

# The pathogenic activation of calpain: a marker and mediator of cellular toxicity and disease states

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Received for publication 5 June 2000

Accepted for publication 14 August 2000

**Summary.** Over-activation of calpain, a ubiquitous calcium-sensitive protease, has been linked to a variety of degenerative conditions in the brain and several other tissues. Dozens of substrates for calpain have been identified and several of these have been used to measure activation of the protease in the context of experimentally induced and naturally occurring pathologies. Calpain-mediated cleavage of the cytoskeletal protein spectrin, in particular, results in a set of large breakdown products (BDPs) that are unique in that they are unusually stable. Over the last 15 years, measurements of BDPs in experimental models of stroke-type excitotoxicity, hypoxia/ischemia, vasospasm, epilepsy, toxin exposure, brain injury, kidney malfunction, and genetic defects, have established that calpain activation is an early and causal event in the degeneration that ensues from acute, definable insults. The BDPs also have been found to increase with normal ageing and in patients with Alzheimer's disease, and the calpain activity may be involved in related apoptotic processes in conjunction with the caspase family of proteases. Thus, it has become increasingly clear that regardless of the mode of disturbance in calcium homeostasis or the cell type involved, calpain is critical to the development of pathology and therefore a distinct and powerful therapeutic target. The recent development of antibodies that recognize the site at which spectrin is cleaved has greatly facilitated the temporal and spatial resolution of calpain activation *in situ*. Accordingly, sensitive spectrin breakdown assays now are utilized to identify potential toxic side-effects of compounds and to develop calpain inhibitors for a wide range of indications including stroke, cerebral vasospasm, and kidney failure.

**Keywords:** age, Alzheimer's disease, excitotoxicity, ischemia, review, spectrin breakdown

### Abbreviations

AMPA,  $\alpha$ -amino-3-hydroxy-5-methylisoxazole-4-propionic acid  
AP5, DL-2-amino-5-phosphonopentanoic acid  
BDP, spectrin breakdown product  
BDP<sub>N</sub>, 147-kDa amino-terminal BDP mediated by calpain I  
BDP<sub>C</sub>, 152-kDa carboxy-terminal BDP mediated by calpain I  
CORT, corticosterone  
KA, kainic acid  
NMDA, *N*-methyl-*D*-aspartate  
PKC, protein kinase C.  
Single letter notation for amino acids are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

A rise in the concentration of intracellular calcium ( $[Ca^{2+}]_i$ ) has been identified as a primary trigger of pathology in most paradigms used to study cellular responses to acute, definable insults (e.g. ischemia, excitotoxicity; see Mattson 1992, 1994). Disturbances in calcium homeostasis also have been observed in tissues from aged humans both with and without associated age-related pathologies such as Alzheimer's disease. Thus, it is likely that some of the actions of calcium in discrete pathological events are also relevant to less defined, and more protracted, degenerative conditions. Calcium-dependent processes which represent points of convergence among a heterogeneous set of degenerative mechanisms are therefore logical targets for intervention strategies, and have the potential to generate valuable markers of pathogenesis.

An observation common to the types of pathologies noted above is the activation of the cysteine protease calpain, a calcium-dependent, nonlysosomal enzyme known to exist widely in animal tissues (see Melloni & Pontremoli 1989 and Croall & DeMartino 1991 for reviews of calpain biochemistry). The activated protease acts on a host of endogenous proteins (Takahashi 1990) including the cytoskeletal element spectrin. Calpain-mediated proteolysis of spectrin leads to the immediate production of breakdown products (BDPs) of ~150 kDa in size. The BDPs are relatively stable and can be readily measured after immunolabelling with antibodies raised to intact spectrin (Siman *et al.* 1984; Seubert *et al.* 1988a, b; Siman & Noszek 1988) or to peptide sequences on either side of the calpain cleavage site (Saido *et al.* 1993; Roberts-Lewis *et al.* 1994; Bahr *et al.* 1995). Rapid and sustained increases in the levels of spectrin BDPs have been observed across a broad spectrum of experimentally induced and insidious pathologies to be reviewed here. Taken together, these studies implicate calpain activation as a primary contributor to the onset and progression of degeneration in vulnerable populations of neurones and

other cell types. Accordingly, assays for calpain activity provide a sensitive technique for detecting the earliest stages of pathogenesis.

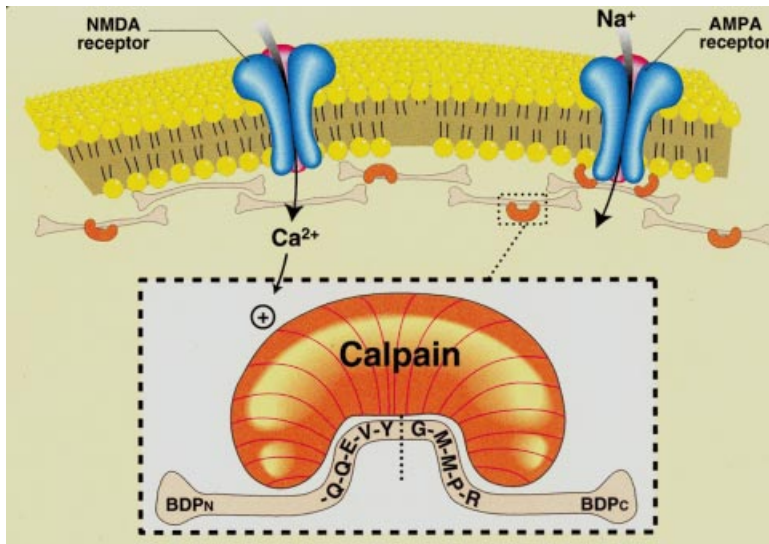
### Calpain-mediated proteolysis of spectrin

Baudry *et al.* (1981) first demonstrated that low concentrations of calcium stimulate leupeptin-sensitive proteolysis of a large protein doublet in brain synaptic membranes. The doublet was later identified as spectrin ( $\alpha$  and  $\beta$  subunits), which is a major actin-binding cytoskeletal component and a preferred substrate for calpain (Siman *et al.* 1984; Boivin *et al.* 1990), particularly in the presence of calmodulin (Seubert *et al.* 1987). The  $\alpha$ -subunit is cleaved by calpain between Tyr<sup>115</sup> and Gly<sup>116</sup> of the 11th spectrin repeat unit (Harris *et al.* 1989; Moon & McMahon 1990) resulting in two BDPs of nearly equal electrophoretic mobility. Polyclonal antibodies developed and affinity-purified against native spectrin recognize these fragments on western blots, and quantification of the immunolabelled cleavage products has been used extensively to measure calpain activation *in situ*.

Spectrin breakdown-specific antibodies also have been developed to epitopes exposed by the calpain cleavage event (Saido *et al.* 1993; Roberts-Lewis *et al.* 1994; Bahr *et al.* 1995). As described in Figure 1, antibodies to the five amino acid sequence (QQEVY) at the carboxyl end of the amino-terminal fragment (anti-BDP<sub>N</sub>) recognize an antigen of ~150 kDa in brain membrane samples that were treated with calcium to activate endogenous calpain. Similarly, anti-BDP<sub>C</sub> antibodies to the calpain-rendered epitope of the carboxy-terminal fragment of spectrin (residues GMMPR) label a 155-kDa antigen in calcium-treated, but not control, membranes. Thus, antibodies against new spectrin termini exposed by calpain proteolysis selectively label distinct proteolytic fragments. These fragments correspond to those described throughout much of the literature as BDP1 and BDP2 (Seubert *et al.* 1988a,b; Siman & Noszek 1988). Occasionally, antibodies to native spectrin also recognize a third calcium-induced cleavage product, forming a triplet pattern with the larger two BDPs (Roberts-Lewis *et al.* 1994; Bahr *et al.* 1995). The smaller proteolytic product may originate from the  $\beta$ -subunit of spectrin, which is also labelled by anti-spectrin antibodies.

### Threshold and subcellular locus of the calpain-spectrin interaction in glutamatergic neurones

The relevance of a particular enzymatic reaction to



**Figure 1.** Excitotoxic activation of calpain. Over-stimulation of excitatory amino acid receptors can lead to a rise in intracellular calcium sufficient to activate calpain. This activation event can be monitored with antibodies selective for new terminal domains produced by the cleavage of key proteins. Shown is calpain targeting its recognition sequence in spectrin, a preferred substrate that makes up a large part of the cytoskeletal network, resulting in the production of aminoterminal (BDP<sub>N</sub>) and carboxyterminal (BDP<sub>C</sub>) breakdown products.

pathology depends largely on such factors as activation threshold, subcellular distribution, substrate selectivity, and the extent to which the proteolytic consequences are reversible. These considerations mirror a set of constraints which initially were used to implicate subtle levels of calpain-mediated spectrin proteolysis in the production of synaptic plasticity (Lynch & Baudry 1984; also see Vanderklish *et al.* 1995, 1996), and which ultimately led to a consideration of calpain over-activation as a critical factor in neuropathology. There are two isoforms of calpain:  $\mu$ -calpain which is activated by micromolar calcium levels and m-calpain activated by millimolar calcium. The  $\mu$ -calpain species, or calpain 1, is found in dendrites (Fukuda *et al.* 1990; Perlmutter *et al.* 1990) and it undergoes autolytic activation (Suzuki *et al.* 1981; Inomata *et al.* 1988; Saido *et al.* 1992) at calcium concentrations (low  $\mu$ M) within the range of those achieved in dendritic spines during intense stimulation of glutamatergic synapses (Muller & Connor 1991; Guthrie *et al.* 1991; Faddis *et al.* 1997). Autolysis occurs at the plasma membrane (Pontremoli *et al.* 1985) so as to influence, for example, pp60<sup>Src</sup>, protein kinase C (PKC), phosphatase 1B, and focal adhesion kinase (Saido *et al.* 1994; Cooray *et al.* 1996; Rock *et al.* 1997) and several types of adhesion molecules (see Bahr *et al.* 1999) during calpain's many roles in cell-signalling cascades. The autolytic activation also allows the protease to contribute in signal transduction events related to cellular structure and synaptic organization (Fox *et al.* 1993; Earnest *et al.* 1995; Inomata *et al.* 1996; Capaldi *et al.* 1997; Kawasaki *et al.* 1997; Bahr, 2000).

The activation threshold of calpain 1 is well above the

resting  $[Ca^{2+}]_i$  ( $\sim 80$  nM), making it unlikely that the protease would be activated by fluctuations in calcium concentrations associated with routine synaptic activity mediated predominantly by AMPA receptors. Indeed, under control conditions BDPs are at low or undetectable levels in immunoblot assays of adult brain homogenate (1–2% of the total antispectrin immunoreactivity), a finding corroborated by immunohistochemical methods. However, western blot analyses have shown that BDPs are produced in the hippocampus during direct (Seubert *et al.* 1988b; del Cerro *et al.* 1994) or indirect (Bahr *et al.* 1995) stimulation of NMDA receptors. That calpain may become activated during physiological NMDA receptor stimulation is suggested by the potent blocking action of calpain inhibitors on long-term potentiation, a NMDA receptor-dependent form of synaptic plasticity (del Cerro *et al.* 1990; Denny *et al.* 1990; Oliver *et al.* 1990). The implied relationship between calpain activation and synaptic transmission conditions that evoke large increases in  $[Ca^{2+}]_i$  (i.e. high frequency activity) was confirmed *in situ* using BDP-specific antibodies (Vanderklish *et al.* 1995). Spectrin BDPs were found to accumulate in postsynaptic elements of CA1 pyramidal cells to an extent that correlated with the number of short, high frequency bursts of afferent stimulation delivered to Schaffer-collateral projections; low-frequency stimulation did not activate calpain. High resolution studies employing a calpain substrate that exhibits fluorescence resonance energy transfer indicate that activation of calpain occurs in dendritic spine heads following stimulation of NMDA receptors (Vanderklish *et al.* 2000). The above results emphasize that calpain activation in adult glutamatergic neurones likely has a

physiological role but is a relatively infrequent event, potentially reserved for specialized synaptic modulation events such as long-term potentiation. The distinction is made here between calpain activation in neurones of the adult nervous system and in developing neurones. Studies conducted *in vitro* and *in vivo* suggest that calpain is involved in the regulation of process outgrowth and differentiation (Oshima *et al.* 1989; Pinter *et al.* 1994). They further indicate that calpain activity is controlled much more dynamically during development, and potentially 'tolerated' at a higher level. They also indicated that spectrin BDPs are induced by modest levels of NMDA receptor activation and associated calcium influx.

### Pathogenic calpain activation

Functionally relevant calpain activation can be expected to be a highly localized and selective event *in vivo* for several reasons: (i) calcium elevation in neurones is tightly regulated by energy-dependent buffering and extrusion systems, and can be compartmentalized by dendritic structure; (ii) routes of calcium entry (e.g. voltage-gated and receptor-operated channels) exhibit distinct distributions, often restricted to particular lamina or neural populations; and (iii) inhibitory (calpastatin) and stimulatory factors (Takeyama *et al.* 1986; Melloni *et al.* 1998) modulate the activity and probably the substrate selectivity of calpain. However, given the broad list of calpain substrates and the irreversible nature of proteolysis, a challenge to any one of the above systems could permit more generalized calpain activation, resulting in inherently pathological rather than functional proteolysis. Release of intracellularly stored calcium and the autocatalytic priming of calpain are also likely to exacerbate the nature of the proteolytic response (Shea 1997a).

Considered next is evidence that over-activation of calpain is, in multiple circumstances, an early and causative event in the development of pathology. As will become evident, assays based on detection of spectrin BDPs can be of great value in describing the link between calpain activation and pathology.

### Excitotoxicity

Intense stimulation of NMDA receptors leads to BDP formation in the hippocampus (Seubert *et al.* 1988b; Siman *et al.* 1989) (see Figure 1). This effect, as shown in studies utilizing organotypic hippocampal slice cultures (Stoppini *et al.* 1991; Bahr 1995), is correlated with the amount of receptor stimulation (del Cerro *et al.* 1994;

Vanderklish *et al.* 1995) and can be detected after only 2 min of NMDA exposure (Bahr *et al.* 1995). This is far in advance of histological signs of degeneration observed in the hippocampus following administration of NMDA for hours to days *in vivo* (Siman & Noszek 1988; Siman *et al.* 1989) and precedes the onset of propidium iodide staining evident after ~30 min of NMDA exposure *in vitro* (see Vornov *et al.* 1991). Both the proteolytic (Seubert *et al.* 1988b; del Cerro *et al.* 1994; Bahr *et al.* 1995) and degenerative (Siman *et al.* 1989) responses to excessive NMDA receptor stimulation are blocked by NMDA receptor channel antagonists. The rapid nature of calpain activation and the resultant BDP induction raises the question of what degree of NMDA receptor-induced proteolysis can be tolerated by neurones. Electrophysiological results suggest that the temporal limit is less than 15 min at saturating concentrations of NMDA (Blake *et al.* 1988). Correspondingly, a 15-min NMDA infusion caused persistent BDP formation in cultured hippocampal slices, while a 5-min infusion produced only transient breakdown of spectrin (Bahr *et al.* 1995). It also was demonstrated in cultured slices that a ~50% reduction in calpain 1 levels using antisense techniques dramatically improved the recovery of evoked synaptic potentials following a 15-min infusion of NMDA (Bednarski *et al.* 1995). The translational suppression of calpain was associated with an expected reduction in NMDA-induced BDPs detected by immunoblot or immunocytochemistry. These studies indicate that calcium-dependent proteolysis induced at a large number of sites becomes pathogenic within a short time. With regard to the intensity of NMDA receptor stimulation, levels of BDP formation and neurodegeneration correlate with the amount of NMDA administered (see Siman *et al.* 1989). Moreover, there is an overlap among areas of unusual NMDA vulnerability, regions of high NMDA receptor density, and loci of spectrin BDP induction. The hippocampal field CA1 exhibits the greatest vulnerability to NMDA (Vornov *et al.* 1995), contains the highest concentration of NMDA receptors in the hippocampus, and manifests the most robust BDP staining following a short exposure to the excitotoxin (Bednarski *et al.* 1995). These data argue that the relative susceptibility of neurones to NMDA is defined by the extent to which calpain is activated by the associated calcium channel response.

An equally reliable, spatially restricted proteolytic response accompanies kainic acid (KA) neurotoxicity. Large accumulations of BDP have been found in the hippocampus and piriform cortex within 3–16 h of KA injection, persisting for as long as 9 days (Siman & Noszek 1988; Siman *et al.* 1989; Najm *et al.* 1992; Bi *et al.* 1996). In the hippocampus, the calpain-mediated

proteolysis was maximal at 24 h, with the highest levels of BDP occurring in the CA3 region, and lesser amounts in CA2/CA1 regions, the dentate, and CA1/subicular regions. This pattern matches the profile of cell loss observed in the hippocampus a few days after the insult (Nadler *et al.* 1978, 1980; Siman & Noszak 1988), and accords well with the distribution of high-affinity KA receptors. It is noteworthy that the magnitude of spectrin proteolysis and the extent of cell damage co-vary with the dosage of KA, much as observed when NMDA is used as the excitotoxin (Siman *et al.* 1989). In conjunction with the temporal and regional correlations between BDP formation and cell death, the findings argue that calpain plays an early and causal role in the development of excitotoxicity.

Of special mention with regard to the KA model of neurotoxicity is its use in understanding how separate, liminally toxic manipulations might act synergistically at the point of calpain activation to produce neuropathology. Elliot *et al.* (1993), in expanding a series of results implicating glucocorticoids in the augmentation of neuronal damage, found that daily injections of corticosterone (CORT) exacerbated KA-induced spectrin proteolysis: spectrin BDP formation was enhanced by 124% in CORT-injected rats relative to vehicle controls. In a complementary study, Lowy *et al.* (1994) demonstrated that adrenalectomy effectively reduced the proteolytic response to KA in the hippocampus and cortex by lowering baseline as well as KA-induced CORT levels. Glucocorticoids are known to inhibit glucose uptake in neurones, and it is thought that secondary metabolic effects, such as compromised calcium and glutamate regulation, are responsible for the amplifying effect of the steroids on other neuropathologies. As expected for this mechanism, mannose supplementation blocked the exacerbating influence of CORT on KA-induced spectrin breakdown (Elliot *et al.* 1993). A more direct influence on KA-induced calpain activation has been shown for polyamines. Spermine, spermidine, and putrescine were found to increase in concentration following KA lesions and other pathological circumstances associated with calpain activation (e.g. ischemia). With the above order of potency, the polyamines stimulated calpain-mediated hydrolysis of endogenous and exogenous protein substrates – most likely through modulation of an endogenous calpain activator (Najm *et al.* 1991a,b). This modulation of calpain by polyamines apparently occurs *in vivo*. As reported by Najm *et al.* (1992), injection of an inhibitor of ornithine decarboxylase, the rate-limiting enzyme in the polyamine metabolic pathway, reduces KA-induced spectrin proteolysis by approximately 75% in the hippocampus. In this experiment, putrescine and

BDP levels were coordinately reduced by DFMO in the first 16 h after KA injection, but not at later time points. At four and seven days post-KA, concentrations of the two products were similarly elevated in DFMO-treated and control animals. It is noteworthy that DFMO was equally ineffective at preventing the delayed neurotoxicity elicited by KA. Binding of the gliosis marker <sup>3</sup>H-RO5–4864, measured 7 days after KA lesions, also was unchanged by DFMO pretreatment; this parameter was highly correlated with the amount of spectrin breakdown whether or not animals received DFMO. Lastly, although DFMO did not completely abolish KA-induced proteolysis after 16 h and its effect at earlier time points remains undetermined, the results obtained with the compound illustrate an important conclusion: there is a clear dissociation when comparing the relationships between neurodegeneration and early vs. late phases of calpain-mediated proteolysis. This dissociation has been made with some experimental precision in paradigms of ischemic brain injury.

#### *Ischemia and hypoxia*

Calpain is one of the main suspected perpetrators in hypoxic/ischemic injury to the brain (see Lee 1995; Lipton 1999) and to other organs such as the liver (Bronk & Gores 1993), heart (Tolnadi & Korecky 1986), and kidney (Edelstein *et al.* 1995, 1996; 1997a,b). The latter is a particularly important subject of study since renal failure is a very common problem affecting about 5% of all hospital admittants and carries a high mortality rate of > 50% (a figure that has not changed significantly in the last 40 years). Additionally, a great deal of research has focused on the brain since the ischemic condition known as stroke affects approximately 500 000 people each year in the United States alone, making it the third leading cause of death and the number one cause of disability. Patterns of regional vulnerability to ischemia in the brain and temporal characteristics of the degenerative response have been described in detail (see Hatakeyama *et al.* 1988). In a typical gerbil model of global forebrain ischemia, transient carotid artery occlusion elicited degeneration in a set of highly vulnerable neural populations. With increasing durations of arterial occlusion, cortical and subcortical structures of lesser susceptibility were recruited among the more vulnerable regions in a reliable sequence. Damage to these structures occurred at a threshold duration of ischemia, beneath which cells recovered, and beyond which neurones died after a delay of a few days. Ischemic lesions also were induced most readily in the CA1 subfield of the hippocampus, a region that, as

noted, contains high levels of NMDA receptors. Channel and agonist blockers that disrupt NMDA receptor function have been shown to ameliorate ischemic injury (Kochhar *et al.* 1991; Kaku *et al.* 1993; Madden *et al.* 1993; Maier *et al.* 1995; Takaoka *et al.* 1997; Mueller *et al.* 1999). Also, given that ischemia leads to massive glutamate efflux, the particularly high density of NMDA receptors in CA1 appears to explain the unusual sensitivity of pyramidal cells in this region. Such results implicating the NMDA receptor as a variable in ischemic brain damage serve to strengthen the longstanding hypothesis that calcium influx is critically involved in pathogenesis.

In a study that linked the NMDA receptor-dependency of ischemia with the known downstream effects of the calcium ion on calpain activation, Seubert *et al.* (1989a) demonstrated that episodes of global ischemia lasting 10 min elicit a rapid proteolysis of spectrin. Using antibodies to native spectrin, BDPs were detected in immunoblot samples collected 15 min after reperfusion. Calpain activity was most pronounced in CA1, while areas of lesser vulnerability (e.g. thalamus and cerebellum) had correspondingly smaller accumulations of the spectrin fragments. At subsequent time points, BDPs were shown to persist only in CA1, and after 4 days this region experienced a second, more intense wave of proteolysis. *In vitro* models of hypoxia also have helped to define the early stages of calcium-dependent proteolysis and to identify which component of ischemia, hypoxia/hypoglycemia or reperfusion, is more influential in eliciting this pathogenic mechanism. Under conditions in which the baseline calpain activation that accompanies acute slice preparation was kept to a minimum, BDP concentrations were roughly doubled in hippocampal slices after 5 min of hypoxia (Arai *et al.* 1991; Lee *et al.* 1991). Thus, the initiating stage of calpain-mediated spectrin proteolysis after global ischemia appears to be unrelated to reperfusion, but rather seems a primary response to hypoxia. Interestingly, the proteolytic response is bi-phasic in nature: an early component initiated during or shortly after ischemia, followed by a later and larger wave correlating with delayed cell death. This general profile holds for multiple models of focal and global ischemia. Using similar techniques, Lee and coworkers (Hong *et al.* 1994b) have shown that focal neocortical ischemia (achieved by occlusion of both carotids and one middle cerebral artery) is associated with a rapid and sustained induction of spectrin BDPs in the infarct zone (also see Lee *et al.* 1991; Bartus *et al.* 1995; Yao *et al.* 1995; Blomgren *et al.* 1997, 1999). Recently, Bartus *et al.* (1998) measured BDP formation in CA1 hippocampus following four-vessel occlusion in the rat and correlated this measure

with histological indices of cell damage and loss of synaptic transmission. Observations made between 6 h and 4 days post-ischemia indicated that calpain activation correlates with the progressive compromise of pyramidal cell structure and function.

The above findings have been corroborated and extended by two groups using BDP-specific antibodies (Saido *et al.* 1993; Roberts-Lewis *et al.* 1994). In immunohistochemical assays, these antibodies labelled only those sites where BDPs accumulated, and thus provided greater spatial resolution of calpain activation, both at the regional and cellular level. Exploiting this specificity, the two groups showed that calpain becomes activated in the dendrites of pyramidal cells in the CA2/CA3 border zone after ischemic episodes as short as 1 min. Longer durations, 5 and 10 min, elicited the immunostaining in CA1 followed by spectrin proteolysis in cortical, thalamic, and other sites. Thus, sampling the post-ischemic brain throughout an extended period revealed that CA1 undergoes an early phase (within 15 min), then a later and more intense phase (between one and two days), of proteolytic activation after an intermediate ischemic duration. The longer episodes of carotid artery occlusion elicited the calpain response within additional cortical and thalamic areas (Roberts-Lewis *et al.* 1994). Analyses with the BDP-specific antibodies provide an important set of observations: (i) the studies document with great acuity the strict spatiotemporal correlations between calpain-mediated proteolysis and cellular pathology in vulnerable brain areas; (ii) they indicate that the proteolysis must persist beyond the initial stimulus-linked phase to irreversibly compromise cells; and (iii) they add to previous data in suggesting that protection of neurones from ischemia is achieved by inhibiting calpain after the primary insult. With regard to the latter, BDP assays have been used to determine the protectant nature of protease inhibitors in target neural populations (see Lee *et al.* 1991) as well as in other highly vulnerable cell types (Edelstein *et al.* 1996).

Pharmacological approaches have been used to test the receptor dependency of the calpain response and the prediction made with the BDP assay that the protease is a primary contributor to pathogenesis in the ischemic brain. Inhibition of NMDA receptors with MK-801 was shown to be effective in reducing both phases of proteolysis in the aforementioned global ischemia paradigm when administered in doses that afford significant neuroprotection (Seubert *et al.* 1990; Roberts-Lewis & Siman 1993; Roberts-Lewis *et al.* 1994). The coordinated reductions in cell death and calpain activation by MK-801 emphasize the importance of NMDA receptor-linked calcium entry in ischemia. Correspondingly, NMDA receptor antagonists also reduce the cellular toxicity

and spectrin breakdown associated with NMDA administration *in vivo* (Siman *et al.* 1989), and prevent BDP formation elicited with the excitotoxin *in vitro* (Seubert *et al.* 1988b; del Cerro *et al.* 1994; Bahr *et al.* 1995). Thus, neuroprotection by NMDA receptor blockade is associated with reductions in calpain activity in two paradigms: ischemia and excitotoxicity.

Direct inhibition of calpain *in vivo* has been shown in several instances to reduce calpain-mediated proteolysis and protect vulnerable cells from hypoxia/ischemia (Lee *et al.* 1991; Rami & Krieglstein 1993; Bartus *et al.* 1994a,b; Hong *et al.* 1994a; Edelstein *et al.* 1996; Li *et al.* 1996; Yuen & Wang 1996; Yokota *et al.* 1999). Such a route for neuroprotection extends to the point of preserving synaptic function as calpain inhibitors improve the recovery of synaptic responses after hypoxia in acute hippocampal (Arai *et al.* 1990; Arlinghaus *et al.* 1991; Chen *et al.* 1997; also see Bednarski *et al.* 1995) and neocortical slices (Hiramatsu *et al.* 1993). Moreover, hippocampal slices prepared from animals treated with the calpain inhibitor leupeptin prior to ischemia retained the ability to express long-term potentiation (Lee *et al.* 1991). This result is of great significance therapeutically as long-term potentiation is thought to underlie the encoding of certain types of memory, and the phenomenon itself relies heavily on the structural and functional integrity of excitatory synapses. As predicted from early data obtained with BDP assays, blockade of the latter, persistent phase of ischemia-induced proteolysis affords significant neuroprotection (Bartus *et al.* 1994a,b). Such reports characterizing the phasic generation of spectrin BDPs after ischemia establish that the second, persistent wave of proteolysis starting within hours of the initial calpain response is correlated with and indicative of eventual cell death. This implies a 'window of opportunity' for therapeutically efficacious inhibition of calpain that may extend many hours after the ischemic insult. In fact, by measuring cerebral infarct volume, Markgraf *et al.* (1998) defined the outer limit of a calpain inhibitor's therapeutic benefit to be as great as six hours postischemia. It stands to reason, then, that a treatment for stroke that targets a point of convergence among many pathological mechanisms has the potential to prevent neuronal damage even when administered several hours after the insult.

#### Trauma and haemorrhage

Calpain-mediated proteolytic responses have been implicated in the neurodegeneration associated with brain trauma and related vascular problems that can lead to cerebral vasospasm and ischemic conditions. Spectrin BDPs were evident regionally after the trauma

of experimental brain injury (Kampfl *et al.* 1996; Saatman *et al.* 1996a; Posmantur *et al.* 1997) and in the cerebrospinal fluid of humans following subarachnoid and intraventricular haemorrhages (see Lee *et al.* 1993). It is not known whether these types of haemorrhage are generated solely by neuronal or vascular tissue. However, increased calpain activation and spectrin fragmentation have been detected in smooth muscle cells of the rabbit basilar artery after vasospasm (Lee *et al.* 1993; Yamaura *et al.* 1993). It is thought that the proteolysis of spectrin, calponin, and regulatory kinases in these cells contributes to the morphological alterations of arterial cells after vasospasm, and perpetuates spastic contraction initiated by haemorrhage (Minami *et al.* 1992; Yamaura *et al.* 1993; Lee 1995). In particular, proteolytic conversion of PKC into a tonically catalytic form by calpain (Sato *et al.* 1997) occurs in the basilar artery after subarachnoid haemorrhage, leading to increased smooth muscle contractility. Evidence suggests that calpain 1 remains activated and levels of its endogenous inhibitor calpastatin are diminished in arterial smooth muscle cells for days after subarachnoid haemorrhage, thereby perpetuating stable contraction (Yamaura *et al.* 1993; Sato *et al.* 1997). With regard to this idea, it has been demonstrated that selective calpain inhibitors attenuate the development and progression of cerebral vasospasm (Minami *et al.* 1992; Lee *et al.* 1997). A dual action of calpain inhibitors on pathological processes in neurones and the vasculature that supports them could account for the efficacy of the inhibitors in models of brain trauma. A brain trauma model developed by McIntosh and colleagues allowed researchers to demonstrate that calpain inhibitors attenuate trauma-induced spectrin breakdown (Posmantur *et al.* 1997) as well as the associated motor and cognitive deficits (Saatman *et al.* 1996b). The above results indicate that measurements of BDP levels in cerebrospinal fluid may be of utility in detecting both the neuronal and vascular aspects of cerebrovascular accidents. They also suggest that calpain inhibition can be useful in decreasing the risk of haemorrhage-induced brain damage associated with certain surgical procedures.

#### Toxins

While the types of *neural* insults considered above – excitotoxicity and ischemia – have the common feature of glutamate receptor hyperstimulation at an early stage, it must be emphasized that routes to pathology which do not directly engage glutamatergic systems also lead to calpain activation. Colchicine, for example, causes neuronal damage by blocking critical transport processes.

Intraventricular injections of the drug caused extensive death developing over several days among granule cells of the dentate gyrus, with much smaller effects in the other hippocampal subdivisions (Sutula *et al.* 1986). Spectrin BDPs appeared within 24 h of intrahippocampal colchicine injection, peaked at 4 days, and remained elevated for weeks (Seubert *et al.* 1989b). As was the case for cell death, the increase in the BDP response was largest in the dentate gyrus, particularly in the molecular layer. Note that these results, together with data on excitotoxicity and ischemia, establish a correlation between selective vulnerability and early appearance of BDPs that includes all of the major hippocampal subfields (see Table 1).

The pathogenic action of some environmental toxins also appears to be mediated by calpain. This includes the action of trimethyltin (see Bahr *et al.* 1995; Munirathinam & Bahr, 2000), a neurotoxic pesticide known to cause seizures, amnesia, and selective damage to the hippocampus, as well as that of the heavy metal mercury (Elliget *et al.* 1994) and maitotoxin (Zhao *et al.* 1999). For example, peripheral injections of trimethyltin into rats produced calpain-mediated spectrin proteolysis in the hippocampus within 24 h (Seubert *et al.* 1988a); the BDP formation steadily increased thereafter for two weeks. The BDP response was more evident in the dentate gyrus relative to field CA1, thus correlating with the regional vulnerability to the toxin (Norberg *et al.* 1998). Exposure of hippocampal slice cultures to trimethyltin, as well as to snake venoms, induced the calpain response and resultant spectrin fragments within 3 h, the shortest time tested (Bahr *et al.* 1995).

*Partial deafferentation*

Brain damaging insults not only cause local degeneration, they can also produce atrophy in neuronal populations that send electrical signals to or receive signals from the injured region. The magnitude and time course of experimentally induced forms of this effect have been measured carefully

**Table 1.** Correlation between cellular vulnerability and early appearance of spectrin BDPs in the hippocampus. Regional selectivity with regards to cellular damage and calpain-mediated spectrin proteolysis is shown for three pathogenic insults

Treatment	Hippocampal region of greatest vulnerability	Region of earliest and largest BDP accumulation
Kainic acid	Field CA3	Field CA3
Ischemia	Field CA1	Field CA1
Colchicine	Dentate gyrus	Dentate gyrus

in a few cases, including the dentate gyrus. Dendritic shrinkage and spine loss were found in granule cells within 48 h of lesioning cortical inputs to the dentate gyrus, and were more substantial in degree by 96 h post-lesion (Ivy *et al.* 1988; Seubert *et al.* 1988a). Spectrin BDPs also appeared in the deafferented zones within 12 h and increased dramatically over the subsequent 24-h period. The calpain response occurred while the severed axons were still functional and prior to any signs of degeneration. These results confirm the sensitivity of the BDP markers as early indicators of pathogenesis, and they demonstrate that BDPs can be used to identify sites where atrophy (as opposed to cell death) has been initiated by a particular insult. Furthermore, in that surgical procedures involving removal or lesioning of brain tissue produce deafferentation, inhibition of calpain may be useful in limiting any associated loss of peripheral function. This application has been tested in an animal model involving ablation of a discrete portion of the visual cortex. Indeed, selective calpain inhibitors attenuated the spread of calpain activation outside the surgical zone, as well as reduced the atrophy in those areas which connected to the removed tissue (Bartus *et al.* 1999).

*Genetic defects*

The brindled mouse mutation is a widely studied example of how genetic defects can lead to the emergence of neuropathology. The mutation involves a problem in the regulation of copper levels that has profound effects on the activity of copper-dependent oxidative enzymes of mitochondria. Death usually occurs in mice bearing the mutation by postnatal day 16 unless the animals are given supplemental doses of CuCl<sub>2</sub>. As mitochondria are an important high-capacity buffering system in neurones, such deficits are expected to lead to altered calcium regulation. Experiments have shown that spectrin BDPs begin to appear between postnatal days 12 and 15 in those brain regions destined to undergo degeneration over the course of further maturation (Seubert *et al.* 1990). Brain regions that are less affected by the mutation, according to alternative criteria, exhibit less calpain activation at the time points assayed. Also in a corresponding manner, peripheral injection of CuCl<sub>2</sub> forestalls both the proteolytic response and the neuropathology that ensues without the copper supplement.

*Ageing*

The gradual degenerative processes characteristic of brain ageing (see Masliah *et al.* 1993) were found to be associated with calpain-mediated proteolysis in a study



using antispectrin antibodies (Bahr *et al.* 1991). Moreover, the study showed that spectrin BDP levels increase with age in the mouse brain in a region-specific manner. While the hindbrain had low concentrations of BDPs across mice of 3–30 months of age, a linear relationship with age was exhibited by BDPs from the telencephalon ( $r = 0.91$ ). This is in correspondence with the fact that telencephalic neurones also selectively express age-related deficits in synaptic markers (Bahr *et al.* 1992, 1993; Masliah *et al.* 1993). Another type of assay showed calpain activity to exhibit only small, statistically insignificant increases in aged human brains (Banay-Schwartz *et al.* 1994). However, this could be due to postmortem effects causing the proteolytic events to fall below detectable levels in many areas. Recent immunocytochemical studies with BDP-specific antibodies (Bahr *et al.* unpublished data) confirmed the region-specific increase in calpain activation in the aged mouse brain, and began to map with precision those cell types of high vulnerability (i.e. cortical and hippocampal neurones). The origins of the regional vulnerability are not known, but it has been proposed that deregulation of the calpain system may occur at a faster rate with age in those regions of the telencephalon that exhibit lasting forms of calcium-triggered synaptic plasticities (see Lynch & Seubert 1989). Overall, the above results suggest that pathogenic processes set in motion by acute neurological insults – such as those discussed earlier – may be recruited in a gradual and selective manner with normal ageing. Such pathogenic insults are probably promoted by age-related disturbances in calcium homeostasis (an issue discussed by Mattson 1992). Indeed, differences in calcium-regulated events including calpain activation have been noted in fibroblasts (Peterson *et al.* 1991) and erythrocytes (Glaser *et al.* 1994) harvested from young vs. aged human subjects. One hypothesis on the contribution of the calpain system to brain ageing posits that other age-related cellular disturbances (for instance, gradual loss of lysosomal function; see Bahr *et al.* 1994a) interact with ongoing but normally subthreshold neuronal 'stresses' (e.g. excitotoxicity), the consequences of which may produce detrimental positive feedback. It is noteworthy that spectrin BDPs increase gradually starting just after adulthood (Bahr *et al.* 1991), perhaps indicating that aberrant activation of calpain is among a set of primary age-related dysfunctions that precede the appearance of overt pathology and symptomology.

#### *Alzheimer's Disease*

Studies with postmortem tissue suggest that spectrin

cleavage events are more pronounced in the hippocampus and cortex of subjects with Alzheimer's disease than in those regions of age-matched control subjects (Masliah *et al.* 1990). Assays for calpain and calpastatin have shown that there are indeed regionally specific increases in the net calpain activity with Alzheimer's disease (Nilsson *et al.* 1990; Saito *et al.* 1993; Saido 1996; also see Haug *et al.* 1996) and in the expression levels of calpain with another age-related disorder, Parkinson's disease (Mouatt-Prigent *et al.* 1996). Histological indices also have shown that spectrin BDPs increase in areas most affected by Alzheimer-type pathogenesis in terms of synapse loss. In this respect, induction of calpain-mediated proteolysis in areas targeted by the disease may involve mechanisms of the type associated with deafferentation (discussed earlier). In one particular study, degradation of spectrin was greatly enhanced in the vulnerable frontal cortex region of a rat model of cholinergic degeneration (Fernandez-Shaw *et al.* 1997). Abnormally high levels of BDPs also have been detected in the cerebrospinal fluid of patients with Alzheimer's or Pick's disease (Vanderklis *et al.* unpublished observation). It is noteworthy that the fundamental cellular disturbance underlying the increased calpain activation in Alzheimers may extend to other somatic contexts. For example, after stimulation with serum or mitochondrial uncoupling agents, skin fibroblasts collected from Alzheimer patients were found to have a significantly higher BDP content than fibroblasts from age-matched or young donors (Peterson *et al.* 1991). This difference was obtained despite indications of equal calpain and calpastatin concentrations among the three donor groups. There was also evidence for an age-related increase in fibroblast BDP content in healthy subjects. The increased BDP levels in Alzheimer's disease and normal ageing are most likely due to the alterations in calcium regulation identified in skin cells (Peterson & Goldman 1986). In another study, increases in membrane-bound calpain and related proteolytic activity were evident in peripheral blood lymphocytes from patients with early onset Alzheimer's disease (Karlsson *et al.* 1995).

Stemming from work by Nixon and colleagues, persistent calpain activation in Alzheimer's disease is thought to contribute to hallmark features of the disease prior to the occurrence of gross synaptic loss and cell death (see Saito *et al.* 1993). These hallmark features of Alzheimer-type pathogenesis refer to (1) the formation of neurofibrillary tangles containing hyperphosphorylated isoforms of the microtubule-associated protein tau, and (2) the abnormal processing of amyloid precursor

protein which can lead to the production of the  $\beta$ -amyloid peptide and related deposition in the form of plaques. With regard to the first event, calpain has been shown to be involved in the proteolytic processing/turnover of tau (Litersky & Johnson 1992; Mercken *et al.* 1995; Yang & Ksiezak-Reding 1995; Shea 1997b; Yang *et al.* 1997; Xie & Johnson 1998) that experiences distinct alterations with age (Bahr *et al.* 1994b; Bahr & Vicente 1998). There is some dispute in regards to whether reduced tau turnover by calpain is a possible mechanism to facilitate neurofibrillary chemistries in Alzheimer's disease. However, calpain was implicated in early stage neurofibrillary degeneration due to an active form of the protease colocalizing with tangles, neuritic plaques, and neuropil threads in Alzheimer brains (Grynspan *et al.* 1997). Further evidence suggests that calpain contributes to the conversion of normal tau to pathogenic, hyperphosphorylated forms of the protein (Shea *et al.* 1996). Interestingly, the action of calpain in this study was determined to be indirect through the proteolytic conversion of PKC into a tonically catalytic kinase, the same event that is thought to underlie vasospasm during subarachnoid haemorrhage (Sato *et al.* 1997). A more recent study indicated that calpain causes the conversion of p35 to p25, a 25-kDa species that accumulates in Alzheimer brains and promotes tau hyperphosphorylation, cytoskeletal disruption, and cell death through the prolonged activation and mislocalization of the cyclin-dependent kinase 5 (cdk5) (see Lee *et al.* 2000). Interestingly, this study also showed that application of the  $\beta$ -amyloid peptide to primary cortical neurones causes corresponding production of p25 and spectrin breakdown products, thus further suggesting a significant role for calpain in the pathogenesis of Alzheimer's disease.

Regarding amyloidogenesis, Siman *et al.* (1990) proposed that calpain is active in the normal, and perhaps pathological, processing of the precursor protein for  $\beta$ -amyloid (also see Li *et al.* 1995). This led to the idea that modulation of calpain may merit consideration as a potential therapeutic strategy against amyloid production. Recent studies, however, found conflicting results using potent calpain inhibitors. Several groups showed that blocking calpain activity causes different effects with regard to the production of  $\beta$ -amyloid: reduction in  $\beta$ -amyloid species (Klafki *et al.* 1996), no change in  $\beta$ -amyloid secretion levels (Figueiredo-Pereira *et al.* 1999), as well as increased secretion of the amyloidogenic peptide from cultured cells (Yamazaki *et al.* 1997; Yamazaki & Ihara 1998). Thus, it is not clear whether inhibitors or activators of the calpain system can influence amyloidogenic chemistries.

$\beta$ -amyloid not only is the major constituent of senile

plaques, the peptide also can cause neuronal apoptosis possibly through calpain (Martin *et al.* 1995; Chi *et al.* 1999; Wolf *et al.* 1999; Lee *et al.* 2000; Ray *et al.* 2000; also see Kluck *et al.* 1997) or through members of another family of cysteine proteases, the caspases (see Chan & Mattson 1999). There is much interest in the relationship between the two types of proteases and their common targets during proteolytic and related pathogenic responses (Porn-Ares *et al.* 1998; Chan & Mattson 1999; Ruiz-Vela *et al.* 1999; Newcomb *et al.* 2000; Wang, 2000). For example, they both cleave AMPA-type glutamate receptors in a way that can potentially regulate excitatory transmission (see Bahr *et al.* 1996; Bahr, 2000) or determine pathogenic consequences (Glazner *et al.* 2000). Interestingly, calpain is thought to contribute to the degradation phase of apoptosis using a caspase-dependent activation mechanism (Wood & Newcomb 1999), possibly through caspase-mediated cleavage of calpastatin and resulting decontrol of calpain activity suppression (Porn-Ares *et al.* 1998; Kato *et al.* 2000). It should be mentioned, however, that there is evidence of caspase-independent involvement of calpain in apoptotic events in platelets (Wolf *et al.* 1999). Calpain inhibition was found to reduce cell death in cortical neurones treated with  $\beta$ -amyloid (Lee *et al.* 2000; also see Jordan *et al.* 1997), although another study showed such inhibition to have no effect on  $\beta$ -amyloid-induced apoptosis (Selznick *et al.* 2000). Calpain also was shown to directly cleave certain caspases and thereby block the activation of the pro-apoptotic species caspase-3 (Chua *et al.* 2000). Such negative regulation of caspase processing by calpain is in contrast to calpain's putative role in apoptosis. Such a discrepancy may stem from isoform-specific actions among calpain species. In fact, the m-calpain isoform was found to selectively cleave and, in effect, activate caspase-3 in a neonatal model of cerebral hypoxia-ischemia where sequential activation of the two proteases occurs (Blomgren *et al.* 2000). This study promotes the idea that the pro-apoptotic caspase-3 is synergistically activated by the early induction of m-calpain, a calpain isoform implicated in Alzheimer's disease (Grynspan *et al.* 1997; Tsuji *et al.* 1998) and Parkinson's (Mouatt-Prigent *et al.* 1996).

#### *Other pathological conditions*

Finally, it is worthwhile to list a variety of other pathogenic circumstances in which calpain has been implicated. The research described above indicates that the protease plays a major role in a wide spectrum of processes including those underlying synaptic plasticity,

neurotoxicity, trauma, and age-related disorders. This spectrum is broadened by additional studies in nervous tissue which suggest that calpain is involved in the inflammatory reactions associated with allergic encephalomyelitis (Shields *et al.* 1998), the demyelination events of multiple sclerosis (see Shields *et al.* 1999), the neuronal damage after spinal cord injury (Li *et al.* 1995, 1998; James *et al.* 1998; Ray *et al.* 1999), as well as in behavioural disorders such as depression (Banay-Schwartz *et al.* 1998) and obsessive-compulsive disorder (Mundo *et al.* 1997). Outside the nervous system, calpain also has been implicated in a great diversity of disorders including: (1) different forms of cardiac pathologies (Atsma *et al.* 1995; Di Lisa *et al.* 1995; Arthur & Belcastro 1997); (2) mechanisms of renal failure (Elliget *et al.* 1994; Kelton *et al.* 1996; Edelstein *et al.* 1997a,b); (3) certain types of respiratory disorders including injury to inspiratory muscles of the diaphragm (see Miller *et al.* 1995; Jiang *et al.* 1998); (4) tissue damage associated with organ transplantation and graft dysfunction (Aguilar *et al.* 1997; Kohli *et al.* 1997; Tijssen *et al.* 1997); (5) collagen destruction and resulting bone erosion in cholesteatoma (Amar *et al.* 1996); (6) cataract formation (Andersson *et al.* 1994; Sanderson *et al.* 1996; Wang *et al.* 1996); (7) the eye inflammatory disorder optic neuritis (see Shields & Banik 1998); (8) a putative cell death mechanism mediated by HIV (Corasaniti *et al.* 1996); (9) breast cancer (Shiba *et al.* 1996; Pink *et al.* 2000); and (10) various disorders involving skeletal muscle (see Kumamoto *et al.* 1995, 1997). With regards to the latter, it should be added that many mutations in calpain genes have been found to co-segregate with certain forms of muscular dystrophy (Richard *et al.* 1995; Beckmann *et al.* 1996; also see Spencer *et al.* 1995). It has been further suggested that calpain is responsible for the early stage muscle degradation in these myopathies (Kumamoto *et al.* 1995). To summarize, the problems associated with unregulated, calpain-mediated proteolysis are not restricted to the central nervous system but are in fact widespread.

### Concluding remarks

Calpain activation is an early event in the cellular response to a variety of stressful and potentially pathogenic conditions. Such a proteolytic response, if sustained, can lead to cell death in vulnerable populations of cells. Studies utilizing the spectrin BDPs as markers of calcium-activated proteolysis have elucidated the commonality of this pathogenic mechanism and described paradigm-specific aspects of its presentation

that correlate with cellular vulnerability. Moreover, data based on BDP measurements have served as the basis of testable predictions and pharmaceutical development concerning the efficacy of calpain inhibition as a therapeutic strategy. It is clear that assays for calpain activity and BDP detection can be exploited as a tool for the study of degenerative mechanisms and related therapeutics.

### Acknowledgements

Editorial work was provided by Ms. Alyson M. Blow and the authors are grateful for her excellent services. This work was supported in part by U.S. Army Medical Research Grant DAMD17-99-C9090.

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