Calpain inhibitors: novel neuroprotectants and potential anticataract agents

Po-wai Yuen[1*] and Kevin KW. Wang [2]

[1]Department of Chemistry and [2]Laboratory of Neurobiochemistr y, Department of Neuroscience Therapeutics, Parke-Davis Pharmaceutical Research, Warner-Lambert Compan y, 2800 Plymouth Road, Ann Arbo r, MI 48105, USA.

*Correspondence

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Introduction

Calpains are a class of cellular cysteine proteases activated by free calcium ion. Several related isoforms have been identified in mammals, chicken, lobster, *Schistosom a* and the fruit fly *Dorsophili a* (1-6). In mammals, there are two categories of calpains cloned: ubiquitous and tissue-specific (3). Ubiquitous calpains are expressed in all cell types (4, 7). They include μ-calpain (previously also known as calpain I) and m-calpain (or calpain II) which differ in their calcium sensitivity under *in vitr o* conditions. Both of these calpain isoforms have distinct large subunits of 80 kDa and 78 kDa, respectively, which are associated with a common small subunit. All three polypeptides have been cloned (8-10). The large subunits have four identifiable domains: domain I is partially autolyzed (to 76 kDa) which lowers its Ca^{2+} requirement (11), domain II is the cysteine protease domain containing the active site cysteine, histidine and asparagine triade (4), domain III has no homology to other proteins, and domain IV is made up of five EF-hand type helix-loop-helix calcium binding structures (8). The small subunit (29 kDa) has two domains: the N-terminal domain V which is truncated off during autolytic activation (to an 18 kDa form), and the C-terminal domain VI which again has five EF-hand structures, based on X-ray crystallography data using recombinant 18 kDa small subunit (12, 13). This data suggested that only the first four EF-hand structures can be occupied with calcium ions while the 5th EF-hand functions to dimerize with the 5th EF-hand in domain IV of the large subunit. Recombinant heterodimeric m- and μ-calpains expressed in *E. col i* and baccullovirus insect cell system, respectively, have been reported. Interestingly, Vilei *et al.* (14) also reported a domain IV deletion mutant. As expected, this protein contains calcium-independent proteolytic activity and exists as a monomeric protein. These new molecular advances should facilitate our understanding of the structure-activity relationship of the holoenzyme.

On the other hand, several tissue-specific isoforms began to emerge in recent years. The skeletal muscle-specific p94 (or CAPN3) (15). Its larger size is due to the presence of two inserts, IS-1 located in the catalytic domain I and IS-2 near the end of domain III. p94 is short-lived (due to rapid hydrolysis) and is located in the nucleus, consistent with its nuclear translocation sequence (Lys-Lys-Lys-Lys-Xaa-Pro) in insert IS-1. Sorimachi *et al.* (16) also identified the smooth muscle-specific calpain nCL-2. It has no inserts and differs from μ/m-calpain only by amino acid diversity. Most recently, Ma *et al.* (17) reported a lens-specific calpain Lp82 in rat, which is derived from alternative splicing of the mRNA for p94 and has a truncated N-terminal NS domain and lacks the IS-1 and IS-2 inserts. Two additional vertebrate calpain genes were identified in mouse and human (CAPN5 and CAPN6) (18). mRNA levels for CAPN5 are highest in testis, liver, trachea, colon and kidney, while CAPN6 mRNA is only expressed in placenta. Both proteins lack homology to the EF-hand domains found in ubiquitous calpains. In addition, CAPN6 appears to lack critical active site residues and thus may not be proteolytically active. At present, it is unclear whether tissue-specific calpains are also associated with the small subunit. In any case, these calpain isoforms probably have highly tissue-specific functions. However, due to space limitation, we will focus only on the ubiquitous calpains.

Calpain was found to prefer Leu or Val as the second residue N-terminal to the cleavage site (P2 position) in small peptide substrates (19). However, this rule is not strictly followed in protein substrates (20). In fact, not all cellular proteins are susceptible to calpain, for example, cytoskeletal proteins, membrane-bound receptor, protein

kinases/phosphatases, PEST-containing proteins, calmodulin binding proteins, myofibrillar proteins and transcription factors (4, 21). Thus, the presence of certain secondary recognition sequences was proposed (21, 22).

In a resting cell, μ/m-calpains are not believed to be significantly activated. The lack of detection (by Western blots) of autolyzed form of calpains or fragmentation of their preferred substrates (such as alpha-spectrin) in unstimulated cells support this notion (23). In fact, the concept generally holds true in uncompromised tissue (*e.g.*, the brain). Calpain activity is apparently tightly regulated by two mechanisms: cytosolic free calcium level and its inhibitory protein, calpastatin (24). A calcium transient due to opening of calcium channel would activate a small fraction of calpain which proceeds to limited prokolysis of one or more of its target substrates. The binding of calpastatin to calpain is calcium-dependent. Thus, how calpain eludes the inhibitory effects of calpastatin is still a subject of debate. One possibility might be that the two proteins occupy different subcellular compartments when calpain is activated. In any case, it has been demonstrated that under various pathological conditions (see below), sustained calcium overload would activate a large fraction of calpain, resulting in extensive degradation of cellular proteins, in an uncontrollable fashion. Compromised structural and functional integrity would ultimately lead to cell death. Therefore, from a pharmaceutical point of view, it would be important to attenuate overactivated calpain during the time of pathology.

Calpain inhibitors

The calpain family of cysteine proteases has been the subject of intense research because of its implicated pathological roles in a number of disease states that involved significant increase in intracellular Ca^{2+} concentrations. A number of calpain inhibitors have been reported in the literature since its discovery some 30 years ago (Fig. 1), the most potent and selective of which is the endogenous inhibitor calpastatin, which exists as both 68 and 105 kDa isoforms (25, 26). Several other proteins, such as heavy chains of L- and H-kininogens and α_2-macroglobulin (α_2-M), have also been reported to inhibit calpain (27, 28). However, these high-molecular weight protein inhibitors have limited therapeutic utility due to their poor cell permeability.

Most of the calpain inhibitors reported are modified peptides containing reactive functional groups to interact with the active-site cysteine thiol of calpain. These inhibitors can be classified into either reversible or irreversible inhibitors (Fig. 1). Reversible inhibitors include the aldehydes, α-keto esters, α-keto acids and α-keto amides, inactivate calpain by forming a hemithioacetal or ketal structure with the active-site cysteine thiol reversibly. Leupeptin (29), antipain (24), calpain inhibitors I and II

(30) and MDL-28170 (30) are widely used peptidyl aldehyde inhibitors (Fig. 1). Fukiage *et al.* (31) recently documented a novel dipeptide aldehyde inhibitor, SJA-6017, which has demonstrated anticataract activity *in vitro*. AK-275 (32, 33) and AK-295 (34) are examples of peptidyl α-keto amides. Leupeptin (35), MDL-28170 (36), AK-275 (33) and AK-295 (34) have also demonstrated neuroprotection in rodent models *in vivo*.

Irreversible calpain inhibitors contain reactive functional groups that can be nucleophilically displaced by the active-site cysteine thiol to form a sulfide, thereby inactivating calpain irreversibly. Examples of inhibitors in this category include chloromethanes (37), diazomethanes (38), haloacetyl hydrazines (39), arylacyloxyketones (40) (*e.g.*, compound **1**, Fig. 1), diphenylphosphinates (41), the E-64 family of peptidyl epoxides (42-44) and disulfides (45) (*e.g.*, compound **2**, Fig. 1).

Although all of these inhibitors have remarkable calpain inhibitory activities, most of them have limited pharmacological values. The two major limitations hindering their development as useful therapeutic agents are poor patent positions and lack of selectivity *versus* other cysteine proteases.

In order to clearly understand the pathological roles that calpain plays in different disease states, it is important to develop potent and selective inhibitors with a reasonable level of membrane permeability. This requirement in turn drives the calpain inhibitor research to a new stage. Most of the peptidyl calpain inhibitors mentioned above are obtained by incorporating calpain preferring P_1 and P_2 residues with reactive functional groups that are borrowed from classical enzyme inhibition research. Therefore, it is not very surprising that these inhibitors also inhibit other cysteine proteases and sometimes even serine proteases. Several groups have tried to modify these active-site targeting inhibitors to increase their calpain selectivity. For example, Li *et al.* (46) found that they can increase the calpain selectivity of dipeptide alpha-keto amides by using Leu-Abu, Leu-Phe or Leu-Nva sequences in the P_2-P_1 position. They have also reported that several alpha-keto amide derivatives such as Z-Leu-Abu-CONHCH$_2$C$_6$H$_3$(3,5-(OMe)$_2$) showed 100-fold selectivity for m-calpain over μ-calpain. Chatterjee *et al.* (47) found that the introduction of a xanthine group at the P_3 position increases inhibitor selectivity for calpain over cathepsin B.

Another approach in dealing with the selectivity problem is to investigate the possibility of inhibiting calpain via other calpain specific allosteric sites. Graybill *et al.* (48) have reported that quinolinecarboxamides are reversible calpain inhibitors that do not target the active site (*e.g.*, compound **3**, Fig. 1). However, the exact inhibition site for these inhibitors is not known. Nonetheless, some of these compounds showed $\geq$ 50-fold selectivity for calpain over both cathepsin B and cathepsin L. Wang *et al.* (49) found that α-mercaptoacrylic acids, such as PD-150606 and
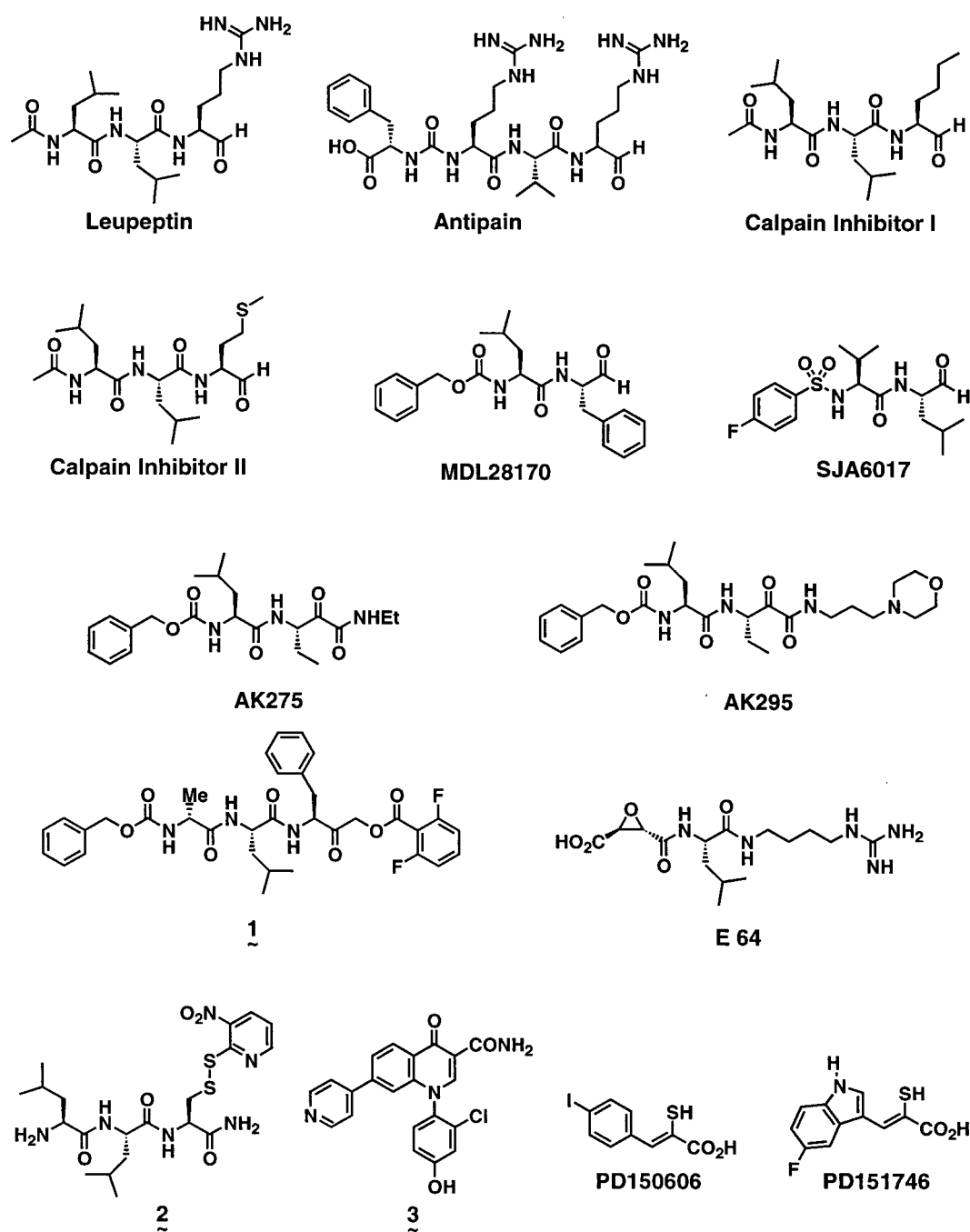
Fig. 1. Examples of calpain inhibitors.

PD-151746, are potent reversible inhibitors of calpain (Fig. 1). These compounds target calpain at its calcium binding domain (12) which accounts for their extremely high selectivity for calpain *versus* cathepsin B (>600-fold) and papain (>2000-fold). Moreover, PD-151746 also showed a 20-fold selectivity for μ-calpain over m-calpain. Both PD-150606 and PD-151746 have been found to be cell permeable.

Neuroprotection

It is now generally accepted that the major central nervous system (CNS) excitatory amino acid glutamate is also a potent neurotoxin when the synaptic concentration goes beyond the safety threshold. Cerebral ischemia (during strokes and cardiac arrest) and traumatic brain injury (TBI) represent the two most common and well-

Cerebral ischaemia

Hypoxia/hypoglycemia
↓
Synaptic glutamate buildup
↓
Glutamate receptor overactivation
↓
$[Ca^{2+}]_i$ overload
↓
Calpain Overactivation
↓
Destruction of cytoskeletal, membrane and cytosolic proteins
↓
Compromised cell functions
↓
Cell death / tissue damage

Cataract

Oxidative/chemical damage to lens cell membrane
↓
$[Ca^{2+}]_i$ elevation
↓
Calpain overactivation
↓
Degradation of crystallin proteins
↓
Precipitation of fragmented proteins
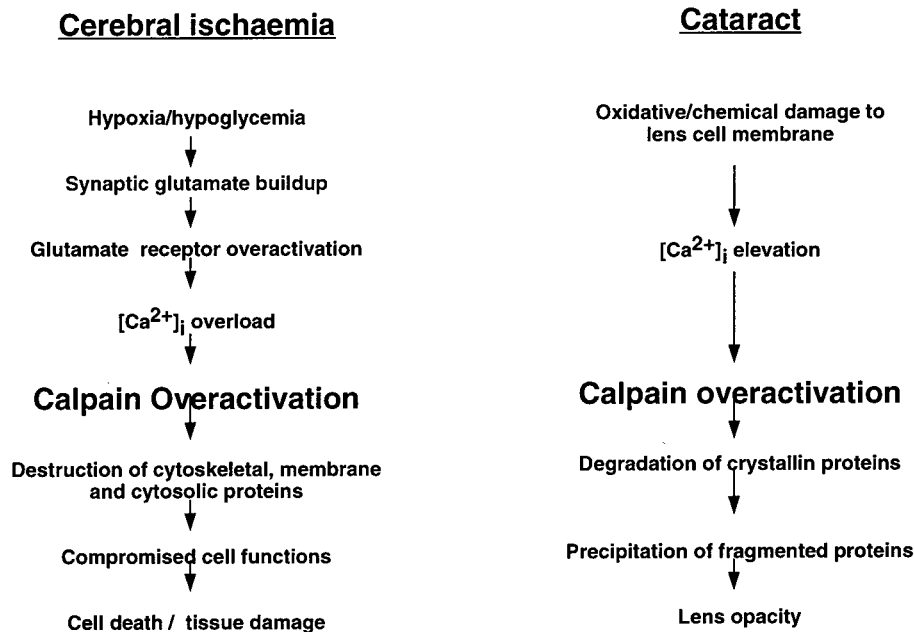↓
Lens opacity

Fig. 2. Parallel pathways of calpain activation in cerebral ischemia and cataract formation. See text for details.

studied manifestations of *in vivo* excitotoxicity (50-52). In these pathological conditions, reduction of blood flow is believed to increase presynaptic vesicular glutamate release and inhibit glutamate reuptake by adjacent astrocytes (49, 50). The resultant excessive levels of glutamate overactivate ionotropic glutamate receptors (*N*-methyl D-aspartic acid [NMDA], (±)-α-amino-3-hydroxyl-5-methylisoxazole-4-propionic acid [AMPA] and kainate receptors) in the postsynaptic membrane, sustaining influx of Na^+ and Ca^{2+}. Due to membrane depolarization, voltage-gated neuronal Ca^{2+} channels are opened, increasing the Ca^{2+} influx. The net result is massive calcium overload which overactivates a number of calcium-dependent enzymes, including calpain. As mentioned above, overactivated calpain could lead to uncontrolled degradation of cytoskeletal proteins, cytosolic and nuclear enzymes and ultimately the loss of cell function (cell death) (Fig. 2).

Evidence for calpain activation in both *in vitro* and *in vivo* models of cerebral ischemia is quite abundant. One common marker for calpain activity is the characteristic 150 kDa alpha-spectrin fragment (SBDP150). It can be detected by either anti-nonerythroid alpha-spectrin antibody or more selectively by antibodies that recognize the neo-N-terminal epitope (*e.g.*, NH_2-GMMPR) generated by the initial calpain cleavage. The SBDP150 has been identified in neuronal culture models of excitotoxicity and hypoxia/hypoglycemia (47, 53-55), as well as hippocampal slices subjected to hypoxia (55, 56).

In terms of *in vivo* models of ischemia, two major types exist: (i) irreversible focal ischemia (*e.g.*, middle cerebral artery occlusion [MCAO]), which mimics the

insult to the brain in a stroke and (ii) transient global ischemia (*e.g.*, four-vessel occlusion [4-VO]), which simulates the selective hippocampal vulnerability in cardiac arrest. Again, based on Western blot and immunohistochemistry data, spectrin breakdown product 150 kDa is readily observed in the ipsilateral cortex in MCAO (57) and in the CA1 neuronal layer in hippocampus in 4-VO (55, 56, 58). In both fluid percussion or mechanical compression models for TBI, SBDP150 is readily observed in areas surrounding the impact site (59, 60). It is worth noting that a number of calpain-sensitive neuronal proteins, including MAP-2 and neurofilament proteins, as well as calpain itself, were also found proteolyzed in several cerebral ischemia and TBI models (61-63). The role of calpain in TBI was recently reviewed (64). In the following section, we will briefly review the studies using calpain inhibitors in acute neurodegeneration.

In vitro *models*

MDL-28170 (10 µM) and leupeptin (100 µM) protected cerebellar Purkinje cells from AMPA toxicity (65). Brorson *et al.* (66) demonstrated that delayed application of MDL-28170 and E-64 (10 µM each) protected cerebellar Purkinje neurons against kainate toxicity and hippocampal neurons against NMDA toxicity. PD-150606 (25 µM) and calpain inhibitor I (1-10 µM) were shown to provide partial protection to fetal rat neocortical neurons against hypoxic/hypoglycemic damage, as well as reducing spectrin breakdown. Moreover, leupeptin and calpain inhibitor I did not protect cultured cerebellar granule cells

against glutamate or NMDA (67, 68), suggesting that granule cells may have adopted a calpain-independent cell death pathway. Two research groups also found that MDL-28170 protected cultured hippocampal neurons against excitotoxicity (69, 70). More recently, calpain was found to be activated in neuronal apoptosis, together with caspase (71). Ironically, both proteases can degrade α-spectrin rapidly, but at different cleavage sites (72). PD-150606, PD-151746 and calpain inhibitor II all partially inhibited low potassium deprivation-induced apoptosis in cerebellar granule neurons (71). Independently, Jordan *et al.* (73) provided evidence that MDL-28170 inhibited β-amyloid peptide 25-35-induced apoptosis in hippocampal cultures.

In a typical hippocampal slice model of hypoxia, the acutely isolated slices are exposed to a brief period of hypoxia which would produce irreversible neuronal loss, especially in the CA1 and CA3 regions. The functional integrity of the CA1 neurons can be monitored by measuring the evoked postsynaptic potential (EPSP). Cell-permeable calpain inhibitor I and MDL-28170 improved the functional recovery of the hippocampal slices from hypoxia (56, 74-76). Spectrin breakdown was attenuated by calpain inhibitor I (76). Hiramatsu *et al.* (77) further showed that synaptic transmission in gerbil neocortical slices subjected to hypoxia was improved by MDL-28170 (50 μM). Freshly isolated cerebellar slices can also be challenged directly with AMPA (200 μM), resulting in high percentage loss of Purkinje neurons in a matter of hours (77). Both MDL-28170 and PD-150606 were shown to attenuate neuronal loss in this model (49, 78).

In vivo *models*

The relative nonselective and low cell permeant, leupeptin, was the first calpain inhibitor used in an *in vivo* ischemia model (56). It was administrated in a prolonged intracerebroventricular infusion paradigm which in fact enhanced hippocampal CA1 neuron survival in gerbils subjected to transient ischemia (56). These results provided the first evidence suggesting that neuroprotection can be achieved by inhibiting calpain. A more recent report showed that administration of MDL-28170 (30-60 mg/kg i.v.) significantly reduced infarct size in a rat MCAO focal ischemia model (57). In parallel, calpain inhibitor I was also found to protect CA1 neurons in the hippocampal CA1 in a 4-VO global ischemia model (79). Supracortical perfusion of AK-275 was shown to markedly reduce infarct size in a rat MCAO model (33). Intraarterial infusion of the more water-soluble AK-295 also reduced infarct size in the same model (34). Additional studies supporting the neuroprotective effects of MDL-28170 in both global and focal cerebral ischemia have been reported (80).

In a rabbit TBI model with mechanical compression, breakdown of spectrin, MAP2 and neurofilaments H and L has been reported (63, 81, 82). Posmantur *et al.* (82) reported that intraarterial infusion of calpain inhibitor II almost completely inhibited neurofilament and spectrin breakdown in the same model. To extend that observation, Saatman *et al.* (59) recently reported that AK-295 reduced brain injury as well as improved functional recovery (as measured by composite neuroscore) from fluid percussion-induced TBI in rat.

Anticataract actions

In the fiber cells of mammalian lens, crystallin proteins (α, β and γ) are highly concentrated and organized to allow light transmission. As the lens ages, various cumulative environmental insults such as UV radiation, high sugar level (diabetes), environmental toxins, free radicals and drugs such as corticosteroids can lead to compromised membrane protein and lipid integrity and increased membrane permeability to ions, including calcium (83, 84). Lens m-calpain is activated under these conditions, resulting in degradation of α- and β-crystallin proteins. Fragmentation of crystallin proteins results in precipitation and hence lens opacity (cataract) (85, 86) (Fig. 2). Calpain-mediated cytoskeletal protein breakdown can further compromise cell permeability. Cataracts (lens opacity) can in fact be modeled by subjecting cultured lens of young rats to chemical oxidants (*e.g.*, sodium selenite, diamide) or calcium ionophoric agents (*e.g.*, A-23187) (87). Addition of xylose in culture also mimics *in vivo* "sugar cataract" (88) due to the production of polyols by aldose reductase (89). The β-crystallin and α-spectrin fragmentation pattern in cultured lens subjected to A-23187 is identical to that formed *in vitro* by calpain digestion (90). Calpain activation in cataract lenses is further supported by the presence of spectrin breakdown products (31, 91). Experimental cataracts can also be chemically induced *in vivo* (*e.g.*, with bolus administration of sodium selenite (20 mol/kg) in young rats (83). In such models, calpain-mediated β-crystallin and α-spectrin (88, 91) have been reported. In fact, N-terminal sequencing revealed that the *in vitro* calpain cleavage sites of β-crystallins matched those found *in vivo* (86, 90). Various studies using calpain inhibitory agents are discussed in the following.

Cultured lens model

Shearer *et al.* examined the effects of several calpain inhibitory agents on cataract formation over the years (92-94). E-64 (5 μM to 1 mM) and MDL-28170 (500 μM) reduced opacity as well as prevented the insolubilization of β-crystallins. In a later study, they found that the membrane-permeable E-64d was more potent (5-20 μM) than

E-64 in reducing A-23187-induced opacity (93). Calpain inhibitors I and II were not protective, possibly due to their cytotoxicity (94). Recently, Sanderson *et al.* (95) reported that these two compounds could inhibit β-crystallin degradation in A-23187-treated lens but not against opacity formation. Most recently, Fukiage *et al.* (31) documented that the novel compound SJA-6017 is superior to E-64 in reducing both spectrin breakdown and lens opacity in A-23187-treated rat lenses.

In vivo *model*

The effects of E-64 on selenite cataract formation have also been investigated *in vivo* in rats. E-64 was injected intraperitoneally (100 mg/kg) 2 h before selenite was administered and the same daily dose was continued for 5 days. In this model, E-64 reduced the frequency of the most severe stage of cataracts, namely, nuclear cataracts (83). It should be noted that in the E-64-treated group, β-crystallin breakdown continued to occur at a slower rate, suggesting that calpain activity was not fully inhibited.

Other indications

Neurologic and neurodegenerative disorders

Excitotoxicity has been reported to contribute in part to several neurodegenerative conditions, including epilepsy, Huntingtons disease, Parkinson disease and amyotrophic lateral sclerosis (51, 52). Likewise, spinal cord injury also involves glutamate receptor activation and calcium influx. Calpain immunoreactivity and neurofilament protein degradation have been demonstrated (96, 97). By inference, calpain inhibitors may have therapeutic values in one or more of these neurological disorders. In addition, calpain may mediate myelin degradation in demyelination disorders (*e.g.*, Wallerian degeneration, multiple sclerosis and peripheral neuropathies) (98). Restricting blood flow after subarachnoid hemorrhage (SAH) is known to induce long-lasting cerebral vasospasm resulting in local ischemia (99). Endothelin receptor and the subsequent calpain activation in the vascular smooth muscle cells are thought to mediate this event. Consistent with that, calpeptin (1 μM) could dilate the exposed spastic basilar artery in a rabbit model (100).

Muscular dystrophy

Calpain has long been proposed to be overactivated in various forms of muscular dystrophy, including Duchenne muscular dystrophy (DMD) in human and mdx mice. In both cases, the actual genetic defect apparently is the lack of expression of the dystrophin protein, which is possibly a calcium leak channel (101). Characteristic features such as myofibrillar protein loss and Z-disc disorganization and increased plasma membrane permeability are potentially mediated through calpain (102).

Recently, the role of calpain in muscular dystrophy took on new meaning as hereditary limb-girdle muscular dystrophy 2A (LGMD2A) was linked to various mutations in the CANP3 gene which encodes the skeletal muscle-specific calpain p94 (103, 104). Most of these mutations would produce dysfunctional p94. In fact, very little mature p94 protein was found in LGMD2A (105). Therefore, it is assumed that p94 has a vital function in maintaining healthy skeletal myocytes. Transgenic mice that bear some of these mutations in the CANP3 gene have recently been made and appear to have LGMD2A-like symptoms. It is possible that in LGMD2A, the lack of p94 triggers the myocytes to overproduce or overactivate μ- and/or m-calpain as a compensatory mechanism (106). Ironically, it could be the excessive activity of these ubiquitous calpains that leads to the observed muscular dystrophy. This attractive hypothesis remains to be tested experimentally.

Miscellaneous

Calpain also appears to have a role in other pathological conditions such as cardiac ischemia, thrombotic platelet aggregation, arthritis and restenosis. These topics have been recently reviewed (24) and will not be discussed here.

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