One notable advantage of calpain as a therapeutic target is that antagonism of increased calpain activation may provide a longer window of opportunity for protecting neurons after the initiation of the neuronal injury.

Potential Contribution of Proteases to Neuronal Damage


Calpain was first discovered as a Ca$^{2+}$-activated protease in rat brain and in skeletal muscle independently 30 years ago. Two major isoforms, m-calpain and n-calpain, with different Ca$^{2+}$ affinities were subsequently isolated and purified from erythrocytes, skeletal muscle and kidney. The presence of an endogenous protein inhibitor, calpastatin, was later discovered in Menchi's laboratory. Both isoforms of calpain appear to be heterogeneous (90kDa and 29 kDa) and can be separated by DEAE-chromatography. Large subunits of calpain can be divided into four domains (I-VI) and the small subunit has two regions (V and VI, Fig. 1). Based on sequence comparison, domain II is associated with a cysteine protease structure. Domain III has no defined function while domain V may be required for interaction with mem-

brane phospholipids. Domains IV and VI each contain four sets of EF-hand Ca$^{2+}$-binding structures, similar to those in calmodulin. These Ca$^{2+}$-bind-
ing structures apparently modulate Ca$^{2+}$-dependency on the catalytic activity. Other calpain isoforms include s-cal-1, the skeletal muscle-specific isoform, and the smooth muscle isoforms, s-cal-2 and s-cal-2.7

Summary

Calpain was first discovered 30 years ago. Two major isoforms were subse-
quent isolated and purified. The presence of an endogenous protein inhibitor, calpastatin, was later discovered. Calpain activity is tightly regulated by Ca$^{2+}$. At physiological levels of Ca$^{2+}$, the role of calpain remains poorly understood but is believed to be involved in mitosis and muscle cell differentiation. Calpain has also been implicated in various membrane fusion events through remodeling of the cytoskeletal network. Calpain activation has been shown to be increased during normal aging and in muscular dystrophy, cardiac, arthritic and Alzheimer's disease, and in acute trauma such as traumatic brain injury (TBI), spinal cord injury and cerebral and cardiac ischemia. Early work on calpain inhibitors was limited to protein inhibitors and other nonselective enzyme inhibitors. Peptidyl alkylboronates such as leucine and antipain are also among the earliest reported calpain in-

activators. Irreversible inhibitors such as the E64 family have also been studied, and peptidyl carboxamides and dipeptidylcarboxamides have long been used as protease inhibitors. A variety of calpain inhibitors are under development. From a ther-
apeutic perspective, calpain inhibitors may have several advantages over other more conventional targets such as ion channel blockers and glutamate antago-
nists, since calpain proteolysis represents a later component of a pathway mediat-
ing cell death initiated by excitotoxicity and elevated Ca$^{2+}$ levels. Although the potential clinical utility of calpain inhibitors appears well established, a number of important considerations remain to be addressed. The role of other proteolytic cascades contributing to neuronal cell damage following TBI must also be con-

sidered. © 1998 Prous Science. All rights reserved.

Drug News Prospect (114), May 1998

Copyright © 1998 Prous Science

CCC 0218-0556/98 215

215
Calpain activity is tightly regulated by Ca²⁺. Although μ- and m-calpain require low mM and high mM concentrations of free Ca²⁺, respectively, in vivo for full activity, the actual Ca²⁺ requirement for activation of calpain in vivo may be much lower due to endogenous activating factors. For example, acidic phospholipids can activate calpain by increasing its Ca²⁺ affinity, and it has also been shown that phosphatidylinositol phosphates are required for calpain activation in intact cells. Furthermore, upon activation by Ca²⁺, the proenzyme forms of calpain (80 kDa + 29 kDa) undergoes autolytis in domains I and V to produce the fully activated (76 kDa + 18 kDa) form. The activated form has a higher affinity for Ca²⁺, and with sustained activation, the large subunit of calpain undergoes further fragmentation, resulting in the loss of enzyme activity. This process could represent a self-regulating mechanism for calpain activity.

Calpain prefers substrates which contain Val or Leu in the P₂ position and is less stringent at the P₁ position. To date, a few dozen proteins have been identified as potential substrates for calpain, at least under in vitro conditions. Substrates for calpain proteolysis include receptor proteins (EGF receptor, IGF receptors, retinoblastoma protein, and TGF-alpha), cytoskeletal and structural proteins (microtubule-associated protein MAP2, talin, dystrophin, and neurofilaments), calmodulin-binding proteins (e.g., n-spectrin plasma membrane Ca²⁺ pump, calcinemins and connexins), and transcription factors (AP-1, c-fos, c-jun, c-myc, AP-2, AP-3, and NFkB). In addition, many enzymes are proteolytically activated by calpain, including phospholipase C, transglutaminase, tyrosine phosphatase B, tyrosine kinase p70, and several calmodulin-regulated enzymes.

At physiological levels of Ca²⁺, the role of calpain remains poorly understood, but it is believed to be involved in mitosis and muscle cell differentiation. Calpain has also been implicated in various membrane fusion events through remodeling of the cytoskeletal network. Perhaps through a similar mechanism, it might be involved in enhancing glutamatergic synapses and long-term potentiation (LTP). When high Ca²⁺ concentrations are reached due to excitotoxicity, however, calpain can be extremely destructive due to its ability to degrade numerous substrates. Calpain activation has been shown to be increased during normal aging and in muscular dystrophy, cataract, arthritis, and Alzheimer's disease, and in acute trauma such as traumatic brain injury (TBI), spinal cord injury, and cerebral ischemia.

**Calpain inhibitors**

Early work on calpain inhibitors was limited to protein inhibitors and other nonselective enzyme inhibitors. The most selective of these is the endogenous inhibitor calpastatin, which binds to calpain in a Ca²⁺-dependent process. The full-length form contains domains I and IV, and the repeat inhibitor domains (I-IV; Fig. 1). A posttranslationally modified form of calpastatin that lacks the first two domains (I and II) (46 kDa, apparent Mr 66 kDa) is found in epididymis. Within the repeat domain, there are three conserved regions (A, B, C). Regions A and C are helical and interact with the EF-hand Ca²⁺ binding domains IV and VI of calpain, respectively. Domain B, however, apparently interacts directly with the catalytic site of calpain and possibly with domain III. Both the 27-mer and the 20-mer peptides derived from the repeat region B have been found to have inhibitory activity as well; however, the large size and resulting low cell permeability of these peptides makes their use rather limited as therapeutic agents. L- and H-kininogens have also been reported in calpain inhibitors. Interestingly, truncation of the kininogens to a key conserved region of both forms (Glu-Val-Val-Ala-Gly-Asp) also retains irreversible calpain-inhibitory properties.

Pepidyl aldehydes such as leupentin (Leu-Leu-Arg-H) and
antipain are also among the earliest reported calpain inactivators.18 Inhibitors of calpain activity also inhibit other proteases, including papain, trypsin, and cathepsin B. Calpain inhibitor 1 (Ac-Leu-Leu-Val-Tyr) and calpain inhibitor II (Ac-Leu-Leu-Val-Tyr), developed in Murachi’s laboratory,22 have been widely used in many investigations and are actively used permeable (Wang, unpublished data); however, calpain inhibitors I and II also inhibit other proteases. A common misconception is that calpain inhibitors I is selective for calpain and calpain inhibitors II is also specific for calpain; they are actually equipotent against both calpains (Wang et al., unpublished data). Calpains (Z-Leu-Leu-Ile) and MLA-28170 (Z-Val-Phe-H) are second-generation peptide aldehydes inhibitors with improved cell permeability. These compounds have limited therapeutic use due to their tendency to stabilize in vivo.17

Irreversible inhibitors such as the E64 family have also been studied. While these peptidyl epoxides showed high specificity for cysteine proteases, they are not particularly selective for calpains. In addition, E64 analogues have poor membrane permeability, which limited their development.23

Peptidyl halomethanes and diazo- methanes have long been used as protease inhibitors. Potent calpain inhibitors were discovered by modifying the P1 and P2 positions with cat- pain-preferred residues.24 Selectivity of these inhibitors for calpain over other cysteine proteases has yet to be achieved. Scientists from Cephalon, Inc. recently published their work on peptidyl fluoromethyl ketone calpain inhibitors. They were able to improve potency slightly by using a tetrahy- droquinolinyl group as the N-terminal coupling group. Their best fluor- omethyl ketone (compound 46 in the article cited) showed only a marginal preference for calpain I (kcat/kM = 276,000 M⁻¹ s⁻¹) over cathepsin H (kcat/kM = 7500 M⁻¹ s⁻¹) and cathepsin L (kcat/kM = 7200 M⁻¹ s⁻¹).25

Peptidyl hydrazones were reported as cysteine protease inhibitors inde- pendently by Graybill et al. (Sandoti Winthrop) and Giordano et al.18,26 These compounds showed little selective and in some cases have higher reactivity for cathepsin B than calpain. A compound of interest among the irreversible calpain inhibitors is B-Loe-Leu-Cys(Nps)S-NH2 (Sankyo compound 3 in the article cited). This compound is much more selective for calpain versus other cysteine proteases such as cathepsin B and papain. It also does not inhibit serum and epidermic pro- teases.27

Specific compounds under development

A variety of compounds are cur- rently under development. Alchorem has developed AK-275, a dipeptidyl α-ketoamide calpain inhibitor,28 and AK-295, a structural analogue of AK-275, which provided a 32% reduction in infarct volume.29 Sandoti Winthrop has designed two peptidyl calpain inhibitors, WIN-66100 and WIN-60217, in addition to Compound 8, a quinolonecarboxamide-based calpain inhibitor, and Compound 69, a tripeptidyl 2,6-difluorobenzoylazain- methyl ketone. In addition, Park-Davis has developed an amencap prolylcarboxypeptidase derivative, PD-150696, that was shown to be neuroprotective in both hypoxic/hypoglycemic injury of cultured cerebrocortical re- dox and AMPA-induced excitotoxic injury of Porcine neurons in cerebellar slices. Furthermore, PD-151746, a structural analogue of PD-150696, showed a 20-fold selectivity between the two calpains (K2 vs K1 = 0.76 vs 5.3 mM) for the cal- pain (K = 5.3 mM).29

Calpain proteolysis in TBI and ischemia

Cytoskeletal alterations after experimental brain injury have pointed to the likelihood of calpain-mediated proteolysis. Increased degradation of MAP2,30,31 the neurofilament triplet proteins, and spectrin32,33 has been reported in cerebral ischemia. In addi- tion, loss of MAP2,34,35 neurofilament 68 (NF 68) and neurofilament 200 (NF-200)36,37 has been reported following TBI in vivo; however, these findings have provided only indirect evidence for calpain activation, since cytoskele- tal protein degradation may be at least partially attributable to proteases other than calpain.

Protease inhibitors that block cal- pains have also been used to further investigate calpain activation in exper- imental brain injury. These inhibitors have been shown to be neuroprotective in cerebral ischemia38 and in vivo and in vitro models of acute CNS injuries.18,39 Because none of these inhibitors is solely selective for calpains and subtle dose-ranging analyses were not conducted, these data have pro- vided relatively indirect evidence for calpain activation after TBI and cere- bral ischemia.

The presence of breakdown prod- ucts (BDPs) to cytoskeletal proteins has also historically been used as an indirect marker of calpain activity. Importantly, TBI has been shown to be associated with the loss of NF 68 and the appearance of low-molecular weight immunoreactive BDPs characteristic of calpain proteolysis.40 Interestingly, the addition of calpain on NF proteins produces similar immuno- positive cleavage products of 57 kDa and 53 kDa in in vivo studies.41 Although other proteases can poten- tially produce immunoreactive BDPs,42 the fragments, such as cathepsin H and D, trypsin, and α-methylyotropin, the mole- cular weights of the proteolytic frag- ments are substantially lower or high- er43,44 and do not resemble the pattern observed post-TBI.

More direct evidence that calpain is involved in neurons following exper- imental brain injury has been shown by the use of antibodies which react specifically to calpain-mediated BDPs of cytoskeletal proteins. The calmod- ulin-binding protein nonerythrocyte α-spectrin (280 kDa; α-spectrin) is per- haps one of the most preferred and well-established endogenous sub- strates for cellular calpains.45 Both α- and γ-calpain attack this protein at two

King, Newl. 15(4), May 1998. 217
one notable advantage of calpain as a therapeutic target is that antagonism of increased calpain activation may provide a longer window of opportunity for protecting neurons after the initiation of the neuronal injury. Importantly, recent reports have provided evidence that delayed antagonist of calpain up to three hours following injury can still reduce neuronal damage in vivo and in vitro. In addition, the studies of calpain-mediated BDPs or e-spectrin following TBI and cerebral ischemia, and studies of ip-calpain,6,17 have provided convergent evidence that calpain is age-regulated for at least 24 hours postinjury. Although the temporal parameters (e.g., time of initiation and duration of treatment following injury) for treatment with calpain inhibitors have yet to be defined, these data suggest that the window of opportunity for calpain inhibitors is at least several hours. Equally important, these studies suggest that the duration of administration (or biological activity) of calpain inhibitors could critically influence the therapeutic efficacy of such agents.

Post studies have consistently documented the efficacy of protease inhibitors in attenuating excitotoxicity-induced cytotoxic damage following TBI in vivo and in vitro. It has been reported that administration of E-64c attenuates the loss of MAP2 in an animal model of focal cerebral ischemia. Further, Lee and colleagues6 reported that a cysteine protease inhibitor, leupeptin, reduced the degradation of spectrin in a hypoxic cortical slice model of hypoxic-anoxic injury. Lastly, a recent report has provided evidence that MDL, 28170 reduces the accumulation of spectrin BDPs following cerebral ischemia in vivo. In addition, several in vitro studies have demonstrated protective effects of other peptide scissile hydrocalpain inhibitors, including calpain inhibitor I and II, against cytotoxic calpain-related protease activity.12-14

Calpain inhibitor administration (the calpain inhibitor AK-295) has also proven to significantly reduce infarct volume in an animal model of cerebral ischemia15 and to attenuate motor and cognitive deficits following TBI in vivo. A recent report also provided evidence that calpain inhibitors II administration, specifically including estrogen-induced cytoskeletal protein loss and neuronal damage following cerebral ischemia. Importantly, this study also demonstrated morphological preservation of dendritic and axonal structures following TBI.

Although the potential clinical utility of calpain inhibitors appears well established, a number of important considerations remain to be addressed. The need to determine the therapeutic window and optimal duration of administration have been mentioned above. The need for relatively specific calpain inhibitors may be greater in clinical applications. In fact, the ability of calpain inhibitors to reduce activity of other proteases may provide additional protection. Assessing issues of toxicity are adequately addressed. Since calpain is a potential mediator of coagulation,19 curc may be used in the prolonged administration of calpain inhibitors to traumatized patients. Lastly, many in vivo studies have demonstrated beneficial effects of antiplatelet drugs in acutely injured animals, and platelet dysfunction has been observed in human traumatic brain injury.20

Potential role of other proteases following TBI

The role of other proteolytic cascades contributing to neuronal cell damage following TBI must also be considered. Intracellular degradation of proteins occurs by several different cellular pathways. One pathway involves a family of lysosomal cysteine proteases known as the cathepsins (for reviews see references 116 and 117) that is responsible for the normal turnover of various pro-
teins.109-21012121 Currently, direct evi-
dence for the contribution of these enzymes to neuro
matic brain injury has not been provided,
which such a role cannot be excluded,
since several cathespin iso-
foms catalyze the neuronal sub-
strates such as neurofilaments that are degraded following TBI.111212

Recently, a new class of proteases
has been implicated in vertebrate apop-
totic cell death (programmed cell death).
These belong to the ICE/CED-3 family of cysteine proteases and con-
sist of at least ten different homologues
including cleavage-sensitive substrates
such as DNA laddering and proteases
in cortical and hippocampal tissue following TBI.12

Another pathway that can con-
tribute to protein degradation in patho-
logic states is the ubiquitin-depend-
tent proteolytic pathway, also known as the proteasome or multicatalytic proteolytic complex (MPC) pathway. The wasting of muscle mass in TBI patients has
been linked to the ubiquitin-dependent
proteolytic pathway.121212 In addi-
tion, past studies have also indirectly
implicated the ubiquitin-dependent
pathway in diffuse axonal injury in humans.1212

Lastly, another family of proteases, the matrix metalloproteinases, which are involved in the maintenance and remodeling of the extracellular matrix, has also been implicated in TBI, specifically by compromising the blood-brain barrier over a period of 15-24 hours following injury.1212

In summary, there are a number of proteases in addition to calpain that can potentially contribute to profound protein
derangements following TBI. These include the cathespins, ICE-like proteases, the ubiquitin-dependent proteases complex and metallo-
proteases, future challenges will lie in

establishing new lines of evidence for the potential role of these still-rela-
atively uncharacterized proteases in TBI.12

References
1. Ichinose, W.A., Stowers, M.H., Goll, D.E.
and Suzuki, A. Calcium-specific removal of
2. Horner, R.B. and Kerks, K.G. Activation of
skeletal muscle phospholipase A by
calcium: II. Identification of a tissue activ-
3. Nishida, L., Takuri, K., Yumoto, T. and
Motokawa, Y. Sagittal-slice electron
microscopic study of the reaction of
Two distinct electron-dense calpain 3
and calpain 2 bands consistently seen from
5. Wainsh, L. Calcium-stimulated proteases in
Cleavage specificity of a cellular calcium-depen-
7. Wang, K.K.W. and Yeon, J.W. Cephalic
fibroblasts, in vitro, osseous tissue and to
8. Seirinuchi, H., Tonogai, S.N., Saiki, T.C.
et al. Muscle-specific calpain, p40K, is degrad-
ed by muscle-induced immediately after trans-
9. Cavallone, S.A. and Hartmann, D.R. Effect of
α-1-antitrypsin deficiency on a mus-
cular system calcium-dependent proteases.
Calcification of the collagen require-
ment for arteries. J Biol Chem 1994; 269:
11627-30.
10. Inagaki, S., Kitazaki, H. and Suzuki, K.
Limited activity of a calcium-stimulated neu-
ronal protease (CAMPS). Evidence of the cal-
improvement is due to the activation of
proteolytic activity of the N-enkephalin.
12. Saiki, T.C., Shimizu, M., Takasawa, T.,
Musashihara, H. and Suzuki, K. Proteolytic
activity of rat brain calpain 1 and calpain 2.
The effects of calpain on the structure of
neurons calpain 2. J Biochem 1977; 248:
579-86.
Limited exocytosis reduces the calcium
requirement of smooth muscle calcium-activated protease. J Biol Chem 1982; 257:
579-92.
15. Coggi, G., Gold, V.D., Petroni, F. and
Kapfham, H.P. The role of anodic activity in
the activity of the Ca2+-dependent proteases (corticotropin and acinar).
16. Wang, K.K.W., Rongaloa, B.D. and
Villalobos, A. Further characterization of
calpain-catalytic activity of actin-catalytic
17. Suzuki, T., Kikuchi, T., Yumoto, N.,
Yoshimura, N. and Motokawa, Y. Comparative
specificity and kinetic studies of
ovarian calpastatin and calpain II with
naturally occurring peptidase and synthetic phosphonoesterase. Biochem J 1984; 226:
1248-50.
18. Comin, R.P. and Ramanathan, G.N.
Calcium-activated neutral protease (cal-
pain) system: Structure, function and regu-
19. Carignan, M., Willis, A.C., Prasad, M.A.
and Crawford, C. The calpain cleavage sites in the cadmium-sensitive receptor
for invasive forms. Eur J Biochem 1994;
20. Wang, K.K.W., Villalobos, A. and
Rongaloa, B.D. Calpastatin inhibits prote-
ase activity of supernatant. Biochem J 1989;
262: 663-70.
Specific degradation of respiratory T and
its recombination in its malnutrition for
260: 57-61.
22. Morice, D.F. and Lee, R.C.S. Membrane
associated calpain 2 as an antitumour of
Victoria deflected neurodegeneration in
the pericellular substantia. Biochem J 1994;
30: 269-74.
Role of phospholipase A in the activation of
the calcium-stimulated neutral protease of human extracellular, J Biol Chem 1985; 260:
389-95.
24. Saiki, T.C., Shimizu, M., Takasawa, T.,
Musashihara, H. and Suzuki, K. Proteolytic
activity of rat brain calpain 1 and calpain 2.
The effects of calpain on the structure of
neurons calpain 2. J Biochem 1977; 248:
579-86.
ANIMAL CLONING TECHNOLOGY APPLIED TO PARKINSON’S DISEASE

Researchers at the University of Colorado Health Sciences Center, Colorado, the University of Massachusetts, Amherst, Massachusetts, and Advanced Cell Technology, Worcester, Massachusetts, have successfully treated parkinsonism in rats by using fetal brain cells from cloned cows. This research is the first demonstration that transgenic cloned animal tissue can be used in the treatment of a disease. Results of the research study appear in the May 1, 1998 issue of the journal Nature Medicine.