991). The protein degradation precedes overt neuronal disintegration and is selective for preferred calpain substrates (Siman and Noszek, 1988). Moreover, levels of intact calpain I decline, consistent with an autoproteolysis that is required for calpain activation (Siman and Noszek, 1988). Fourth, many of the preferred calpain substrates are major structural proteins (Burgoyne and Cumming, 1982; Siman et al., 1984; Zimmerman and Schlaepfer, 1984; Siman and Noszek, 1988; Johnson et al., 1989), suggesting that uncontrolled calpain activation could be involved in neuronal structural failure. Finally, protease inhibitors that block calpain have been shown to be neuroprotective in animal models of ischemia (Lee et al., 1991; Rami and Kriegstein, 1993), suggesting that calpain activation may be a pivotal link between ischemia-induced calcium overload and neuronal degeneration.

In spite of the considerable evidence associating calpain I activation with ischemic neuronal damage, it has not yet been possible to demonstrate definitively that calpain I activation occurs in ischemic neurons and is causally involved in neuronal death. For example, it cannot be ruled out that the ischemia-induced protein degradation may take place at least partly in non-neuronal cells or be mediated by proteases other than calpain. The reduction in proteolysis and neuronal death by protease inhibitors that block calpain is suggestive, but must be viewed with caution since the compounds that have been used are known to target several proteases, and may have other physiological effects as well. Furthermore, although the enhanced protein degradation precedes overt neuronal degeneration, it remains uncertain whether proteolysis may be stimulated as a secondary consequence of neuronal disintegration. It has also been reported that, in some systems, calpain inhibition does not protect neurons from toxin-induced death (Manev et al., 1991). Finally, calcium-independent mechanisms can contribute under certain circumstances to neuronal injury (Dubinsky and Rothman, 1991; Goldberg and Choi, 1993).

In order to address questions concerning the role of calpain

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activation in mediating the neuronal degeneration caused by transient global ischemia, we have generated antibodies selective for the calpain-produced cleavage products of brain spectrin. The use of antibodies specific for protease cleavage products is a novel approach to evaluating proteolytic activity in the brain *in vivo*, and has been successfully applied to the autolytic activation of calpain in platelets (Saïdo et al., 1992, 1993) and to the calpain-mediated turnover of protein kinase C in human monoblasts (Kikuchi et al., 1993). Using *in situ* detection of calpain-mediated proteolysis, we have demonstrated that calpain I is rapidly activated following global cerebral ischemia. The method has been used to map the calpain activation to distinct cell types and subcellular compartments, to identify neurotransmitter receptors responsible for calpain activation, and to investigate the time course of calpain activation relative to neurodegeneration.

Materials and Methods

Antibody production and immunoblotting. The peptides CQQQEVY and GMMPRDGC were synthesized by solid-phase methods and their structures confirmed by fast atom bombardment-mass spectrometry. Each peptide was conjugated through its cysteine residue to keyhole limpet hemocyanin with the heterobifunctional coupling agent maleimidobenzoyl-*N*-hydroxysuccinimide ester (Calbiochem, San Diego, CA; Hårlow and Lane, 1988). Conjugates were used to immunize rabbits. Abs 37 and 38 were directed against CQQQEVY, and gave essentially identical results, while Ab39 was prepared using GMMPRDGC. An antibody against purified rat brain spectrin (Ab212) has been described previously (Siman and Noszek, 1988; Siman et al., 1989a).

Antibodies 37, 38, 39, and 212 were evaluated by immunoblotting for reactivity with intact brain spectrin and the spectrin breakdown products generated upon cleavage by nine proteases. A crude membrane fraction was prepared from gerbil brain by homogenization in 10 vol of 50 mM Tris-HCl (pH 7.4), 4 mM EDTA at 4°C. Following centrifugation at $500 \times g$ for 5 min, the pellet was discarded and the supernatant centrifuged at $40,000 \times g$ for 20 min. The pellet was taken as the crude membrane fraction. Membranes were washed with one of four buffers: (I) 50 mM Tris-HCl (pH 7.4); (II) I + 10 mM CaCl₂; (III) 50 mM sodium acetate (pH 6.0), 1 mM EDTA, 5 mM 2-mercaptoethanol; (IV) II + 5 mM 2-mercaptoethanol. Membranes (50 μ l) were incubated with the following proteases (obtained from Sigma, St. Louis, MO, or Boehringer Mannheim, Indianapolis, IN) and additions for 1 hr at 37°C: human cathepsin B (50 mU) or cathepsin L (1.25 mU) in buffer III; human neutrophil elastase (250 ng), bovine α -chymotrypsin (50 ng), bovine trypsin (100 ng, TPCK-treated), human plasmin (10 mU), or human kidney urokinase (60 mU) in buffer I; human thrombin (0.4 U) + 200 μ M E64 in buffer II. In addition, the calpain endogenous to brain membranes (Baudry et al., 1981; Siman et al., 1983) was activated by incubation in buffer IV. Reactions were stopped with SDS-PAGE sample buffer and boiling. Samples were run on 5% gels, transferred to nitrocellulose, and immunostained (Siman and Noszek, 1988; Siman et al., 1989a) with either anti-brain spectrin (Ab212; 1:800), Ab38 (1:500), or Ab39 (1:300).

Ischemic surgery. Male Mongolian gerbils weighing 50–80 gm (Tumblebrook Farms, West Brookfield, MA) were anesthetized with Nembutal (50 mg/kg, i.p.). An anterior ventral midline incision was made in the neck, right and left common carotid arteries were isolated from the vagus nerve, and suture thread was tied around each artery to achieve total occlusion of blood flow for a period of 1, 5, or 10 min. Control animals were anesthetized but were not subjected to the carotid occlusion. At the end of the ischemic period, the suture threads were removed, and the incision was closed with wound clips after ascertaining that blood flow was completely restored to both carotid arteries. Body temperature was monitored with a rectal probe and maintained within normal physiological limits by the use of a heat lamp throughout the surgery and an incubator during the postoperative recovery period. After the surgery, the gerbils were anesthetized and killed at 30 min, or 1, 2, 5, or 10 d following ischemia. Those animals to be processed for silver staining or immunocytochemistry were perfused transcardially and the brains prepared as described below; animals to be processed for immunoblot analyses were decapitated and the dorsal hippocampus ho-

mogenized and prepared as described above. One group of gerbils received the noncompetitive NMDA receptor antagonist MK-801 (3 mg/kg, i.p.) immediately following a 5 min occlusion of the carotid arteries, and was processed for immunocytochemistry 2 d later. Three gerbils were used for each experimental condition.

Histology. The indirect immunoperoxidase histochemical procedure, used here to localize calpain-generated fragments of brain spectrin, has been described previously (Siman et al., 1989b). Briefly, at various times after ischemia, gerbils were deeply anesthetized with Nembutal and perfused with 0.1 M sodium phosphate buffer (pH 7.4) followed by 4% paraformaldehyde in phosphate buffer. Brains were postfixed in paraformaldehyde for 2–3 hr and cryoprotected overnight in 20% sucrose in 0.1 M sodium phosphate at 4°C. Sagittal sections were prepared from frozen brains at 45 μ m and incubated in 50% methanol containing 1% H₂O₂ for 30 min. After rinses in 20 mM Tris-HCl/150 mM NaCl (pH 7.4, TBS), sections were incubated for 30 min in 5% horse serum in TBS with 0.1% Triton X-100, and then with Ab37 (1:10,000) overnight at 4°C. After washes, sections were transferred to goat anti-rabbit IgG (Cappel; 1:75), followed by a rabbit peroxidase-antiperoxidase complex (Cappel; 1:1000). Diaminobenzidine (500 μ g/ml) and H₂O₂ (0.0015%) in TBS were used for color development. For preabsorption, 50 μ g of peptide immunogen (CQQQEVY) was combined with 1 ml of Ab37 (1:10,000) and incubated for 1 hr at room temperature. This primary antibody solution was then used as above. Additional control sections were handled as above, except for the absence of primary antibody.

Silver staining for degenerating neurons was performed by a modified Gallyas method, as described previously (Nadler and Evenson, 1983; Siman et al., 1989b).

Results

Production of antibodies that selectively react with calpain-generated spectrin breakdown products

Calpain I-mediated proteolysis of brain spectrin is limited, producing a single cleavage at an identified site that splits the α -subunit approximately in half (Harris et al., 1988). We used this information to design antibodies against short peptides corresponding to the COOH-terminus of the NH₂-terminal α -spectrin fragment (CQQQEVY) and the NH₂-terminus of the COOH-terminal α -spectrin fragment (GMMPRDGC) (Fig. 1). The reactivity of antibodies to the peptides was assessed in hippocampal membranes prepared from gerbil brain 2 d following transient global ischemia (Fig. 1). This insult has previously been shown to elicit an increase in spectrin degradation in hippocampus (Seubert et al., 1989; Lee et al., 1991). Anti-CQQQEVY (Abs 37 and 38) and anti-GMMPRDGC (Ab39) labeled 150 kDa polypeptides that were confirmed as spectrin breakdown products by their immunoreactivity with Ab212, raised against purified brain spectrin (Fig. 1). Unlike Ab212, however, the anti-peptide antibodies exhibited little or no reactivity with intact α -spectrin. Therefore, the epitopes recognized by the anti-peptide antibodies are inaccessible in intact spectrin, but become exposed by the proteolytic degradation of spectrin evoked by cerebral ischemia.

Because calpain I is known to cleave brain spectrin in the domain used to generate the anti-peptide antibodies, it is a candidate for the hippocampal protease activated *in situ* by cerebral ischemia. To investigate this possibility further, the ability of calpain and other proteases to cleave spectrin and form proteolytic fragments reactive with Ab38 and Ab39 was determined by immunoblot analysis. Gerbil brain membranes were treated with either calcium to activate the endogenous calpain (Baudry et al., 1981; Siman et al., 1983) or with eight other mammalian proteases (Fig. 2). The antibody to brain spectrin (Ab212) labeled the intact >240 kDa α -subunit as well as a number of smaller breakdown products generated by many of the proteases. Addition of calcium, cathepsin L, elastase, chy-

Figure 1. Design of antibodies that react with α -spectrin fragments generated by calpain I cleavage. The *top panel* shows the sequence of the domain in human α -spectrin that is cleaved by human calpain I (Harris et al., 1988). The peptide immunogens designed on the basis of this sequence are illustrated, linked to carrier protein (KLH, keyhole limpet hemocyanin). Antibody 38 was designed to react with the extreme COOH-terminus of the NH₂-terminal fragment; antibody 39, with the extreme NH₂-terminus of the COOH-terminal fragment. Antibody 212 was raised against purified rat brain spectrin. The *bottom panel* shows Western blots of gerbil hippocampal membranes prepared 3 d after a 5 min episode of cerebral ischemia. Note that Ab212 labels the α -subunit of spectrin as well as a closely spaced set of breakdown products. In contrast, Ab38 and Ab39 each label a single polypeptide comigrating with the breakdown products, but fail to react with intact spectrin. Molecular weight markers are shown on the *left* of each blot to aid alignment.

motrypsin, trypsin, thrombin, plasmin, and urokinase all led to spectrin cleavage and production of Ab212-immunoreactive fragments. The effect of calcium was blocked by the cysteine protease inhibitor E64, consistent with activation of calpain. In sharp contrast, Abs 38 and 39 labeled a 150 kDa polypeptide generated only by calcium treatment and, in the case of the former antibody, by chymotrypsin. Thus, *in vitro* activation of calpain, but not eight other mammalian proteases, generated polypeptide fragments similar to those generated *in vivo* by the ischemia-activated hippocampal protease. The polypeptides labeled by Abs 38 and 39 likely represent the two halves of calpain-cleaved α -spectrin. Higher-resolution SDS-PAGE confirmed that Ab38 labels the second largest spectrin fragment, while Ab39 reacts with the largest spectrin derivative (Boryczko-Coyne et al., unpublished observations).

Time course and localization of calpain activation following transient cerebral ischemia

Using Ab212 to intact brain spectrin, Western blot analysis revealed that spectrin breakdown products were present in the hippocampus as early as 30 min following a 5 min bilateral occlusion of the carotid arteries (Fig. 3). The magnitude of ischemia-induced spectrin degradation in the hippocampus peaked at 1–5 d, depending on the spectrin fragment, and was minimal by 10 d following the carotid occlusion. To investigate further

Figure 2. Specificity of Ab38 and Ab39 for α -spectrin fragments generated by calpain I, but not by other mammalian proteases. Gerbil hippocampal membranes were incubated with the indicated proteases, or with calcium to activate the calpain endogenous to brain membranes, as described in Materials and Methods. Spectrin and its fragments were identified by Western blotting with Abs 212, 38, and 39. Note that addition of calcium, cathepsin L, elastase, chymotrypsin, trypsin, thrombin, plasmin, or urokinase all produce smaller polypeptides immunoreactive with Ab212. In contrast, spectrin fragments immunoreactive with Ab38 are generated only by calcium and chymotrypsin, and a derivative immunoreactive with Ab39 is produced only by calcium. The calcium effects are blocked by E64, a cysteine protease inhibitor.

the identity of the hippocampal protease(s) activated at the various time points by transient ischemia, the same samples were immunolabeled with Abs 38 and 39, which are selective for calpain-generated spectrin derivatives. Like Ab212, these Abs labeled 150 kDa spectrin fragments present in ischemic hippocampus and barely detectable in control hippocampus, but unlike Ab212 exhibited little or no reactivity with intact spectrin subunits (>240 kDa). Generally, levels of the fragments were increased at 30 min, remained elevated for the next 2 d, and then declined. More specifically, the spectrin derivative reactive with Ab38 was present at high levels between 30 min and 2 d and apparently was further degraded to the third and smallest spectrin fragment, while levels of the Ab39-reactive fragment peaked at 2 d. The former pattern is similar to that of the second

largest spectrin fragment labeled by Ab212, while the latter pattern resembles that of the top (largest) Ab212-reactive fragment, again consistent with the labeling of distinct calpain-derived spectrin fragments by Abs 38 and 39.

The antibodies reactive with calpain-derived fragments of brain spectrin were used as neuroanatomical tools to localize calpain activation following cerebral ischemia. The pattern of immunohistochemical staining in sagittal sections of the gerbil brain for calpain-generated spectrin fragments was determined at 30 min, and 1, 2, and 10 d following ischemia (Fig. 4). Brain sections from control (nonischemic) animals exhibited only very light immunostaining of neuronal perikarya with Ab38 (Fig. 4A), whereas by 30 min following a 5 min bilateral occlusion of the carotid arteries, intense immunoreactivity was apparent in the parietal and frontal cortex (layers I, III, and V), piriform cortex, tenia tecta, striatum, ventral thalamus, and hippocampus (Fig. 4B). Hippocampal immunoreactivity at this time point was primarily confined to the CA3 sector, with some staining of neurons in the posterior CA1 subfield and at the CA2–CA3 border. The dentate gyrus was unstained. Immunoreactivity for calpain-degraded spectrin was neuron specific, and present in perikarya as well as basal and apical dendrites of hippocampal neurons (Figs. 4F, 5; see also Fig. 7) and the long primary dendrites of neocortical pyramidal neurons (Fig. 5).

At 24 hr following ischemia, cortical, thalamic, and striatal immunostaining was still present, but the hippocampal staining was more intense, with a broader distribution of immunoreactivity in the CA1 region (Fig. 4C). At this time, staining of apical and basal dendrites of pyramidal neurons in area CA1 became prominent. By 2 d following the 5 min ischemic insult, only the CA1 region of the hippocampus was intensely stained; the CA3 subfield of the hippocampus, and the cortex, thalamus, and striatum no longer exhibited significant immunoreactivity for calpain-degraded spectrin (Fig. 4D). By 10 d following ischemia, virtually all immunoreactivity had disappeared, and sections from ischemic brain were indistinguishable from those from control brain (Fig. 4E).

Preabsorption of Ab38 with an excess of the peptide immunogen totally blocked immunostaining of the tissue sections (Fig. 4, G vs F), consistent with specific labeling of calpain-degraded spectrin epitopes.

Activation of calpain following short or long duration of ischemia

In order to differentiate between the effects of nondamaging versus severely damaging periods of ischemia on calpain activation, we compared the results of a short (1 min), intermediate (5 min), or long (10 min) interruption of blood flow. One minute of occlusion of the carotid arteries, which does not lead to neuronal degeneration (data not shown), resulted in the appearance of a narrow band of Ab38-immunoreactive neurons in the CA2–CA3 border region of the hippocampus by 30 min postocclusion (Fig. 6A). There was no other immunoreactivity apparent at 30 min, and by 3 d following carotid occlusion the brain was devoid of immunoreactivity (Fig. 6B).

In sharp contrast, 10 min of occlusion of the carotid arteries produced a pattern of staining in the hippocampus (CA1 and CA2–CA3 border zone), cortex (layers III and V), tenia tecta, and thalamus that was manifested at 30 min and persisted for 3 d following the ischemic insult (Fig. 6D). Some cases also exhibited prominent immunoreactivity in the striatum and olfactory tubercle. At 3 d following an intermediate (5 min) ische-

Figure 3. Time course of calpain-mediated spectrin degradation triggered by ischemia. Gerbil hippocampal membranes were prepared at the indicated times after a 5 min episode of cerebral ischemia. Shown here are Western blots stained with Abs 212, 38, and 39. Ab212 labels three smaller immunoreactive fragments whose content increases following ischemia, peaking at different times postischemia. Ab38 labels a polypeptide whose content is high from 30 min to 2 d and then declines thereafter, while the spectrin derivative labeled by Ab39 peaks at 2 d and then decreases.

mic insult the immunoreactivity in the cerebral cortex, tenia tecta, and thalamus was either less robust (Fig. 6C) or, more commonly, completely absent (Fig. 4D). Thus, a short ischemic episode produced no prolonged calpain activation, whereas an intermediate episode led to sustained calpain activation confined primarily to hippocampal area CA1, and a long episode induced sustained calpain activation that invariably extended outside of hippocampus to include regions of cerebral cortex and thalamus.

Silver impregnation histochemistry performed 3 d after a 5 min ischemic insult demonstrated degeneration of neurons in

Figure 4. Immunohistochemical mapping of regional distribution and time course of calpain-mediated spectrin breakdown. Sagittal sections of control, untreated gerbil brain (*A*), or brains of gerbils subjected to 5 min of global cerebral ischemia (*B–F*) were immunostained with anti-CQQQEVY, which reacts with the second largest calpain-derived spectrin fragment, as described in Materials and Methods. *B*, 30 min postischemia; *C*, 1 d postischemia; *D*, 2 d postischemia; *E*, 10 d postischemia. *F*, Higher-power view of area CA1 of the hippocampus, 2 d postischemia. *G*, Same as in *F*, except that the antibody to calpain-degraded spectrin was preabsorbed with peptide immunogen. *PC*, parietal cortex; *FC*, frontal cortex; *TT*, tenia tecta; *Pir*, piriform cortex; *S*, striatum; *VT*, ventral thalamus; *H*, hippocampus; *CA1*, hippocampal CA1 region. Scale bars: *A–E*, 1 mm; *F* and *G*, 60 μ m.

the CA1 and CA2–CA3 border regions of the hippocampus (Fig. 6*E,F*). This pattern of silver degeneration corresponded with the pattern of intense hippocampal immunoreactivity for calpain-degraded spectrin seen under the same conditions of ischemia (Fig. 6*C*).

Attenuation of ischemia-induced calpain activation with MK-801

To begin to define the receptor-based mechanisms by which calpain activation and spectrin degradation are triggered, gerbils

Figure 5. Localization of calpain-degraded spectrin in hippocampal and cerebral cortical neurons. The brains of gerbils subjected to 5 min of ischemia were immunostained for calpain-degraded spectrin as described in Materials and Methods. *A*, Hippocampal CA2–CA3 border, 30 min postischemia. Asterisk denotes a pyramidal cell body. Arrowheads denote immunoreactive basal dendrites, while the arrows denote labeled apical dendrites. *B*, Parietal cortex, layer V, 30 min postischemia. Immunoreactivity for calpain-degraded spectrin is present in the cell bodies and primary dendritic arbor of pyramidal neurons. Scale bar, 10 μ m.

were treated with the noncompetitive NMDA receptor antagonist MK-801 immediately following a 5 min occlusion of the carotid arteries. Treatment of gerbils with a dose of MK-801 (3 mg/kg, i.p.) that partially blocks hippocampal neuronal death (Roberts-Lewis and Siman, 1993) resulted in a partial attenuation of immunoreactivity for calpain-degraded spectrin in the CA1 region of hippocampus 2 d following ischemia (Fig. 7, *A* vs *B*). Thus, a treatment that reduces ischemia-induced hippocampal neurodegeneration results in a parallel decrease in calpain activation and spectrin degradation. At 1 d following the ischemia, MK-801 treatment produced an essentially complete blockade of immunostaining in the cortex (Fig. 7, *C* vs *D*), thalamus (Fig. 7, *E* vs *F*) and striatum (data not shown), suggesting that the rapid and more widespread calpain-mediated spectrin degradation results primarily from activation of NMDA receptors.

Discussion

We have developed antibodies selective for the two calpain I-generated cleavage products of brain spectrin, and have used immunoblotting and immunohistochemistry to provide direct evidence that calpain I is activated in neurons following cerebral ischemia. Immunogens were designed that mimic epitopes at the extreme COOH-terminus of the NH₂-terminal α -spectrin calpain-generated fragment, and the extreme NH₂-terminus of the COOH-terminal fragment, but which are inaccessible in the spectrin holoprotein (Fig. 1). Moreover, the antibodies selectively react with α -spectrin fragments generated by calpain I cleavage (Harris et al., 1988), but not with spectrin derivatives produced by a variety of other mammalian proteases (Fig. 2), which apparently cleave α -spectrin at different sites than calpain. While the results do not prove definitively that calpain I is the endogenous protease responsible for enhanced spectrin degradation, the immunological evidence that the endogenous protease and calpain I both cleave α -spectrin at the same site,

coupled with previous observations linking calpain to excitotoxic- and ischemia-evoked protein degradation (Siman and Noszek, 1988; Seubert et al., 1989; Siman et al., 1989a), strongly argues for the involvement of calpain I. The antibody-based approach described here is a useful method for the *in situ* detection of calpain activation and protein degradation in mammalian brain.

Immunoreactivity for calpain-degraded spectrin was evident in hippocampus, cortex, thalamus, and striatum of the gerbil brain at 30 min following a 5 min occlusion of the carotid arteries, consistent with previous immunoblot analyses showing spectrin degradation in these areas (Seubert et al., 1989). Unlike prior studies, however, the present method permitted neuroanatomical localization of calpain activation within discrete cell populations and subcellular compartments. Immunoreactivity for calpain-cleaved spectrin was neuron specific, and present in both cell bodies and the basal and apical dendrites of hippocampal pyramidal cells as well as the primary dendrites of neocortical pyramidal neurons, extending into layer I (Figs. 4–6). A laminar distribution of staining was evident in cortical neurons (layers III and V), and in the striatum (rostral and caudal poles), corresponding to neuronal populations that possess greater vulnerability to ischemic damage (Kirino, 1982; Pulsinelli et al., 1982; Smith et al., 1984; Crain et al., 1988). In contrast to the staining at short postischemia times, by 2 d following a 5 min occlusion immunoreactivity for calpain-degraded spectrin was restricted to the CA1/CA2 region of hippocampus (Fig. 4). Although it cannot be ruled out that temporal differences in the regional distribution of calpain-degraded spectrin may be partly due to rapid clearance of spectrin fragments from all brain regions except for hippocampal area CA1, additional observations argue for the contribution of a sustained calpain activation in hippocampus. Immunoblot experiments with antibodies 212 and 39 indicate that levels of hippocampal calpain-degraded spectrin continue to increase between 1 and 2 d postischemia (Fig. 3).

Figure 6. Effects of varying the period of ischemia on calpain-mediated spectrin degradation. *A*, Hippocampus, 1 min of ischemia, 1 d postinsult. Note the intense immunoreactivity for calpain-degraded spectrin restricted to neuronal perikarya and dendrites at the CA2-CA3 border (arrow).

Figure 7. Attenuation of calpain-mediated spectrin breakdown by the NMDA antagonist MK-801: gerbil brain subjected to 5 min of cerebral ischemia, immunostained for calpain-degraded spectrin. *A*, CA1 region of the hippocampus, 2 d postischemia. *B*, Same as *A*, except MK-801 was administered at time of ischemia. *C* and *D*, Layers I–III of the parietal cortex, 1 d postischemia. *E* and *F*, Thalamus, 1 d postischemia. *C* and *E*, control; *D* and *F*, MK-801. Note that MK-801 reduces immunoreactivity for calpain-degraded spectrin in hippocampus at a time when this brain region is the only one consistently immunopositive. At earlier times, MK-801 completely blocks immunostaining for calpain-degraded spectrin. Scale bars: *A* and *B*, 100 μ m; *C* and *D*, 50 μ m; *E* and *F*, 25 μ m.

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B, One minute of ischemia, 3 d postinsult. There is no sustained hippocampal immunoreactivity for calpain-degraded spectrin. *C*, Five minutes of ischemia, 3 d postinsult. Immunostaining for calpain-degraded spectrin persists in the hippocampus. *D*, Ten minutes of ischemia, 3 d postinsult. Immunoreactivity for calpain-degraded spectrin is prominent in hippocampus, cortical layers III and V, tenia tecta (*TT*), and olfactory tubercle (*Tu*). *E*, CA1 region of hippocampus after 5 min of ischemia, 3 d postinsult. Silver impregnation histochemistry to localize degenerating neurons. *Arrowheads* denote healthy neuronal cell bodies, while the *arrows* denote degenerating neurons. *F*, CA2–CA3 border region of hippocampus after 5 min of ischemia, 3 d postinsult. Silver staining is as in *E*. Scale bars: *A* and *B*, 200 μ m; *C* and *D*, 1 mm; *E*, 25 μ m; *F*, 50 μ m.

Furthermore, the intensity of immunoreactivity for calpain-degraded spectrin markedly increases in hippocampal area CA1 during this time period (Fig. 4). Collectively, the results suggest that in response to cerebral ischemia, calpain activation is both rapidly initiated and arrested in many forebrain regions, but is sustained for at least 48 hr in area CA1 of hippocampus.

Pyramidal neurons in the CA1 subfield of hippocampus exhibit sudden, massive degeneration at 3–4 d following a mild, transient (5 min) ischemic insult, a phenomenon known as “delayed neuronal death” (Kirino, 1982; Pulsinelli et al., 1982). In contrast, the CA3 region and granule cell layer of dentate gyrus are spared under the same ischemic conditions. Neurons in neocortex, primarily layer III, dorsolateral striatum, and ventral thalamus, have also been shown to degenerate following moderate transient ischemia, with cortical and thalamic neurons showing a delayed death similar to that occurring in hippocampus, whereas striatal neurons appear to degenerate more rapidly (Pulsinelli et al., 1982; Smith et al., 1984; Crain et al., 1988; Lin et al., 1990). Under our conditions, only CA1 neurons invariably die after a 5 min period of ischemia, beginning to degenerate at 2 d after the insult based on silver impregnation histochemistry (Fig. 6). Frank neuronal necrosis is not seen until the third and fourth days. Thus, the calpain activation and spectrin degradation occur prior to neuronal death, and are not likely to result simply from the disintegration of these cells. Moreover, in brains of gerbils subjected to a 1 min period of ischemia, which does not produce neuronal damage, calpain activation is not sustained for 2 d in any brain region (Fig. 6). On the other hand, a 10 min ischemic insult, which results in more widespread neuronal damage, leads to persistent accumulations of calpain-degraded spectrin in neocortex, thalamus, and striatum, as well as hippocampus, at 3 d following the ischemia. The results are consistent with the hypothesis that calcium overload and calpain I activation are early and obligatory events contributing to neuronal degeneration (Siman and Noszek, 1988; Seubert and Lynch, 1990). Further supporting this hypothesis, protease inhibitors that block calpain are reportedly neuroprotective in animal models of ischemia (Inuzuka et al., 1990; Lee et al., 1991; Rami and Kriegstein, 1993).

The underlying mechanisms that account for the delayed and selective vulnerability of neurons to transient ischemia are not completely understood, but excessive release of the excitatory neurotransmitter glutamate has been clearly linked to ischemia-induced neurotoxicity, and blockade of the NMDA class of glutamate receptors can reduce ischemic neuronal damage (Simon et al., 1984; Gill et al., 1987; Boast et al., 1988; Rod and Auer, 1989). Although regions of the brain that are selectively vulnerable to transient ischemia are enriched in NMDA receptors (Monaghan and Cotman, 1985), there is not a consistent relationship between NMDA receptor density and vulnerability to ischemia-induced neurodegeneration. In the present study, treatment of gerbils with a neuroprotective dose of the noncompetitive NMDA antagonist MK-801 (Gill et al., 1987; Roberts-Lewis and Siman, 1993) completely eliminated immunoreactivity for calpain-degraded spectrin in the neocortex, thalamus, and striatum at 24 hr following a 5 min ischemic insult, and partially blocked hippocampal area CA1 immunoreactivity at 2 d. This indicates that, after a 5 min ischemic episode, calpain activation is mediated at least partly by NMDA receptor activation in hippocampal area CA1, and almost exclusively by NMDA receptors in other regions of the forebrain. It is noteworthy that the incomplete blockade of hippocampal calpain

activation by MK-801 in the present study is consistent with the incomplete or variable anti-ischemic effects obtained with this drug by other investigators (Warner et al., 1991). Clearly, sustained activation of NMDA receptors alone cannot account for the selective vulnerability of neurons to ischemia. Non-NMDA receptors, particularly of the AMPA class, probably contribute to the calpain activation (Caner et al., 1993) and ischemic damage (Sheardown et al., 1990; Buchan et al., 1991). The distribution of calpain I also appears not to be a major risk factor for vulnerable neurons, since the level of calpain I-like immunoreactivity in pyramidal neurons of area CA1 is relatively low compared with other neuronal populations (Siman et al., 1985; Hamakubo et al., 1986; Perlmutter et al., 1988). Nevertheless, our finding of a sustained calpain activation confined to a vulnerable neuronal population suggests that either a selective calcium overload, or a selective sensitivity to calcium overload, is a key feature of neuronal susceptibility to degeneration.

In addition to containing elevated levels of the degradation products of the actin-binding protein spectrin, neurons vulnerable to cerebral ischemia have reduced content of neurofilament polypeptides NF-H and NF-M (Ogata et al., 1989) and the microtubule-associated protein MAP2 (Kitagawa et al., 1989). The fact that these polypeptides are preferred calpain I substrates (Zimmerman and Schlaepfer, 1984; Siman and Noszek, 1988), coupled with the evidence presented here and elsewhere for activation of calpain I following ischemia (Seubert et al., 1989), suggests that calpain I activation mediates the ischemia-triggered degradation of all of these proteins. The activation of calpain in hippocampal dendrites following ischemia (Figs. 5, 6) is consistent with the reported decrease in dendritic proteins such as MAP2, and supports the proposal that dendrites are particularly sensitive to ischemic stress (Kitagawa et al., 1989). Moreover, spectrin and the neurofilament and microtubule polypeptides are components of the three major cytoskeletal systems of neurons, raising the possibility that one contribution of sustained calpain activation to neurodegeneration may be in the structural collapse of the neuron.

Calpain I activation in the dendrites of forebrain neurons immediately following ischemic insults not leading to degeneration of these neurons (Figs. 4, 6), and the demonstrated involvement of NMDA receptors in this activation have important implications for the functional role of calpain I under nonpathological conditions. NMDA receptors participate in phenomena associated with synaptic plasticity, such as long-term potentiation and the formation of ocular dominance columns during visual cortical development (for reviews, see Bekkers and Stevens, 1990; Collingridge and Singer, 1990). It has been proposed that calpain might be involved in synaptic structural changes underlying learning and memory processes (Lynch and Baudry, 1984; Siman et al., 1987). The results described here provide evidence that nonpathological NMDA receptor stimulation leads to dendritic calpain activation, consistent with a role for calpain in signal transduction processes contributing to neuronal plasticity. Future investigations of the role of calpain I in events involving NMDA receptor-mediated neuronal plasticity should be facilitated by the use of the antibody-based method described here for *in situ* detection of protease activation.

In conclusion, we have used antibodies selective for spectrin fragments generated by calpain cleavage to demonstrate that neurons exhibiting prolonged calpain activation following tran-

sient cerebral ischemia are destined to die. The results support the hypothesis that the sustained activation of calpain is causally related to delayed neuronal death, and emphasize that inhibitors of calpain should be investigated further as potential therapeutic agents in the treatment of ischemic or related neurological disorders.

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