

See discussions, stats, and author profiles for this publication at: <https://www.researchgate.net/publication/14316042>

Wang KKW, Yuen P Development and therapeutic potential of calpain inhibitors. Adv Pharmacol 37:117-152

ARTICLE in ADVANCES IN PHARMACOLOGY (SAN DIEGO, CALIF.) · FEBRUARY 1997

DOI: 10.1016/S1054-3589(08)60949-7 · Source: PubMed

CITATIONS

76

READS

19

2 AUTHORS:



[Kevin KW Wang](#)

University of Florida

310 PUBLICATIONS 11,147 CITATIONS

SEE PROFILE



[Po-wai Yuen](#)

Pharmaron

54 PUBLICATIONS 2,063 CITATIONS

SEE PROFILE

*Department of Neuroscience Therapeutics
Parke-Davis Pharmaceutical Research
Warner-Lambert Company
Ann Arbor, Michigan 48105

†Department of Chemistry
Parke-Davis Pharmaceutical Research
Warner-Lambert Company
Ann Arbor, Michigan 48105

Development and Therapeutic Potential of Calpain Inhibitors

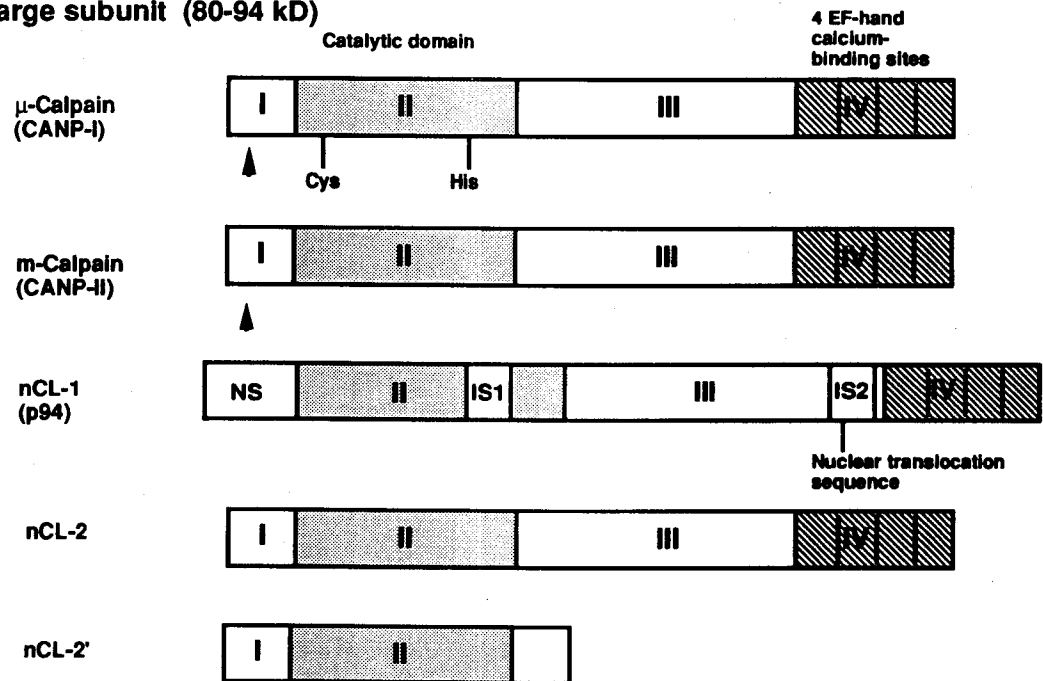
I. Overview on Calpain

A. The Structure of Calpain and Its Isoforms

Calpain, the Ca^{2+} -activated neutral protease (CANP), was independently identified in rat brain and skeletal muscle (Guroff, 1964; Huston and Krebs, 1968). This class of proteases has two major isoforms that appear to be almost universally distributed in most mammalian tissues. μ -Calpain (previously also known as calpain I) and m-calpain (or calpain II) are the two isoforms that differ in their calcium sensitivity under *in vitro* conditions. As their names imply, μ -calpain and m-calpain require low and high micromolar free calcium for activation, respectively (Croall and Demartino, 1991; Saido *et al.*, 1994; Wang and Yuen, 1994). The calpains can be found in the cytosolic compartment and can also be identified in the plasma membrane and other organelle membranes (Banik *et al.*, 1992). Both isoforms contain two subunits. Although the large subunit for each isoform is distinct, the

small subunit is identical (Fig. 1). The large subunit is 80 kDa in size and generally can be divided into four domains based on homology to other proteins. Domain II is the catalytic domain that is homologous to other members of the cysteine protease family. Domain I and domain III have no homology to other proteins. Domain IV is a calmodulin-like domain containing four calcium-binding EF-hand structures (Aoki *et al.*, 1986) (Fig. 1). The small subunit (29 kDa) has two domains: the N-terminal half (domain V) contains a glycine-rich region that interacts with biological membranes as well as phospholipids (e.g., PIP₂) that lower its Ca²⁺ requirement (Imajoh *et al.*, 1986), and the C-terminal half (domain VI) is another calcium-binding domain with four EF-hand structures. It is assumed that

Large subunit (80-94 kD)



Small subunit (29 kD)



FIGURE 1 Model of calpain isoforms and subunits. At least four distinct isoforms of the large calpain subunit have been cloned. (about 80–94 kDa). The large subunit can be divided into four domains. Domain II is a cysteine protease domain containing the catalytic cysteine (Cys) and histidine (His). Domain III has unknown functions and is not homologous to other proteins. Domain IV is made up of four EF-hand Ca²⁺-binding sites. μ -Calpain and m-calpain are ubiquitous, whereas nCL-1 is skeletal muscle specific, and smooth muscles possess nCL-2 and nCL-2' produced by alternative splicing. μ - and m-Calpains contain a 29-kDa small subunit, which has a glycine-rich domain V and another EF-hand Ca²⁺-binding domain (domain IV'). The arrowheads indicate position of initial autolytic cleavage sites. Domain nomenclature is according to Suzuki (1987). Modified from Figure 1 in Wang and Yuen (1994).

these Ca^{2+} -binding sites impose a strict Ca^{2+} requirement on the catalytic activity. It is generally agreed that μ -calpain and m-calpain exist as proenzymes (80 kDa + 29 kDa) in the resting state. On activation by Ca^{2+} , calpain autolyzes by truncation of the N-terminal portion of domain I and most of domain V from the small subunit. The catalytic activity of this truncated form (76 kDa + 18 kDa) seems to have greater Ca^{2+} sensitivity (Suzuki *et al.*, 1981a). More recently, skeletal muscle-specific (nCL-1; p94, CANP-3) and stomach smooth muscle-specific (nCL-2) forms of the large subunit were identified by molecular cloning approaches (Sorimachi *et al.*, 1989, 1993). nCL-1 has a larger molecular weight, since it contains two inserts: IS-1 located in catalytic domain I and IS-2 near the end of domain III (Fig. 1). Interestingly, IS-1 has a nuclear translocation-like sequence (Lys-Lys-Lys-Lys-Xaa-Pro) in its catalytic domain. nCL-2 can be alternatively spliced to generate a form (nCL-2') that totally lacks the Ca^{2+} -binding domain. Ironically, this protein product could be a calcium-independent form of calpain. Whether any of these new forms of the large subunit are associated with the small subunit is unknown at present.

B. Substrates and Physiological Functions

Based primarily on *in vitro* studies with purified enzyme, a number of cellular proteins have been identified as potential calpain substrates (Table I). These included a large number of cytoskeletal proteins (e.g., spectrin, MAP-2), a growing list of membrane receptors (e.g., EGF receptor), G-proteins, calmodulin-binding proteins, enzymes involved in signal transduction (such as protein kinase C, IP3 kinase), and metabolic pathways (e.g.,

TABLE I Substrates of Calpain

<i>Class</i>	<i>Examples of target proteins</i>
Cytoskeletal and structural proteins	Spectrin, MAP-2 ^a , tau factor, neurofilament H, M and L, α -actinin
Membrane-bound receptors and proteins	EGF receptor, AMPA-receptor, calcium pump, anion channel, calcium release channel, L-type calcium channel, G-proteins
Calmodulin binding proteins	Calcium pump, calcineurin, CaM-dependent protein kinase II, myosin light chain kinase, neuromodulin, connexins, IP3 kinase
Enzymes	Protein kinase C, HMG-CoA reductase, cAMP-dependent kinase, pyruvate kinase, phosphorylase kinase
Myofibrillar proteins	Troponin I, troponin T, tropomyosin, myosin
Transcription factors	c-fos, c-jun, Pit-1, Oct-1, CP1a, and b, c-Myc

^a MAP-2, microtubule-associated protein-2; EGF, epidermal growth factor; CaM, calmodulin; IP3, inositol 1,4,5-trisphosphate; HMG, 3-hydroxy-3-methylglutaryl.

phosphorylase kinase) (Wang *et al.*, 1989; Croall and Demartino, 1991; Greenwood and Jope, 1994; Bi *et al.*, 1994). Various transcription factors (e.g., c-fos, c-jun) have also been identified as endogenous calpain substrates (Hirai *et al.*, 1991; Watt and Molloy, 1993).

Using synthetic peptides as substrates, calpain was found to prefer Leu or Val as the second residue N-terminal to the cleavage site. However, this rule was not strictly followed when protein substrates were used instead (Sakai *et al.*, 1987). It was proposed that secondary recognition sequences may come into play. Hydrophilic sequences enriched in proline, glutamate, aspartate, serine, and threonine, the so-called PEST sequences, appear to exist in most calpain substrates and are usually located near the cleavage sites (Wang *et al.*, 1989). Calmodulin-binding proteins have also been reported to be preferred substrates of calpain. A recent report showed that a synthetic peptide corresponding to the calmodulin-binding region of the plasma membrane calcium pump can indeed interact with the calcium-binding domain in the large subunit of μ -calpain (Molinari *et al.*, 1995). This study confirms the presence of a secondary interaction between calpain and its substrates.

II. Development of Calpain Inhibitors

The quest to find inhibitors for calpain began when the Ca^{2+} -dependent protease was discovered 30 years ago (Guroff, 1964; Huston and Krebs, 1968). Early inhibitors were Ca^{2+} chelators, such as EDTA and EGTA, or sulfhydryl group-specific reagents, such as iodoacetate and 5,5'-dithiobis(2-nitrobenzoic acid). As the knowledge of calpain substrate specificity increased and calpain inhibitors from natural products screening programs became available, more selective inhibitors were designed and synthesized. We currently have an arsenal of calpain inhibitors at our disposal. These inhibitors include protein inhibitors, such as calpastatin, irreversible peptide inhibitors, such as E64 analogs, reversible peptide inhibitors, such as peptidyl α -keto amides, and several nonpeptide inhibitors (Wang and Yuen, 1994). With the recent emergence of several cell-permeable calpain selective inhibitors, the goal of understanding the physiological roles of calpain may soon be achievable.

A. Protein Inhibitors

Calpastatin is the endogenous inhibitor that strongly and specifically inhibits both isoforms of calpain. It consists of a unique N-terminal domain (domain L) and four repetitive calpain-inhibiting domains (repeats 1–4). Most tissues express either this full-length protein (68 kDa) or a shortened form, which contains only repeats 2–4 (45 kDa), or both (Takano *et al.*, 1986; Murachi, 1989). Each domain consists of about 140 residues. All

four internally repetitive domains show similar inhibitory activities against μ -calpain and m-calpain. However, no inhibition is observed in the case of domain L (Maki *et al.*, 1987). Each repetitive domain has three well-conserved regions located on separated exons. Of the three regions, A, B, and C, region B is essential and strongly inhibits both μ -calpain and m-calpain. Ishima *et al.* (1991) also demonstrated that a synthetic 27-residue peptide corresponding to exon 1B has the ability to inhibit calpain specifically. It has been shown that the synthetic oligopeptides of regions A and C had no calpain inhibition activity, whereas region B oligopeptide showed weak inhibition activity (Yang *et al.*, 1994). Takano *et al.* (1995) further showed that regions A and C interact in a Ca^{2+} -dependent manner with the calcium-binding domains IV and VI, respectively. They proposed that these interactions enhance the inhibitory effect of calpastatin.

Calpain is also inhibited by the heavy chains of L-kininogen and H-kininogen (Salvesen *et al.*, 1986; Schmaier *et al.*, 1986). Puri *et al.* (1993b) used the highly conserved sequence, Gln-Val-Val-Ala-Gly-NH₂, present in domains II and III of human kininogens to produce an irreversible inhibitor of plate calpain. Another reported protein inhibitor for calpain is α_2 -macroglobulin (α_2 -M) (Sasaki *et al.*, 1983). However, the calpain- α_2 -M complex still retained some calpain activity (Crawford, 1987).

B. Irreversible Peptide Inhibitors

Peptidyl chloromethanes are irreversible inhibitors that inhibit both cysteine and serine proteases. The mechanism of inhibition involves nucleophilic attack of the active-site cysteine thiol on the chloromethane fragment of the inhibitor. Potent chloromethane inhibitors were obtained by incorporating calpain-preferring residues at the P₁ and P₂ positions. For example, Sasaki *et al.* (1986) found that with Phe at P₁ and Leu at P₂, the compound dansyl-Leu-Leu-Phe-CH₂Cl inhibited μ -calpain and m-calpain, with IC₅₀ values of 0.12 and 0.18 μM , respectively. The compound also showed 10-fold selectivity for calpains over papain.

Peptidyl difluoromethyl and trifluoromethyl ketones are effective inhibitors of serine but not cysteine proteases (Imperiali and Abeles, 1986; Stein *et al.*, 1987; Peet *et al.*, 1990). However, peptidyl monofluoromethanes (Rasnick, 1985) are more than one order of magnitude more reactive with cysteine proteases than with serine proteases (Anglikier *et al.*, 1987). The mechanism by which monofluoromethanes inhibit cysteine proteases may not simply be an irreversible nucleophilic displacement of the α -fluorine by the active-site cysteine thiol. The formation of a reversible hemithioketal intermediate between the keto carbonyl group of the inhibitor and the active-site thiol of the cysteine protease may also play a role.

Giordano *et al.* (1993) discovered a new class of cysteine protease inhibitors, the peptidyl haloacetyl hydrazines. At concentrations of 0.1–

0.4 mM, these compounds do not inhibit trypsin, chymotrypsin, or porcine pancreatic elastase after incubation for 5 hs. The nonspecific alkylating properties are relatively low, as demonstrated in tests using glutathione as the nucleophile. These inhibitors have higher reactivity for cathepsin B than for calpain.

Peptidyl diazomethanes are irreversible inhibitors of cysteine proteases. They were previously thought to inhibit only cysteine proteases and not serine proteases (Leary *et al.*, 1977; Green and Shaw, 1981; Shaw, 1990). However, Zumbrunn *et al.* (1988) found that Z-Phe-Arg-CHN₂ inhibits plasma kallikrein, and more recently, Stone *et al.* (1992) found that [³H]Ac-Ala-Ala-Pro-CHN₂ was a competitive slow-binding inhibitor of the serine protease prolyl endopeptidase. Therefore, one cannot make the general assumption that peptidyl diazomethanes are selective for cysteine proteases. Crawford *et al.* (1988) studied a number of these diazomethanes to probe their selectivity for m-calpain relative to cathepsin L and cathepsin B. The P₁ position specificity for m-calpain was found to follow the order of Leu < Trp < Tyr(I) < Tyr < Met < homoPhe. It was also observed that calpain prefers Leu in the P₂ position, confirming the earlier Sasaki study on calpain substrates (Sasaki *et al.*, 1984). Leu in P₃ provided a better calpain inhibitor, which was also favorable to cathepsin L. Finally, Anagli *et al.* (1991) found that Z-Leu-Leu-Tyr-CHN₂ is the fastest reacting calpain inhibitor, and Boc-Val-Lys(Z)-Leu-Tyr-CHN₂ is the only inhibitor that reacts more rapidly with m-calpain than with cathepsin L or cathepsin B. However, Z-Leu-Leu-Tyr-CHN₂ is a better inhibitor for cathepsin L, and for Boc-Val-Lys(Z)-Leu-Tyr-CHN₂, the rate difference is only 1.6 times favoring calpain.

Peptidyl arylacyloxyketones are known to be potent inactivators of lysosomal cysteine proteases (Krantz *et al.*, 1991). Harris *et al.* (1995) recently investigated their use for inhibiting calpain. A number of peptidyl arylacyloxyketones with different aryl groups were synthesized. Although μ -calpain prefers hydrophobic amino acid residues at P₁ and P₂, Harris *et al.* (1995) found that the leaving group structure on these inhibitors has a profound influence on the potency, which can even override the putative P₁-P₂ specificity preferences of calpain. They discovered that Z-D-Ala-Leu-Phe-OCO-2,6-F₂-Ph (compound 1, Fig. 2) is a selective, time-dependent inhibitor of m-calpain with >100-fold selectivity over cathepsin B and L. Moreover, this compound has a human plasma half-life of 85 min. It is interesting to note that peptidyl arylacyloxyketones are orally bioavailable and lack gross toxicological effects (Wagner *et al.*, 1994).

In another study by Dolle *et al.* (1995), it was observed that the diphenylphosphinate leaving group can be used to replace benzoate groups. In this case, the compound (compound 2, Fig. 2) is more than 50-fold selective for μ -calpain relative to both interleukin-1 β (IL-1 β) converting enzyme and cathepsin B.

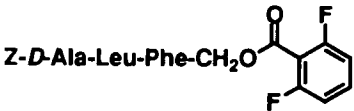
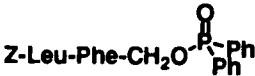
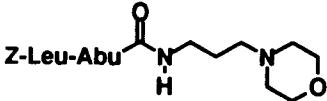
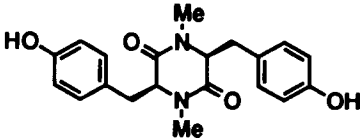
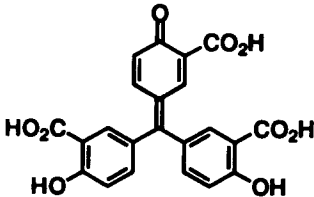
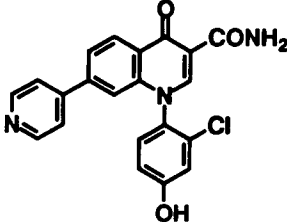
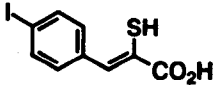
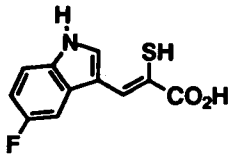
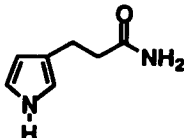
Compound #	Name	Structure
1		
2		
3	AK295	
4		
5		(<i>N</i> -Me)-Tyr-(<i>N</i> -Me)-Tyr-Leu-Ala-OH
6	ATA	
7		
8	PD 150606	
9	PD 151746	
10	Cystamidin A	

FIGURE 2 Recently identified calpain inhibitors. For selectivity refer to text. Z, carboben-zoxy; Abu, 2-aminobutyric acid.

The peptidyl epoxides, *trans*-epoxysuccinyl-L-leucylamido-4-guanidino) butane (E64) and 2S,3S)-*trans*-epoxysuccinyl-L-leucylamido-3-methylbutane (E64c), are another class of irreversible calpain inactivators. These inhibitors all have a common epoxysuccinyl unit, which can form a covalent bond with the active-site cysteine thiolate. E64 was first isolated from solid cultures of *Aspergillus japonicus* TPR-64 (Hanada *et al.*, 1978). Studies of synthetic analogs of E64 showed that the guanidino group was not essential for inhibitory activity. It was found that E64c, an analog with an isopentyl group replacing the charged guanidino group of E64 at P₃, was equally effective in inhibiting calpain. Furthermore, Parkes *et al.* (1985) characterized a new inhibitor. EP-460 which had a threefold increase of the rate constant relative to E64. It was produced by replacing the guanidino group of E64 with a 4-benzyloxycarbonylaminobutyl group. Although E64 analogs showed high specificity for cysteine proteases (Hanada *et al.*, 1978; Barrett *et al.*, 1982), they possessed no selectivity for calpain relative to other cysteine proteases (Barrett *et al.*, 1982; Inaba *et al.*, 1979; Sugita *et al.*, 1980; Towatari *et al.*, 1978). An additional problem with these compounds is their poor membrane permeability. Little calpain inhibitory activity was observed in intact cells despite their high potency in broken cell assays (Wilcox and Mason, 1992). Esterification of the free carboxylic acid group of E64c did produce an inhibitor, E64d (Tamai *et al.*, 1987), that is able to cross plasma membranes and inhibit intracellular calpains (McGowan *et al.*, 1989). E64d is 100-fold less effective in lysed cells than E64c. However, once inside cells, it can be hydrolyzed readily by cellular esterases to give E64c, thus, inhibiting calpain *in situ* (Huang *et al.*, 1992). Other compounds of interest include peptidyl sulfonium methyl ketones, which according to Pliura *et al.* (1992) are more potent inhibitors of calpain than fluoromethyl ketones, diazomethyl ketones, and acyloxymethyl ketones. The sulfonium methyl ketones did show some preference toward calpain. In fact, Z-Leu-Leu-Phe-CH₂S⁺(CH₃)₂ · Br⁻ is a 57-fold faster inhibitor of calpain than of cathepsin B.

Matsueda *et al.* (1990) reported that peptide disulfides are selective calpain inhibitors. These inhibitors all contain a S-(3-nitro-2-pyridinesulfonyl) group (Npys) as a common structural feature. Compounds with Leu-Leu or Val-Val sequences were found to be highly potent inhibitors of calpain. For example, H-Leu-Leu-Cys(Npys)-NH₂ strongly inhibited calpain (IC₅₀ = 0.18 μM) but only weakly inhibited cathepsin B (IC₅₀ = 170 μM) and papain (IC₅₀ = 190 μM). Additionally, this compound did not inhibit serine and aspartic proteases.

C. Reversible Peptide Inhibitors

Peptidyl aldehydes are reversible calpain inhibitors that also inhibit serine proteases. These inhibitors inactivate cysteine proteases by reacting

reversibly with the active-site thiol via their aldehyde moiety to form a hemithioacetal intermediate (Schröder *et al.*, 1993). The peptidyl aldehydes were initially discovered from natural product sources. Leupeptin and antipain are among the earliest reported calpain inactivators. (Toyo-Oka *et al.*, 1978; Suzuki *et al.*, 1981b). Leupeptin, Ac-Leu-Leu-Arg-H, is isolated from the filtrate of *Streptomyces* cultures (Aoyagi and Umezawa, 1975). It inhibits μ -calpain and m-calpain, with K_i values of 0.32 and 0.43 μ M, respectively (Sasaki *et al.*, 1984). However, it also inhibits plasmin, trypsin, papain, and cathepsin B. Antipain ([*(S)*-1-carboxy-2-phenylethyl]-carbamoyl-L-Val-Arg-H) is produced by *Streptomyces michigaensis* and *Streptomyces yokosukaensis* (Sasaki *et al.*, 1984). However, like leupeptin, it also inhibits other proteases, including papain, trypsin, cathepsin A, and cathepsin B. Strepin P-1 (N-*i*-valeryl-Tyr-Val-Arg-H), isolated from the culture fluid of *Streptomyces tanabeensis* (SAB-934), inhibits calpain potently but also inhibits papain and trypsin (Ogura *et al.*, 1985). Staccopins P1 and P2 are natural products isolated from *S. tanabeensis* (SAM-0019) (Saito *et al.*, 1987). They inhibit calpains and papain strongly and were not active against serine proteases, such as trypsin and chymotrypsin. Staccopins P1 and P2 are pentapeptides with the structures Val-Val-Val-Val-Phe-H and Val-Val-Val-Val-Tyr-H, respectively.

All the inhibitors mentioned have limited pharmacological value because of their poor cell permeability. Therefore, a series of studies was undertaken to modify these peptides. The changes that most improved the activity of the compounds were substitutions on the N-terminal with a lipophilic moiety, such as a benzyloxycarbonyl group. Calpeptin (Z-Leu-Nle-H) was one such second-generation peptidyl aldehyde inhibitor (Yano *et al.*, 1993). It inhibits human platelet μ -calpain, with an IC_{50} of 40 nM (Tsujioka *et al.*, 1988). Sasaki *et al.* found that 4-phenylbutyryl-Leu-Met-H inhibited both μ -calpain ($K_i = 36$ nM) and m-calpain ($K_i = 50$ nM), with only weak inhibition of trypsin, chymotrypsin, and cathepsin H (Sasaki *et al.*, 1990). They also discovered two new inhibitors, calpain inhibitor I (Ac-Leu-Leu-Nle-H) and calpain inhibitor II (Ac-Leu-Leu-Met-H). However, calpain inhibitor I is a better inhibitor of cathepsin L ($K_i = 0.5$ nM) than of calpains, and calpain inhibitor II also inactivated cathepsin B ($K_i = 100$ nM). Another cell-penetrating calpain inhibitor, MDL28170 (Z-Val-Phe-H) (Mehdi *et al.*, 1988), was used in the study conducted by Arlinghaus *et al.* (1991). This compound greatly improved the posthypoxic recovery of synaptic potentials of transient hypoxia-treated rat brain hippocampal slices. Although these second-generation peptidyl aldehydes have improved cell permeability relative to their predecessors, they still have limited use in *in vivo* pharmacological studies. The major obstacle is that calpain specificity still has not been achieved. In addition, these peptidyl aldehydes are readily oxidized under physiological conditions (Imperiali, 1988; Shaw, 1990).

Peptidyl α -keto esters were initially used as serine protease inhibitors (Hori *et al.*, 1985). Li *et al.* (1993) extended their use to inhibit calpain and other cysteine proteases. A number of peptidyl α -keto esters, α -keto acids, and α -keto amides have been synthesized. The α -keto esters (e.g., Z-Leu-Phe-COOEt) were found to have some selectivity for μ -calpain (around $K_i = 1.8 \mu\text{M}$) and m-calpain (around $K_i = 0.4 \mu\text{M}$) relative to cathepsin B (around $K_i = 340 \mu\text{M}$) and papain (around $K_i = 75 \mu\text{M}$). The α -keto esters were, however, rapidly degraded *in vivo* by plasma esterases. The peptidyl α -keto acids were the better inhibitors among the peptidyl α -keto family of compounds. Indeed, Z-Leu-Phe-COOH was the best inhibitor for calpains ($K_i = 8.5 \text{ nM}$, 5.7 nM , $4.5 \mu\text{M}$, and $7.0 \mu\text{M}$ for μ -calpain, m-calpain, cathepsin B, and papain, respectively) discovered in this study. Unfortunately, the α -keto acids have rather poor membrane permeability. Li *et al.* (1993) then studied the α -keto amides to improve both plasma stability and membrane permeability. Z-Leu-Phe-CONH(CH₂)₂Ph was the best α -keto amide inhibitor ($K_i = 52 \text{ nM}$ and 24 nM for μ -calpain and m-calpain, respectively) in their purified enzyme assays. One of the α -keto amide analogs, AK275 (Z-L-Leu-L-Abu-CONHEt) (Fig. 2), was found to protect against focal ischemic brain damage in rats when administered post-ischemia (Harbeson *et al.*, 1994; Bartus *et al.*, 1994a). To improve the solubility of the α -keto amides, analogs of AK275 were further modified. AK295, a compound with a morpholinopropyl group replacing the ethyl group on the side chain of AK275, was found to be more soluble in aqueous media while preserving the potency, selectivity, and neuronal permeability of the peptidyl α -keto amides. It was found also to be efficacious in the rat middle cerebral artery occlusion (MCAO) model (Bartus *et al.*, 1994b). Several peptidyl α -keto amides showed selectivity for μ -calpain vs m-calpain. For example, Z-Leu-Abu-CONH-*i*-Bu and Z-Leu-Phe-CONHEt showed a 28-fold and 18-fold selectivity for m-calpain over μ -calpain (Li *et al.*, 1993).

Alvarez *et al.* (1994) reported the isolation and structure elucidation of two new calpain inhibitors from *Streptomyces griseus* (SC488). The diketopiperazine (compound 4, Fig. 2) inhibited calpain, with an IC₅₀ of $0.8 \mu\text{M}$, and the tetrapeptide (compound 5, Fig. 2), with an IC₅₀ of $1.2 \mu\text{M}$.

D. Nonpeptide Calpain Inhibitors

Most of the calpain inhibitors reported in the literature are peptides or modified peptides (Wang, 1990; Wang and Yuen, 1994). Nonpeptide calpain inhibitors are limited to general protease inactivators, such as N-ethylmaleimide (Fox *et al.*, 1983), iodoacetic acid (Suzuki, 1983), mersalyl (Croall *et al.*, 1986), isocoumarins (Bartus *et al.*, 1992), and diisopropyl phosphorofluoridate (Gupta and Abou-Donia, 1995). Most of these inhibitors are neither potent nor selective considering their inhibition of other proteases. These properties severely restrict their usefulness and predictive value in the study of calpain-mediated events.

Recently, a number of nonpeptide calpain inhibitors began to be mentioned in the literature. Posner *et al.* (1995) demonstrated that aurintricarboxylic acid (ATA) (compound 6, Fig. 2) inhibited both μ -calpain and m-calpain with IC_{50} of 22 μM and 10 μM , respectively. They also reported that ATA reduced N-methyl-D-aspartate (NMDA)-induced spectrin breakdown and neuronal death. Similar to all other previously reported nonpeptide inhibitors, ATA also inhibited a number of enzymes, such as endonucleases, DNA polymerases, glucose 6-phosphate dehydrogenase, and phosphofructose kinase. Considering that ATA also blocks apoptotic cell death (McConkey *et al.*, 1989, 1990), it may be an interesting compound for further evaluation.

Graybill *et al.* (1995) discovered that quinolinecarboxamides are reversible inhibitors. Compound 7 (Fig. 2) showed approximately 50-fold selectivity for human erythrocyte μ -calpain over both cathepsin B and cathepsin L. Experiments with multiple concentrations of [3H]casein and inhibitor demonstrate that these quinolinecarboxamides do not inhibit calpain competitively, indicating that they may bind at a site other than the active site.

Wang *et al.* (1996) found that derivatives of α -mercaptoacrylic acid, PD150606 and PD151746 (Compounds 8 and 9, Fig. 2), are reversible calpain inhibitors. The more potent analog PD150606 inhibits μ -calpain and m-calpain, with apparent K_i of 0.21 and 0.37 μM , respectively. Calpain inhibitors reported in the literature, with the exception of some peptidyl α -keto amides, show minimal differentiation between the two calpain isoforms. PD151746 is an interesting exception that shows a 20-fold selectivity for μ -calpain ($K_i = 0.26 \mu M$) over m-calpain ($K_i = 5.33 \mu M$). PD151746 and the peptidyl α -keto amide Z-Leu-Abu-CONH-*i*-Bu are new and important tools for the study of calpain because of their complementary selectivity toward the two isoforms. The mercaptoacrylates are cell permeable and are extremely selective for calpain (>600-fold relative to cathepsin B and >2000-fold for papain). Furthermore, they inhibit calpain uncompetitively with respect to casein. These findings, together with the fact that PD150606 also shows low affinity inhibition of calmodulin-stimulated calcineurin activity, led to the proposal that these inhibitors may bind to the Ca^{2+} -binding domain of calpain.

Another compound of interest because of its structural simplicity is cystamidin A (Compound 10, Fig. 2). It is a natural product isolated from *Streptomyces* sp. KP-1241 culture. Omura *et al.* (1993) claimed that it has calpain inhibitory properties, although biological data were not reported.

III. Therapeutic Areas

A. Cerebral Ischemia/Excitotoxicity

Glutamate is a major excitatory amino acid (EAA) in the central nervous system (CNS). The release of glutamate from vesicular stores of presynaptic

neurons evokes the activation of different glutamate receptors in the postsynaptic membrane (Meldrum and Garthwaite, 1990). Glutamate receptors can be either ionotropic or metabotropic. The ionotropic glutamate receptors have three different subtypes named after their selective agonists: NMDA, (\pm)- α -amino-3-hydroxyl-5-methylisoxazole-4-propionic acid (AMPA), and kainate receptors. Physiological activation of these ionotropic receptors usually leads to transient calcium or sodium influx, or both, thus completing the neurotransmission. However, excessive synaptic glutamate levels are neurotoxic (termed "excitotoxicity"). Excitotoxicity has been implicated in various acute neurodegenerative disorders, such as epilepsy, cerebral ischemia, and traumatic head injury (Choi, 1988; Meldrum and Garthwaite, 1990; Dingledine *et al.*, 1990; Lipton and Rosenberg, 1994). Our current understanding of this phenomenon suggests the following sequence of events: (1) overactivation of ionotropic glutamate receptors in the postsynaptic membrane leading to sustained influx of Na^+ and Ca^{2+} , (2) Na^+ influx-induced membrane depolarization, which in turn activates voltage-gated neuronal Ca^{2+} channels, further increasing Ca^{2+} influx, (3) sustained elevation of neuronal Ca^{2+} to high nanomolar concentrations, (4) Ca^{2+} -dependent systems, including calmodulin, protein kinase C (PKC), phospholipase C and A_2 , calmodulin-dependent kinase II, nitric oxide synthase, and calpains, are overactivated, and (5) one or perhaps all of these processes produce irreversible damage to the neurons, which eventually leads to cell death (Wang and Yuen, 1994) (Fig. 3). Indeed, excitotoxicity was found to mediate neuronal injuries in cerebral ischemia (e.g., in stroke or cardiac arrest), epilepsy, and chronic neurodegenerative disorders (Meldrum and Garthwaite, 1990). Excessive presynaptic release and impaired reuptake of glutamate are the main causes of the synaptic buildup of glutamate during ischemic events (Meldrum and Garthwaite, 1990; Lipton and Rosenberg, 1994). It is now well established in both *in vitro* and *in vivo* models of cerebral ischemia that antagonists of either the NMDA receptor [e.g., dizocilpine (MK-801)] or the AMPA/kainate receptor [e.g., 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(f)quinoxaline (NBQX)] can provide significant neuroprotection (Meldrum and Garthwaite, 1990; Lipton and Rosenberg, 1994). However, NMDA antagonism can lead to undesirable psychotomimetic side effects (Olney *et al.*, 1991). An alternative approach is intervention at the level of intracellular Ca^{2+} -dependent mechanisms that account for neuronal death. In this section, we focus only on the role of calpain.

As an initial step to show that calpain is involved in excitotoxicity or ischemic events, one needs to demonstrate that calpain is activated in pathological situations. One universal marker for calpain activation is the breakdown of α -spectrin (280 kDa) to two characteristic fragments of 150 kDa and 145 kDa. In a neuronal culture model of excitotoxicity and hypoxia (Siman and Noszek, 1988; Hajimohammadreza *et al.*, 1995; Wang *et al.*, 1996), as well as both *in vitro* (hippocampal slices) and *in vivo* models

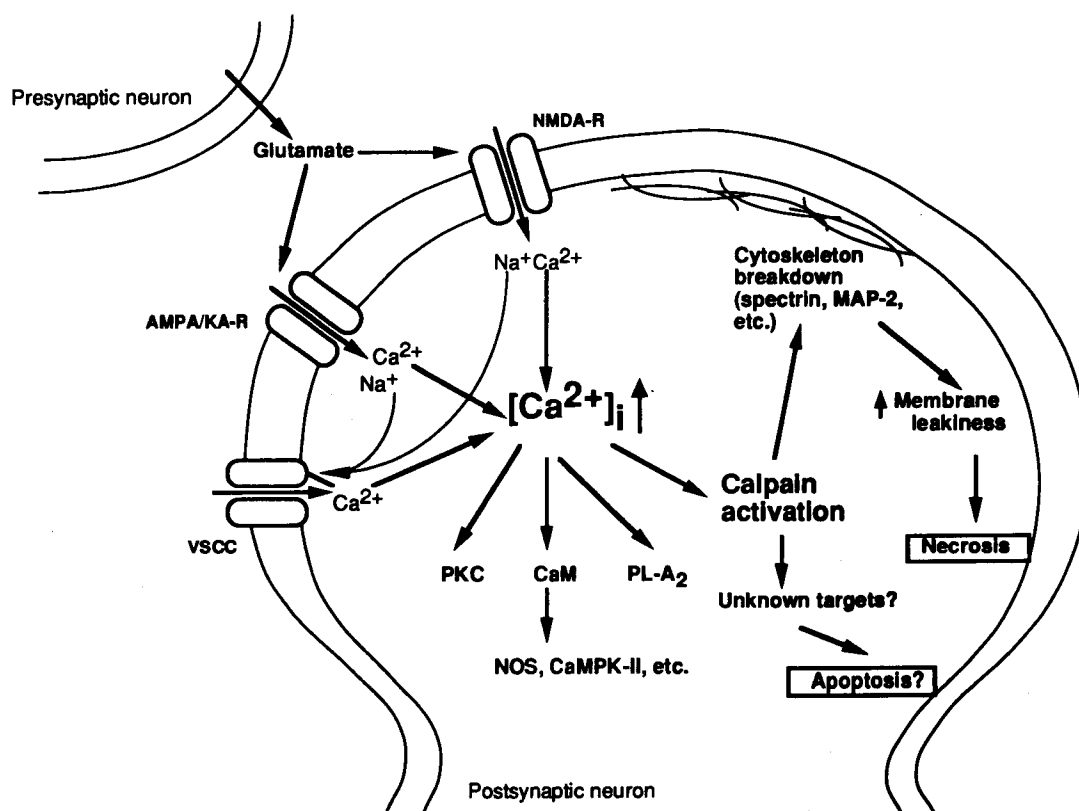


FIGURE 3 Calpain in excitotoxicity/cerebral ischemia. Excessive buildup of synaptic glutamate results in overactivation of both the NMDA receptor (NMDA-R) and AMPA/kainate receptor (AMPA/KA-R), which allow Ca^{2+} or sodium influx, or both. The elevated free intracellular sodium level could further trigger the activation of the voltage-sensitive Ca^{2+} channel (VSCC). The resultant intracellular Ca^{2+} elevation could activate a number of Ca^{2+} -dependent systems, including protein kinase C (PKC), calmodulin (CaM)-dependent nitric oxide synthase (NOS), and protein kinase II (CaMPK-II), phospholipase A₂ (PL-A₂), and calpain. The damaging effects of calpain overactivation are most obvious as the breakdown of cytoskeleton, which leads to compromised membrane integrity and necrotic death. It is possible that calpain activation could also trigger apoptosis, which contributes to tissue damage. Modified from Figure 3 in Wang and Yuen (1994).

of ischemia, spectrin breakdown products were readily observed (Arai *et al.*, 1991; Siman and Noszek, 1988; Hajimohammadreza *et al.*, 1995). A number of other neuronal proteins, including MAP-2 and neurofilament proteins, are susceptible to activated calpain (Johnson and Foley, 1993; Kamakura *et al.*, 1985; Schlaepfer *et al.*, 1985). Therefore, one can envision that calpain activation can lead to cytoskeleton/plasma membrane damage, followed by a general loss of cell function and the eventual death of neurons. Additional evidence supporting the contributory role of calpain in neuronal loss came from studies using various calpain inhibitors. These studies comprise three categories based on biological complexity: primary neuronal cultures, brain slices, and *in vivo* models.

1. Neuronal Cultures

Several primary CNS neuronal cultures are susceptible to either direct excitotoxic insults (e.g., glutamate, NMDA, AMPA) or hypoxia/hypoglycemia (Choi *et al.*, 1987; Goldberg and Choi, 1993). The neuronal cultures are usually harvested from the brains of fetal or neonatal animals. These cells include neocortical, hippocampal, cerebellar Purkinje, and cerebellar granule neurons (Choi *et al.*, 1987; Goldberg and Choi, 1993; Brorson *et al.*, 1994, 1995; Hajimohammadreza *et al.*, 1995; Di Stasi *et al.*, 1991).

Rami and Krieglstein (1993), using NaCN to induce hypoxia in chick embryonic neuronal cultures, found that the neurons are partially protected by leupeptin and calpain inhibitor I. E64, MDL28170 (10 μM each), and leupeptin (100 μM) also protected cerebellar Purkinje cells from AMPA toxicity (Brorson *et al.*, 1994). Brorson *et al.* (1995) have further shown that MDL28170 (10 μM) protected cerebellar Purkinje neurons against kainate toxicity and hippocampal neurons against NMDA toxicity even after delayed application. A similar protective effect was found with E64 (10 μM) in the cerebellar cultures. This delayed antagonism is consistent with calpain's being a downstream intracellular mediator of cell death. Wang *et al.* (1996) recently demonstrated that PD150606 protects fetal rat neocortical neurons from hypoxic/hypoglycemic damage and reduces spectrin-breakdown. Moreover, they also found a similar protective effect with 1–10 μM calpain inhibitor I. Calpain inhibitors (leupeptin or calpain inhibitor I) did not protect cultured cerebellar granule cells against glutamate or NMDA challenge (Manev *et al.*, 1991; Di Stasi *et al.*, 1991). This finding suggests that granule cells may have adopted a calpain-independent cell death pathway.

2. Brain Slices

Hippocampal slices are perhaps one of the most widely used *in vitro* model for excitotoxicity and ischemia (Siman *et al.*, 1990; Arai *et al.*, 1991). For instance, exposure of hippocampal slices to a brief period of hypoxia produces irreversible neuronal loss, especially in the CA1 and CA3 regions. The functional loss in the CA1 neurons can be monitored by measuring the evoked postsynaptic potential (EPSP). A decrease or loss of this response indicates neuronal damage in the CA1 region. Using this model, several groups have demonstrated that cell-permeable calpain inhibitor I and MDL28170 improved the functional recovery of the hippocampal slices from hypoxia (Lee *et al.*, 1991; Arai *et al.*, 1990; Arlinghaus *et al.*, 1991). As expected, spectrin breakdown can be observed in hypoxia-treated slices, and the spectrin breakdown can be attenuated by calpain inhibitor I (Arai *et al.*, 1991). Similarly, Hiramatsu *et al.* (1993) showed that synaptic transmission in gerbil neocortical slices was also improved by MDL28170 (50 μM). Another model involves a direct challenge of brain slices with

excitotoxin. For example, cerebellar slices can be challenged with AMPA, and Purkinje neurons will die within a few hours (Caner *et al.*, 1993). Both MDL28170 and PD150606 have been shown to be neuroprotective in this model (Caner *et al.*, 1993; Wang *et al.*, 1996).

3. In Vivo Cerebral Ischemia Models

In vivo models for cerebral ischemia can be categorized into two major types: irreversible focal ischemia (e.g., MCAO) and transient global ischemia [e.g., four-vessel occlusion (4-VO)]. The first model mimics the insult to the brain in stroke, and the latter simulates cardiac arrest. Glutamate receptor antagonists and N-type calcium channel blockers ω -conotoxin MVIIA (SNX111) are proven neuroprotective agents in these models (Meldrum and Garthwaite, 1990; Li and Buchan, 1993; Valentino *et al.*, 1993). Several investigators have characterized the cytoskeletal protein breakdown that occurs in these preclinical models. For example, spectrin breakdown was observed in the hippocampus in both the global (Seubert *et al.*, 1989; Lee *et al.*, 1991) and the focal ischemia (Hong *et al.*, 1994) models. Leupeptin, when administered in a prolonged intracerebroventricular infusion paradigm, enhanced hippocampal CA-1 neuron survival in gerbils subjected to transient ischemia (Lee *et al.*, 1991). These results suggest that in spite of poor cell permeability, neuroprotection can be achieved if sufficient compound is accumulated over time. A more recent report showed that administration of MDL28170 (30–60 mg/kg i.v.) significantly reduced infarct size in a rat focal ischemia model (Hong *et al.*, 1994). Similarly, calpain inhibitor I also reduced neuronal damage of the hippocampal CA1 subfield in global ischemia (Rami and Kriegstein, 1993). Another article reported that supracortical perfusion of AK275 also markedly reduced infarct size in a rat MCAO model (Bartus *et al.*, 1994a). A related but more water-soluble compound AK295 also reduced infarct size in a rat MCAO model when infused intraarterially (Bartus *et al.*, 1994b).

As noted previously, glutamate receptor antagonists are proven neuroprotectants in animal models of cerebral ischemia. However, these antagonists may also elicit adverse side effects, considering the critical neurotransmitter role glutamate plays in the CNS. Psychotomimetic effects have been demonstrated with NMDA receptors blockers (Olney *et al.*, 1991). In this regard, calpain inhibitors may be strategically superior, since most exist in a latent, nonactivated proenzyme form (80 kDa + 29 kDa). It is likely that only a very small fraction of calpains (e.g., subplasma membrane located) could be activated as a signal transduction pathway while the majority remains inactive. This is implied from the lack of spectrin breakdown or calpain autolysis in neurons stimulated with nontoxic doses of NMDA or AMPA. It appears, therefore, that only at the time of a pathological event would one expect pronounced activation of calpain. Thus, it is a reasonable

hypothesis that calpain inhibition would not lead to untoward CNS side effects.

B. Traumatic Brain Injury

Traumatic brain injury (TBI) refers to impact-induced acute neurodegenerations, such as those that occur after a fall, automobile accident, and so on. Excessive intracellular Ca^{2+} accumulation in TBI is well documented (Fineman *et al.*, 1993). Glutamate receptor overactivation has been identified as the cause of such Ca^{2+} influx (Hayes *et al.*, 1992). Animal models of TBI have been developed, such as the one that involves using a metal tip to produce a compression in the brain (Dixon *et al.*, 1991). Evidence of calpain translocation to the membrane fraction in a rabbit brain trauma model (Arrigoni and Cohadon, 1991), together with the breakdown of MAP-2 and neurofilaments H and L, has been reported in an animal model of TBI (Taft *et al.*, 1992; Posmantur *et al.*, 1994). Since the tissue damage in TBI is thought to be mediated through glutamate excitotoxicity (Hayes *et al.*, 1992), inhibition of calpain activation should conceivably reduce the amount of neuronal damage.

C. Subarachnoid Hemorrhage/Vasospasm

Subarachnoid hemorrhage (SAH) results from a rupture of a saccular intracranial aneurysm. SAH is also known to induce long-lasting cerebral vasospasm by restricting blood flow, thus triggering ischemic events similar to those in stroke (Kassell *et al.*, 1985). Endothelin was proposed to be released from endothelial cells in response to the invasion of oxyhemoglobin from the blood clots (Foley *et al.*, 1993). This leads to endothelin receptor activation in vascular smooth muscle cells, which produces a long-lasting spastic response. L-type calcium channel blockers (e.g., nimodipine) have been used successfully in SAH, suggesting the involvement of calcium influx into the vascular smooth muscle cells (Findlay *et al.*, 1991). In a canine SAH model, vasospasm is induced by successive intracisternal injection of autologous blood near the basilar artery. The resulting blood clot induces a spastic response on the blood vessel (Minami *et al.*, 1992). In this model, topical application of calpeptin ($1\ \mu\text{M}$) dilated the exposed spastic basilar artery (Minami *et al.*, 1992). Although the mechanism is unclear, possible involvement of proteolytic activation of PKC by calpain was suggested because the PKC inhibitor 1-(5-isoquinolinyisulfonyl)-2-methylpiperazine (or H7) also had the same vasodilating effect. The calpain hypothesis was further supported by the observed increase of spectrin breakdown in the smooth muscle cells of spastic basilar artery in a rabbit model of SAH (Foley *et al.*, 1993). Thus, calpain inhibitors can be potentially useful agents in SAH,

especially when the use of L-type calcium channel blocker is contraindicated (Findlay *et al.*, 1991).

D. Other Neurological Disorders

1. Chronic Neurodegeneration

Several chronic neurodegenerative disorders, such as Huntington's disease, Parkinson's disease, and amyotrophic lateral sclerosis (ALS), have been linked in part to excitotoxicity (Meldrum and Garthwaite, 1990; Lipton and Rosenberg, 1994). Consequently, calpain inhibitors may have the potential to alleviate the accompanying neurological deficits.

2. Alzheimer's Disease

In Alzheimer's disease (AD), abnormal processing of the amyloid precursor protein (APP) produces a 4 kDa β -amyloid peptide (β -A4), which is suggested to be a critical event in the pathogenesis of AD (Ashall and Goate, 1994). The β -amyloid peptide apparently self-aggregates and, after decades of deposition, forms the major component of senile plaques. Calpain, among other proteases, is found to cleave APP (Siman *et al.*, 1990). Calpain cleaves APP at three different sites, all located extracellularly. Therefore, if calpain were to attack APP, it is likely that internalized APP would be cleaved. The most C-terminal cleavage could produce a fragment that contains the entire β -amyloid peptide (amyloidogenic), which can be further processed by another protease. Incidentally, calpain immunoreactivity was also found in senile plaques (Iwamoto *et al.*, 1991). There is also evidence of calpain activation in the brains of Alzheimer's patients, as judged by the increased ratio of the activated 76 kDa form to the inactive 80 kDa form of μ -calpain (Saito *et al.*, 1993). Besides the potential processing of β -amyloid peptide, calpain has been proposed to be a calcium effector that is altered in AD. Ironically, abnormal homeostasis may be produced by an imbalanced ratio of β -A4/APP (Mattson *et al.*, 1993). The impact of activated calpain on cytoskeleton stability and protein kinase activities could translate into abnormal membrane protein trafficking, altered processing of APP and secretion, as well as synaptic dysfunction (Nixon *et al.*, 1995).

3. Demyelination Disorders and Peripheral Nerve Injury

a. Demyelination Disorders Calpain is capable of degrading myelin sheath proteins (such as myelin basic proteins) and axonal proteins (e.g., neurofilament proteins) (Banik *et al.*, 1984, 1985) and has been found to be colocalized with fragmented myelin in myelinated nerve fibers in rabbits (Persson and Karlsson, 1991). It has thus been suggested that calpain may mediate myelin degradation, as seen in demyelination disorders, such as wallerian degeneration, multiple sclerosis, and peripheral neuropathies. In wallerian degeneration, influx of calcium into the axon distal to the site of injury has

been reported (Schlaepfer and Micko, 1979; Zimmerman and Schlaepfer, 1984). Since m-calpain is localized in axons (Mata *et al.*, 1991) and degrades axonal proteins, such as neurofilaments H, M, and L *in vitro* (Schlaepfer and Micko, 1979; Banik *et al.*, 1985; Kamakura *et al.*, 1985), it can potentially participate in the axonal degeneration process.

b. Spinal Cord Injury Although the pathology of spinal cord injury is still not well established, it appears to involve excitotoxins, much like TBI (Meldrum and Garthwaite, 1990). In experimental spinal cord injury, Ca^{2+} accumulates within the injured axons (Waxman, 1993). Calpain is thought to be activated and hence to contribute to the degeneration of axons and myelins (Banik *et al.*, 1984, 1986). In support of this observation, leupeptin reduces axonal damage in experimental spinal cord injury (Iizuka *et al.*, 1986).

c. Peripheral Nerve Injury Peripheral nerve injury may also involve calpain activation, since administration of leupeptin improved functional recovery after peripheral nerve repair in a monkey model (Badalamente *et al.*, 1989, 1992). Interestingly, the authors noted that intramuscular administration of leupeptin for months did not appear to produce any adverse effects.

E. Cardiac Ischemia

In myocardial infarction, the injury sustained by cardiomyocytes is likely similar to that of neurons in cerebral ischemia. However, cardiomyocytes appear to suffer from a secondary form of oxidative injury during coronary reperfusion. It has been well documented that myofibrillar proteins, including myosin heavy chain, troponin T and I, tropomyosin, and α -actinin, are susceptible to calpain degradation *in vitro* (Ishiura *et al.*, 1980). In isolated neonatal rat myocytes, Iizuka *et al.* (1991, 1993) demonstrated that calpain activity was elevated during hypoxia, as suggested by the presence of spectrin breakdown products. They further showed that the ischemia-induced spectrin breakdown was blocked by 10 μM of calpain inhibitor I or E64. Also, although hypoxia alone caused about 60% cell death in 6 h, the presence of either inhibitor reduced cell death to slightly above the normoxic level. In a recent study, Atsma *et al.* (1995) found that both intracellular calcium levels and calpain activities were elevated during metabolic inhibition (with NaCN and 2-deoxyglucose) in neonatal rat cardiomyocytes. They demonstrated that calpain inhibitor I and leupeptin could inhibit intracellular calpain activity but did not attenuate cell death induced by metabolic inhibition. It is possible that metabolic inhibition does not completely mimic ischemia. Isolated hearts have been used to model cardiac ischemia (Matsumura *et al.*, 1993). For example, such hearts can be subjected to a temporary global ischemia (e.g., 15 min), followed by reperfusion. Generally, this

reversible insult leads to compromised myocardial functions. Thus, the model is generally referred to as myocardial stunning. Matsumura *et al.* (1993) showed that leupeptin significantly enhanced functional recovery following stunning. In a similar model, Yoshida *et al.* (1995) found increased μ -calpain and m-calpain activity after ischemia/reperfusion. They also observed spectrin breakdown and found that E64d partially reduced creatine kinase release during reperfusion. Finally E64c was used as a potential protective agent in a dog model where acute myocardial infarction was induced by occlusion of the left anterior descending artery (Toda *et al.*, 1989). The combination of E64c and reperfusion significantly reduced infarct size. However, E64c alone did not show significant reduction of infarct size. It appears that calpain is activated during myocardial ischemia, and several cysteine protease inhibitors that inhibit calpains showed beneficial effects. Obviously, this area is still in its infancy and deserves additional studies.

F. Muscular Dystrophy

The absence of dystrophin in Duchenne muscular dystrophy (DMD) patients as well as mdx mice (due to genetic mutations) has been identified as the key component for these forms of hereditary muscular dystrophy. Dystrophin is a protein in the sarcolemma that regulates the stretch-sensitive Ca^{2+} leak channels and thus may be important in regulating intracellular calcium levels (Franco and Lansman, 1990). Myofibrillar protein loss and Z-disc disorganization and plasma membrane defects are some of the features of mdx and DMD. In mdx mice, intracellular Ca^{2+} levels in skeletal muscle fibers were found to be significantly raised, with a concomitant increase in the rate of protein degradation (Turner *et al.*, 1988). The rate of protein degradation could be reduced to normal levels by leupeptin (Turner *et al.*, 1993). Myofibrillar protein turnover, both normal and abnormal, is likely mediated by calpain (van der Westhuyzen *et al.*, 1981; Kumamoto *et al.*, 1995). It was also shown that m-calpain level apparently was higher in dystrophic hamster skeletal muscles (Johnson and Hammer, 1988). A recent study further examined the roles of calpain in mdx mice (Spencer *et al.*, 1995). The authors noted that although the mRNA levels for μ -calpain and m-calpain do not differ between mdx mice and controls, immunoblots indicated μ -calpain autolytic activation (presence of 76 kDa fragment). Interestingly, culturing of mdx mouse myotubes in the presence of leupeptin (50 μM) was found to prevent the elevation of the resting Ca^{2+} level normally seen in mdx muscles (Turner *et al.*, 1993). It is postulated that calpain degrades a sarcolemmal protein that leads to further increased opening of the Ca^{2+} leak channels. Thus, these results suggest at least two active roles of calpain in promoting muscular dystrophy: myofibrillar protein degradation and altered Ca^{2+} homeostasis. Another intriguing twist in the area of

muscular dystrophy is the recent finding that the inherited disorder limb-girdle muscular dystrophy is linked to mutations in the gene for the skeletal muscle-specific form of calpain (p94, gene product of CANP-3) (Richard *et al.*, 1995). Interestingly, the authors reported that many of those mutations should produce dysfunctional p94 protein, suggesting that the expression of p94 is important for normal muscle maintenance/turnover.

G. Cataracts

The mammalian lens consists of densely packed fiber cells that are terminally differentiated. The fiber cells contain a very high concentration of crystallin proteins (α , β_H , β_L , β_s , and γ), which are organized to allow light transmission. Over its lifetime, the lens receives many forms of insults such as UV radiation, hyperglycemia (diabetes), and exposure to environmental toxins, free radicals, and drugs (corticosteroids). The cumulative effect of these insults is oxidative damage to membrane proteins and lipids (Shearer, 1987). This leads to compromised membrane integrity and increased membrane permeability to ions, such as calcium. Elevated free Ca^{2+} concentrations (as high as $600 \mu\text{M}$) have been demonstrated in chemically induced experimental cataract (Azuma *et al.*, 1991). Lens m-calpain is thought to be activated and, hence, to degrade α -crystallin and β -crystallin proteins. This process leads to crystallin precipitation and, ultimately, lens opacity (cataract) (Yoshida *et al.*, 1984; David and Shearer, 1993) (Fig. 4).

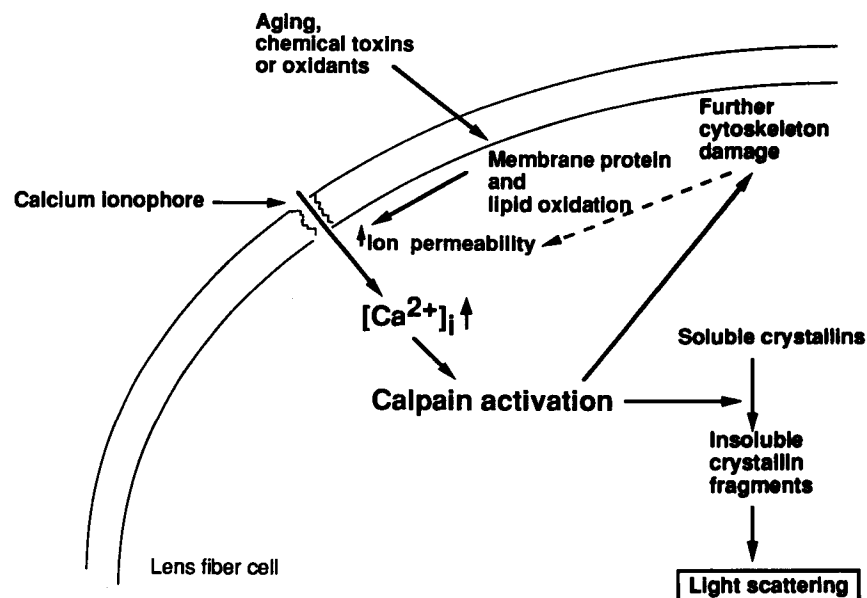


FIGURE 4 Calpain in cataract formation. In the lens fiber cell, aging, chemical toxins, and other environmental insults induce oxidation of plasma membrane proteins and lipids, resulting in increased ion (e.g., Ca^{2+}) permeability. Ca^{2+} ionophore also mimics this process. Such calcium concentration elevations activate calpain, which degrades crystallin proteins into insoluble products. The result is light scattering and lens opacity. Calpain could also attack the cytoskeleton, further increasing membrane permeability.

Additional evidence for calpain activation in cataractic lens is the presence of spectrin breakdown products (Marcantonio and Duncan, 1991). As mentioned previously experimental cataract can be chemically induced both in cultured lens *in vitro* and in several animal models.

1. Cultured Lens

The thiooxidative agents sodium selenite and diamide, as well as calcium ionophore A23187, are all effective agents in inducing lens opacity (Shearer *et al.*, 1987). Addition of xylose in culture also mimics *in vivo* sugar cataract due to the production of polyols by aldose reductase (Van Heyningen, 1959). m-Calpain can hydrolize β -crystallins *in vitro*, causing their eventual insolubilization (David *et al.*, 1993). Shearer's laboratory has examined the effect of several calpain inhibitory agents on cataract formation (Shearer *et al.*, 1991; Lampi *et al.*, 1992; Azuma *et al.*, 1992). E64 (5 μ M–1 mM) and MDL28170 (500 μ M) reduced opacity prevented the insolubilization of β -crystallins. High concentrations of these compounds were necessary to allow sufficient penetration into the nuclear region of the lens. Subsequently, the authors found that the membrane-permeable E64d was more potent (as little as 5–20 μ M) than E64 in reducing opacity in A23187-treated lens (Azuma *et al.*, 1992). Calpain inhibitor I and calpain inhibitor II were not protective, most likely due to their cytotoxicity (Lampi *et al.*, 1992).

2. In Vivo Cataract Models

Similar to the cultured lens model, a single i.v. dose of selenite induced cataract in rats (Shearer *et al.*, 1987). Sugar cataract can be induced by high dietary consumption of sugars, such as galactose (Azuma *et al.*, 1995). Alternatively, a hereditary cataract mouse model is available (Nakano mouse). In selenite-induced cataract in rats, α -crystallin and β -crystallin were found to be extensively proteolyzed. Also, N-terminal sequencing revealed that the *in vitro* calpain cleavage sites of β -crystallins matched those found *in vivo* (David and Shearer, 1993; David *et al.*, 1993). In the Nakano mouse, calcium levels were elevated before the onset of nuclear cataract, and evidence of calpain autolysis was also noted (Yoshida *et al.*, 1985b). Most recently, in galactose-induced sugar cataract in rats, the lenses were found to have marked calcium accumulation, decreased calpain immunoreactivity a reflection of sustained calpain activation and autolysis, and α -crystallin and β -crystallin degradation (Azuma *et al.*, 1995).

In parallel with the cultured lens studies, the effects of E64 on selective cataract formation have been investigated *in vivo*. Rats were given i.p. injection of E64 2 h before selenite was given. A daily dose of E64 was continued for 5 days. In this model, E64 reduced the frequency of the most severe stage of cataract, nuclear cataract (Azuma *et al.*, 1991). However, the authors noted that even in the E64-treated group, β -crystallin breakdown

continued to occur at a slower rate. It was concluded that the amount of E64 was still not sufficient to neutralize all calpain activities.

In brief, calpain appears to play an important role in many forms of experimental cataracts. Besides the effect of crystallins, calpain may also be actively destroying cytoskeleton structure and thus further compromising membrane permeability of lens cells (Fig. 4). To date, no therapeutic agents can significantly prevent, impede, or block cataract formation. The only medical intervention is lens replacement surgery. If the calpain hypothesis is validated in human cataract, topical applications of calpain inhibitors as anticataractogenic agents could be an economical alternative to lens replacement surgery.

H. Thrombotic Platelet Aggregation

μ -Calpain and m-calpain are found in platelets (Sakon *et al.*, 1981). Platelet activation is one of the few physiological situations where calpain activation and translocation to plasma membrane were clearly demonstrated (Ariyoshi *et al.*, 1992; Kuboki *et al.*, 1992; Saido *et al.*, 1993). Many platelet proteins including several calmodulin-binding proteins (talin, spectrin filamin, caldesmon, calcineurin), were found to be degraded by calpain during platelet activation (O'Halloran *et al.*, 1985; Onji, *et al.*, 1987; Tallant *et al.*, 1988). Several studies also further linked calpain to platelet aggregation, an event that leads to formation of blood clots (thrombosis) (Brass and Shattil, 1988; Ishii *et al.*, 1990; Takano *et al.*, 1991; Ariyoshi *et al.*, 1991; Puri *et al.*, 1992, 1993a,b). The key event that causes platelet aggregation is the binding of fibrinogen to fibrinogen receptors on the platelet surface. This interaction occurs only when platelets are first activated by stimuli, such as ADP, thrombin, or plasmin. The current understanding is that membrane-bound ADP receptor (aggregin) is responsible for the latency of the fibrinogen receptor. When platelets are stimulated with thrombin, intracellular free Ca^{2+} can rise to 1–5 μM , which triggers calpain activation. Calpain was found to degrade aggregin, resulting in exposure of the fibrinogen receptors to the platelet surface through an unknown mechanism (Baldassare *et al.*, 1985; Puri *et al.*, 1989), which facilitates fibrinogen binding to platelets and promotes aggregation. Consistent with this hypothesis, calpain has been found to be activated during platelet aggregation, as evidenced by the proteolysis of talin and filamin (Fox *et al.*, 1983). Calpain inhibitors leupeptin, EST (E64c), antipain, and Phe-Gln-Val-Val-Cys-(3-nitro-2-thiopyridine)-Gly-NH₂ were found to block aggregin breakdown and platelet aggregation (Brass and Shattil, 1988; Ishii *et al.*, 1990; Takano *et al.*, 1991; Ariyoshi *et al.*, 1991; Puri *et al.*, 1992, 1993a,b).

I. Restenosis

Percutaneous transluminal coronary angioplasty is a widely used medical procedure to expand the inner diameter of clotted arteries to improve

blood flow in patients who suffer from atherosclerotic coronary artery disease. Unfortunately, the success of this procedure is limited by the spontaneous and gradual renarrowing of the arteries (termed "restenosis") (McBride *et al.*, 1988). It was shown that calpeptin (100 μM) and calpain inhibitor I (50 μM) inhibited angioplastic restenosis possibly by controlling proliferation and migration of smooth muscle cells to the neointimal layer of the blood vessels (Wilensky *et al.*, 1991; March *et al.*, 1993).

J. Joint Inflammation/Arthritis

Both μ -calpain and m-calpain have been found in the synovial fluid of the knee joint, presumably through externalization, especially in patients with rheumatoid arthritis (Suzuki *et al.*, 1990; Fukui *et al.*, 1989; Yamamoto *et al.*, 1992). The major cartilage matrix component proteoglycan was also reported to be susceptible to calpain (Suzuki *et al.*, 1992). It is hypothesized that externalized calpain damages the extracellular matrix and contributes to the inflammation process, in addition to other matrix proteases (cysteine and metalloproteases). Recently, using collagen-induced arthritis in mice, Szomor *et al.* (1995) described a correlation of the histological grade of arthritis and the immunohistochemical staining of m-calpain in the knee joint. Interestingly, in the arthritic areas, they identified increased m-calpain

TABLE II Therapeutic Areas in Which Calpain Overactivation Is Implicated

<i>Disorders</i>	<i>Proposed mechanism</i>
Cerebral ischemia/ excitotoxicity	Breakdown of cytoskeletal proteins and other cellular proteins, leading to neuronal death and loss of neuronal functions
Brain trauma	Similar mechanism to excitotoxicity
Spinal cord injury/ demyelination	Degradation of neurofilament proteins and myelin proteins
Subarachnoid hemorrhage	Proteolytic activation of protein kinase C, leading to sustained cerebral vasospasm
Alzheimer's disease	Amyloidogenic fragments of APP produced calpain
Cardiac ischemia	Breakdown of myofibril proteins causing cardiac myocyte death
Muscular dystrophy	Imbalanced Ca^{2+} homeostasis, triggering proteolysis of myofibril proteins by calpain
Cataract	Crystallin fragmentation by calpain in aging lens, leading to precipitation and lens opacity
Thrombotic platelet aggregation	Calpain-mediated proteolysis of aggregin that promotes platelet aggregation
Restenosis	Calpain-mediated proliferation and migration of smooth muscle cells, leading to renarrowing of blood vessel after angioplasty
Arthritis	Breakdown of cartilage/extracellular matrix component proteoglycan

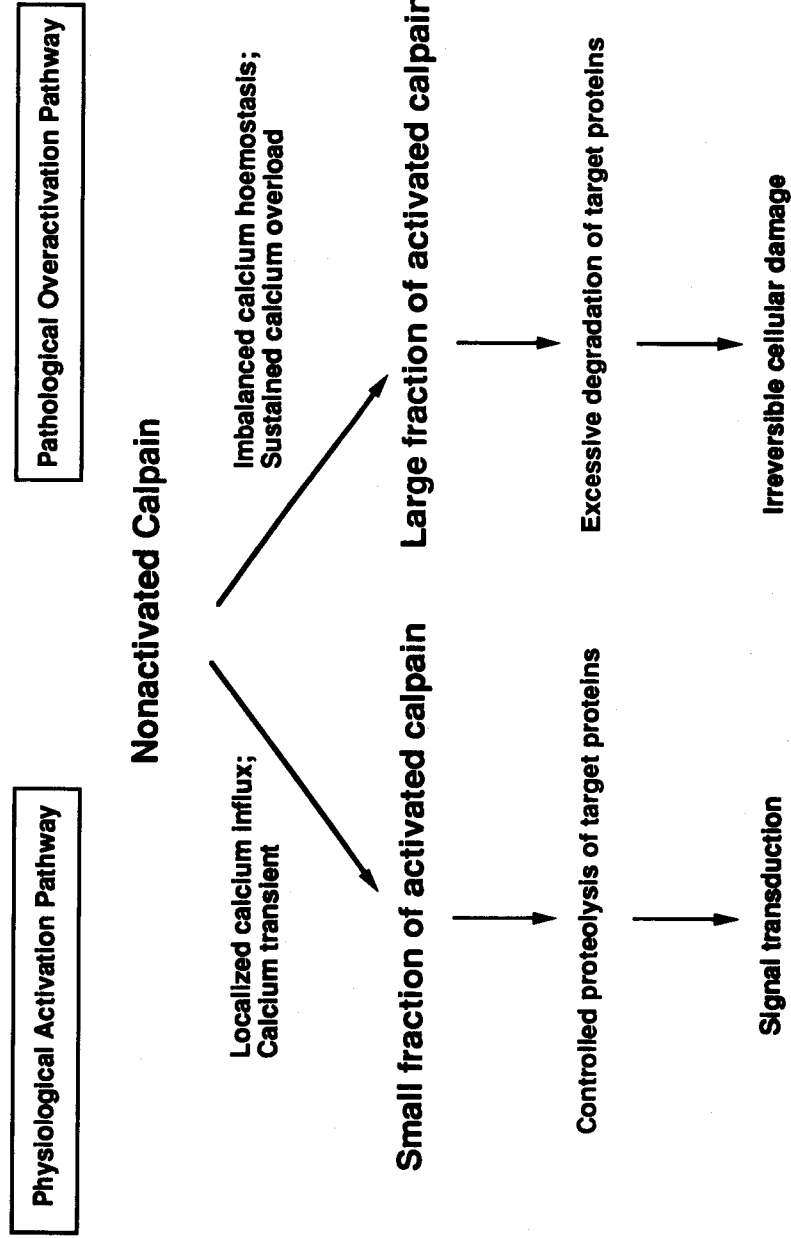


FIGURE 5 Physiological activation and pathological overactivation of calpain. Calpain in resting cells is generally nonactivated. A physiological stimulus could trigger calcium transients or localized calcium influx. A small fraction of calpain molecules could be activated in a controlled manner. This may lead to limited proteolysis of certain target proteins for calpain, thus completing the signal transduction mechanism. However, under pathological conditions where calcium homeostasis is perturbed or a sustained intracellular calcium elevation is experienced, a large fraction of calpain molecules may be activated, which may lead to excessive proteolysis of target proteins and significant structural and functional damage to the cell.

levels both intracellularly in inflammatory cells and extracellularly on the surface of cartilage and synovium. This indicates that calpain could potentially participate in two ways: (1) involvement in a proinflammatory reaction intracellularly and (2) degradation of matrix proteins directly. To date, it remains to be determined if calpain inhibitors have beneficial effects when applied directly to inflamed joints.

K. Other Diseases

Calpain has also been found to play a contributory role in apoptosis in thymocytes and T cells (Sarin *et al.*, 1993, 1994; Squier *et al.*, 1994). In these studies, agents such as calpain inhibitor I are antiapoptotic. Apoptosis has been implicated as a mechanism by which CD4⁺ T cells are killed during HIV infection. Therefore, calpain inhibition could potentially be beneficial (Sarin *et al.*, 1994). Calpain has been suggested as a mediator of hypoxia-induced injury in rat renal proximal tubules (Edelstein *et al.*, 1995a,b).

IV. Perspectives

In this review, we have presented evidence that calpain overactivation may be a key component in a number of disorders (Table II). The common theme for most of these disorders is cellular Ca²⁺ overload. Physiologically, in a resting cell, the major fraction of calpain is not activated. As a result of either a transient or localized accumulation of calcium (e.g., next to the cytosolic side of a calcium channel), a small fraction of the calpain is activated to produce limited proteolysis of one or more of its target substrates, fulfilling its role in signal transduction (Fig. 5). Under pathological conditions, sustained calcium overload would activate a large fraction of calpain, resulting in uncontrolled and extensive degradation of cellular proteins. Compromised cell structure and functions would ultimately lead to cell death (Fig. 5). Calpain also appears to be an ideal pharmaceutical target, since this protease is most active during pathological events. The current challenge is to identify cell-permeable and selective calpain inhibitors for evaluation in various *in vivo* disease models.

References

- Alvarez, M. E., Houck, D. R., White, C. B., Brownell, J. E., Bobko, M. A., Rodger, C. A., Stawicki, M. B., Sun, H. H., Gillum, A. M., and Cooper, R. (1994). Isolation and structure elucidation of two new calpain inhibitors from *Streptomyces griseus*. *J. Antibiot.* 47, 1195-1201.

- Anagli, J., Hagmann, J., and Shaw, E. (1991). Investigation of the role of calpain as a stimulus-response mediator in human platelets using new synthetic inhibitors. *Biochem. J.* 274, 497–502.
- Angliker, H., Wikström, P., Rauber, P., and Shaw, E. (1987). The synthesis of lysylfluoromethanes and their properties as inhibitors of trypsin, plasmin and cathepsin B. *Biochem. J.* 241, 871–875.
- Aoki, K., Imajoh, S., Ohno, S., Emori, Y., Koike, M., Kosaki, G., and Suzuki, K. (1986). Complete amino acid sequence of the large subunit of the low Ca^{2+} -requiring form of human Ca^{2+} -activated neutral protease (μCANP) deduced from its cDNA sequence. *FEBS Lett.* 205, 313–317.
- Aoyagi, T., and Umezawa, H. (1975). In "Proteases and Biological Control" (E. Reich, D. B. Rifkin, and E. Shaw, eds.), pp. 429–454. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Arai, A., Kessler, M., Lee, K., and Lynch, G. (1990). Calpain inhibitors improve the recovery of synaptic transmission from hypoxia in hippocampal slices. *Brain Res.* 532, 63–68.
- Arai, A., Vanderklish, P., Kseeler, M., Lee, K., and Lynch, G. (1991). A brief period of hypoxia causes proteolysis of cytoskeletal proteins in hippocampal slices. *Brain Res.* 555, 276–280.
- Ariyoshi, H., Shiba, E., Kambayashi, J., Sakon, M., Tsujinaka, T., Uemura, Y., and Mori, T. (1991). Characteristics of various synthetic peptide calpain inhibitors and their application for the analysis of platelet reaction. *Biochem Int.* 23, 1019–1033.
- Ariyoshi, H., Shiba, E., Sakon, M., Kambayashi, J., Kawasaki, T., Kang, J., Kawashima, S., and Mori, T. (1992). Membrane binding and autolytic activation of calpain-I in human platelets. *Biochem. Int.* 27, 335–341.
- Arlinghaus, L., Mehdi, S., and Lee, K. S. (1991). Improved posthypoxic recovery with a membrane-permeable calpain inhibitor. *Eur. J. Pharmacol.* 209, 123–125.
- Arrigoni, E., and Cohadon, F. (1991). Calcium-activated neutral protease activities in brain trauma. *Neurochem. Res.* 16, 483–487.
- Ashall, F., and Goate, A. M. (1994). Role of the β -amyloid precursor protein in Alzheimer's disease. *Trends Biochem. Sci.* 19, 42–46.
- Atsma, D. E., Bastiaanse, E. M. L., Jerzewski, A., Van der Valk, L. J. M., and Van der Laarse, A. (1995). Role of calcium-activated neutral protease (calpain) in cell death in cultured neonatal rat cardiomyocytes during metabolic inhibition. *Circ. Res.* 76, 1071–1078.
- Azuma, M., David, L. L., and Shearer, T. R. (1991). Cysteine protease inhibitor E64 reduces the rate of formation of selenite cataract in the whole animal. *Curr. Eye Res.* 10, 657–666.
- Azuma, M., David, L. L., and Shearer, T. R. (1992). Superior prevention of calcium ionophore cataract by E64d. *Biochim. Biophys. Acta* 1180, 215–220.
- Azuma, M., Inoue, E., Oka, T., Shearer, T. R. (1995). Proteolysis by calpain is an underlying mechanism for formation of sugar cataract in rat lens. *Curr. Eye Res.* 14, 27–34.
- Badalamentle, M. A., Hurst, L. C., and Stracher, A. (1989). Neuromuscular recovery using calcium protease inhibition after median nerve repair in primates. *Proc. Natl. Acad. Sci. USA* 86, 5983–5987.
- Badalamentle, M. A., Hurst, L. C., and Stracher, A. (1992). Recovery after delayed nerve repair: Influence of a pharmacologic adjunct in a primate model. *J. Reconstr. Microsurg.* 8, 319–397.
- Baldassare, J. J., Bakshian, S., Knipp, M. A., and Fisher, G. J. (1985). Inhibition of fibrinogen receptor expression and serotonin release by leupeptin and antipain. *J. Biol. Chem.* 260, 10531–10535.
- Banik, N. L., Hogan, E. L., Whetstine, L. J., and Balentine, J. D. (1984). Changes in myelin and axonal proteins in CaCl_2 -induced myelopathy in rat spinal cord. *CNS Trauma* 1, 131–137.
- Banik, N. L., McAlhaney, W. W., and Hogan, E. L. (1985). Calcium stimulated proteolysis in myelin: Evidence for a Ca^{2+} -activated neutral proteinase associated with purified myelin of rat CNS. *J. Neurochem.* 45, 581–588.

- Banik, N. L., Hogan, E. L., Powers, J. M., and Smith, K. P. (1986). Proteolytic enzymes in experimental spinal cord injury. *J. Neurol. Sci.* 73, 245-256.
- Banik, N. L., Chakrabarti, A. K., Konat, G. W., Gantt, W. G., and Hogan, E. L. (1992). Calcium-activated neutral proteinase (calpain) activity in C6 cell line: Compartmentation of mu and m calpain. *J. Neurosci. Res.* 31, 708-714.
- Barrett, A. J., Kembhawi, A. A., Brown, M. A., Kirschke, H., Knight, C. G., Tamai, M., and Hananda, K. (1982). L-trans-Epoxy succinyl-leucylamido(4-guanidino)butane (E-64) and its analogues as inhibitors of cysteine proteinases including cathepsins B, H and L. *Biochem. J.* 201, 189-198.
- Bartus, R. T., Eveleth, D. D., Jr., Lynch, G. S., and Powers, J. C. (1992). Use of calpain inhibitors in the inhibition and treatment of neurodegeneration. PCT Int. Appl., WO 9211850 A1.
- Bartus, R. T., Baker, K. L., Heiser, A. D., Sawyer, S. D., Dean, R. L., Elliott, P. J., and Straub, J. A. (1994a). Postischemic Administration of AK275, a calpain inhibitor, provides substantial protection against focal ischemic brain damage. *J. Cereb. Blood Flow Metab.* 14, 537-544.
- Bartus, R. T., Hayward, N. J., Elliott, P. J., Sawyer, S. D., Baker, K. L., Dean, R. L., Akiyama, A., Straub, J. A., Harbeson, S. L., Li, Z., and Powers, J. (1994b). Calpain inhibitor AK295 protects neurons from focal brain ischemia: Effects of postocclusion intra-arterial administration. *Stroke* 25, 2265-2270.
- Bi, X., Tocco, G., and Baudry, M. (1994). Calpain-mediated regulation of AMPA receptors in adult rat brain. *Neuroreport* 6, 61-64.
- Brass, L. F., and Shattil, S. J. (1988). Inhibition of thrombin-induced platelet activation by leupeptin. Implications for the participation of calpain in the initiation of platelet activation. *J. Biol. Chem.* 263, 5210-5216.
- Brorson, J. R., Manziolillo, P. A., and Miller, R. L. (1994). Ca^{2+} entry via AMPA/KA receptors and excitotoxicity in cultured cerebellar Purkinje cells. *J. Neurosci.* 14, 187-197.
- Brorson, J. R., Marceccilli, C. L., and Miller, R. J. (1995). Delayed antagonism of calpain reduces excitotoxicity in cultured neurons. *Stroke* 26, 1259-1267.
- Caner, H., Collins, J. L., Harris, S. M., Kassell, N. F., and Lee, K. S. (1993). Attenuation of AMPA-induced neurotoxicity by a calpain inhibitor. *Brain Res.* 607, 354-356.
- Choi, D. W. (1988). Glutamate neurotoxicity and diseases of the nervous system. *Neuron* 1, 623-634.
- Choi, D. W., Maulucci-Gedde, M., and Kriegstein, A. R. (1987). Glutamate neurotoxicity in cortical culture. *J. Neurosci.* 7, 357-368.
- Crawford, C. (1987). Inhibition of chicken calpain II by proteins of the cystatin superfamily and α_2 -macroglobulin. *Biochem. J.* 248, 589-594.
- Crawford, C., Mason, R. W., Wikstrom, P., and Shaw, E. (1988). The design of peptidyl diazomethane inhibitors to distinguish between the cysteine proteinases calpain II, cathepsin L and cathepsin B. *Biochem. J.* 253, 751-758.
- Croall, D. E., Morrow, J. S., and DeMartino, G. N. (1986). Limited proteolysis of the erythrocyte membrane skeleton by calcium-dependent proteinases. *Biochim. Biophys. Acta* 882, 287-296.
- Croall, D. E., and Demartino, G. N. (1991). Calcium-activated neutral protease (calpain) system: Structure, function, and regulation. *Physiol. Rev.* 71, 813-847.
- David, L. L., and Shearer, T. R. (1993). Beta-crystallins insolubilized by calpain II *in vitro* contain cleavage sites similar to beta-crystallins insolubilized during cataract. *FEBS Lett.* 324, 265-270.
- David, L. L., Shearer, T. R., and Sahih, M. (1993). Sequence analysis of lens beta crystallins suggests involvement of calpain in cataract formation. *J. Biol. Chem.* 268, 1937-1940.
- Di Stasi, A. M., Gallo, V., Ceccarini, M., and Petrucci, T. C. (1991). Neuronal fodrin proteolysis occurs independently of excitatory amino acid-induced neurotoxicity. *Neuron* 6, 445-454.

- Dingledine, R., McBain, C. J., and McNamara, J. O. (1990). Excitatory amino acid receptors in epilepsy. *Trends Pharmacol. Sci.* 11, 334-338.
- Dixon, C. E., Clifton, G. L., Lighthall, J. W., Yaghmai, A., and Hayes, R. L. (1991). A controlled cortical impact model of traumatic brain injury in the rat. *J. Neurosci. Methods* 39, 253-262.
- Dolle, R. E., Singh, J., Whipple, D., Osifo, I. K., Speier, G., Graybill, T. L., Gregory, J. S., Harris, A. L., Helaszek, C. T., Miller, R. E., and Ator, M. A. (1995). Aspartyl α -((diphenylphosphinyl)oxy)methyl ketones as novel inhibitors of interleukin-1 β converting enzyme. Utility of the diphenylphosphinic acid leaving group for the inhibition of cysteine proteases. *J. Med. Chem.* 38, 220-222.
- Edelstein, C. L., Wieder, E. D., Yaqoob, M. M., Gengaro, P. E., Burke, T. J., Nemenoff, R., and Schrier, R. W. (1995a). Calpain is a mediator of hypoxia-induced rat renal proximal tubular injury. *J. Invest. Med.* 43, 351A.
- Edelstein, C. L., Wieder, E. D., Yaqoob, M. M., Gengaro, P. E., Burke, T. J., Nemenoff, R. A., and Schrier, R. W. (1995b). The role of cysteine proteases in hypoxia-induced rat renal proximal tubular injury. *Proc. Natl. Acad. Sci. USA* 92, 7662-7666.
- Findlay, J. M., Macdonald, R. L., and Weir, B. K. (1991). Current concepts of pathophysiology and management of cerebral vasospasm following aneurysmal subarachnoid hemorrhage. *Cerebrovasc. Brain Metab. Rev.* 3, 336-361.
- Fineman, I., Hovda, D., Smith, M., Yoshino, A., and Becker, D. (1993). Concussive brain injury is associated with a prolonged accumulation of calcium: A ^{45}Ca autoradiographic study. *Brain Res.* 624, 94-102.
- Foley, P. L., Kassell, N. F., Hudson, S. B., and Lee, K. S. (1993). Hemoglobin penetration in the wall of the rabbit basilar artery after subarachnoid hemorrhage and intracisternal hemoglobin injection. *Acta Neurochir. Wien* 123, 82-86.
- Fox, J. E., Reynolds, C. C., and Phillips, D. R. (1983). Calcium-dependent proteolysis occurs during platelet aggregation. *J. Biol. Chem.* 258, 9973-9981.
- Franco, A., Jr., and Lansman, J. B. (1990). Calcium entry through stretch inactivated ion channels in mdx myotubes. *Nature* 344, 670-673.
- Fukui, I., Tanaka, K., and Murachi, T. (1989). Extracellular appearance of calpain and calpastatin in the synovial fluid of the knee joint. *Biochem. Biophys. Res. Commun.* 162, 559-566.
- Giordano, C., Calabretta, R., Gallina, C., Consalvi, V., Scandurra, R., Chiaia Noya, F., and Franchini, C. (1993). Synthesis and inhibiting activities of 1-peptidyl-2-haloacetyl hydrazines toward cathepsin B and calpains. *Eur. J. Med. Chem.* 28, 297-311.
- Goldberg, M. P., and Choi, Q. W. (1993). Combined oxygen and glucose deprivation in cortical cell culture: Calcium-dependent and calcium-independent mechanism of neuronal injury. *J. Neurosci.* 13, 3510-3524.
- Graybill, T. L., Dolle, R. E., Osifo, I. K., Schmidt, S. J., Gregory, J. S., Harris, A. L., and Miller, M. S. (1995). Inhibition of human erythrocyte calpain I by novel quinolinecarboxamides. *Bioorg. Med. Chem. Lett.* 5, 387-392.
- Green, G. D. J., and Shaw, E. (1981). Peptidyl diazomethyl ketones are specific inactivators of thiol proteinases. *J. Biol. Chem.* 256, 1923-1928.
- Greenwood, A. F., and Jope, R. S. (1994). Brain G-protein proteolysis by calpain: Enhancement by lithium. *Brain Res.* 636, 320-326.
- Gupta, R. P., and Abou-Donia, M. B. (1995). Diisopropyl fluorophosphate (DFP) treatment alters calcium-activated proteinase activity and cytoskeletal proteins of the hen sciatic nerve. *Brain Res.* 677, 162-166.
- Guroff, G. (1964). A neutral calcium-activated proteinase from the soluble fraction of rat brain. *J. Biol. Chem.* 239, 149-155.
- Hajimohammadreza, I., Probert, A. W., Coughenour, L. L., Borosky, S. A., Boxer, P. A., Marcoux, F. W., Wang, K. K. W. (1995). Inhibition of calmodulin-dependent protein

- kinase-II attenuates excitotoxic amino acid mediated neuronal death. *J. Neurosci.* 15, 4093–4101.
- Hanada, K., Tamai, M., Morimoto, S., Yamagishi, M., Ohmura, S., Sawada, J., and Tanaka, I. (1978). Studies on thiol protease inhibitors. Part I. Isolation and characterisation of E64, a new thiol protease inhibitor. *Agric. Biol. Chem.* 42, 523–528.
- Harbeson, S. L., Abelleira, S. M., Akiyama, A., Barrett, R., III, Carroll, R. M., Straub, J. A., Tkacz, J. N., Wu, C., and Musso, G. F. (1994). Stereospecific synthesis of peptidyl α -keto amides as inhibitors of calpain. *J. Med. Chem.* 37, 2918–2929.
- Harris, A. L., Gregory, J. S., Maycock, A. L., Graybill, T. L., Osifo, I. K., Schmidt, S. J., and Dolle, R. E. (1995). Characterization of a continuous fluorogenic assay for calpain I. Kinetic evaluation of peptide aldehydes, halomethyl ketones and (acyloxy)methyl ketones as inhibitors of the enzyme. *Bioorg. Med. Chem. Lett.* 5, 393–398.
- Hayes, R. L., Jenkins, L. W., and Lyeth, B. G. (1992). Neurotransmitter-mediated mechanisms of traumatic brain injury. *J. Neurotrauma* 9, S173–S187.
- Hirai, S., Kawasaki, H., Yaniv, M., and Suzuki, K. (1991). Degradation of transcription factors, c-Jun and c-Fos, by calpain. *FEBS Lett.* 287, 57–61.
- Hiramatsu, K.-i., Kassell, N. F., and Lee, K. S. (1993). Improved posthypoxic recovery of synaptic transmission in gerbil neocortical slices treated with a calpain inhibitor. *Stroke* 24, 1725–1728.
- Hong, S. C., Goto, Y., Lanzino, G., Soleau, S., Kassell, N. F., and Lee, K. S. (1994). Neuroprotection with a calpain inhibitor in a model of focal cerebral ischemia. *Stroke* 25, 663–669.
- Hori, H., Yasutake, A., Minematsu, Y., and Powers, J. C. (1985). Inhibition of human leukocyte elastase, pancreatic elastase and cathepsin G by peptide ketones. In "Peptides: Structure and Function. Proceedings of the Ninth American Peptide Symposium (C. M. Deber, V. J. Hruby, and K. D. Kopple, eds.), pp. 819–822. Pierce Chem. Co., Rockford, IL.
- Huang, Z., McGowan, E. B., and Detwiler, T. C. (1992). Ester and amide derivatives of E64c as inhibitors of platelet calpains. *J. Med. Chem.* 35, 2048–2054.
- Huston, R. B., and Krebs, E. G. (1968). Activation of skeletal muscle phosphorylase kinase by Ca^{2+} . II. Identification of the kinase activating factor as a proteolytic enzyme. *Biochemistry* 7, 2116–2121.
- Iizuka, H., Iwasaki, Y., Yamamoto, T., and Kadoya, S. (1986). Morphometric assessment of drug effects in experimental spinal cord injury. *J. Neurosurg.* 65, 92–98.
- Iizuka, K., Kawaguchi, H., and Yasuda, H. (1991). Calpain is activated during hypoxic myocardial cell injury. *Biochem. Med. Metab. Biol.* 46, 427–431.
- Iizuka, K., Kawaguchi, H., and Kitabatake, A. (1993). Effects of thiol protease inhibitors on fodrin degradation during hypoxia in cultured myocytes. *J. Mol. Cell Cardiol.* 25, 1101–1109.
- Imajoh, S., Kawasaki, H., and Suzuki, K. (1986). The amino-terminal hydrophobic region of the small subunit of calcium-activated neutral protease (CANP) is essential for its activation by phosphatidylinositol. *J. Biochem.* 99, 1281–1284.
- Imperiali, B. (1988). Synthetic fluoropeptides as a pharmacologically useful compound. In "Synthetic Peptides in Biotechnology," pp. 97–129, Alan R. Liss, New York.
- Imperiali, B., and Abeles, R. H. (1986). Inhibition of serine proteases by peptidyl fluoromethyl ketones. *Biochemistry* 25, 3760–3767.
- Inaba, T., Hirayama, Y., and Fujinaga, N. (1979). Inhibition of cathepsin B1 by E-64, a thiol proteinase inhibitor, and its derivatives. *Agric. Biol. Chem.* 43, 655–656.
- Ishii, H., Kuboki, M., Fujii, J., Hiraishi, S., and Kazama, M. (1990). Thiolprotease inhibitor, EST, can inhibit thrombin-induced platelet activation. *Thromb. Res.* 57, 847–861.
- Ishima, R., Tamura, A., Akasaka, K., Hamaguchi, K., Makino, K., Murachi, T., Hatanaka, M., and Maki, M. (1991). Structure of the active 27-residue fragment of human calpastatin. *FEBS Lett.* 294, 64–66.
- Ishiura, S., Nonaka, I., and Sugita, H. (1980). Calcium-activated neutral protease: Its degradative role in muscle cells. In "Muscular Dystrophy" (S. Ebashi, ed.), pp. 265–282. University of Tokyo Press, Tokyo.

- Iwamoto, N., Thangnipon, W., Crawford, C., and Emson, P. C. (1991). Localization of calpain immunoreactivity in senile plaques and in neurones undergoing neurofibrillary degeneration in Alzheimer's disease. *Brain Res.* 561, 177-180.
- Johnson, G. V., and Foley, V. G. (1993). Calpain-mediated proteolysis of microtubule-associated protein 2 (MAP-2) is inhibited by phosphorylation by cAMP-dependent protein kinase, but not by Ca^{2+} /calmodulin-dependent protein kinase II. *J. Neurosci. Res.* 34, 642-647.
- Johnson, P., and Hammer, J. L. (1988). Calpain and calpastatin levels in dystrophic hamster skeletal muscles. *Int. J. Biochem.* 20, 1227-1230.
- Kamakura, K., Ishiura, S., Suzuki, K., Sugita, H., and Toyokura, Y. (1985). Calcium-activated neutral protease in the peripheral nerve, which requires microM order Ca^{2+} , and its effect on the neurofilament triplet. *J. Neurosci. Res.* 13, 391-403.
- Kassel, N. F., Sasaki, T., Colohan, A. R. T., and Nazar, G. (1985). Cerebral vasospasm following aneurysmal subarachnoid hemorrhage. *Stroke* 16, 562-572.
- Krantz, A., Copp, L. J., Coles, P. J., Smith, R. A., and Heard, S. B. (1991). Peptidyl (acyloxy)-methyl ketones and the quiescent affinity label the departing group as a variable structural element in the design of inactivators of cysteine proteinases. *Biochemistry* 30, 4678-4687.
- Kuboki, M., Ishii, H., Horie, S., and Kazama, M. (1992). Procalpain I in cytoplasm is translocated onto plasma and granule membranes during platelet stimulation with thrombin and then activated on the membranes. *Biochem. Biophys. Res. Commun.* 185, 1122-1127.
- Kumamoto, T., Ueyama, H., Watanabe, S., Yoshioka, K., Miike, T., Goll, D. E., Ando, M., and Tsuda, T. (1995). Immunohistochemical study of calpain and its endogenous inhibitor in the skeletal muscle of muscular dystrophy. *Acta Neuropathol.* 89, 399-403.
- Lampi, K. J., Kadoya, K., Azuma, M., David, L. L., and Shearer, T. R. (1992). Comparison of cell-permeable calpain inhibitors and E64 in reduction of cataract in cultured rat lenses. *Toxicol. Appl. Pharmacol.* 117, 53-57.
- Leary, R., Larsen, D., Watanabe, H., and Shaw, E. (1977). Diazomethyl ketone substrate derivatives as active-site directed inhibitors of thiol proteinases: Papain. *Biochemistry* 16, 5857-5861.
- Lee, K., Frank, S., Vanderklish, P., Arai, A., and Lynch, G. (1991). Inhibition of proteolysis protects hippocampal neurons from ischemia. *Proc. Natl. Acad. Sci. USA* 88, 7233-7237.
- Li, H., and Buchan, A. M. (1993). Treatment with an AMPA antagonist 12 hours following severe normothermic forebrain ischemia prevents CA1 neuronal injury. *J. Cereb. Blood Flow Metab.* 13, 933-939.
- Li, Z., Patil, G. S., Golubski, Z. E., Hori, H., Tehrani, K., Foreman, J. E., Eveleth, D. D., Bartus, R. T., and Powers, J. C. (1993). Peptide α -keto ester, α -keto amide, and α -keto acid inhibitors of calpains and other cysteine proteases. *J. Med. Chem.* 36, 3472-3480.
- Lipton, S. A., and Rosenberg, P. A. (1994). Excitatory amino acids as a final common pathway for neurologic disorders. *N. Engl. J. Med.* 330, 613-622.
- Maki, M., Takano, E., Mori, H., Sato, A., Murachi, T., and Hatanaka, M. (1987). All four internally repetitive domains of pig calpastatin possess inhibitory activities against calpains I and II. *FEBS Lett.* 233, 174-180.
- Maki, M., Bagci, H., Hamaguchi, K., Ueda, M., Murachi, T., and Hatanaka, M. (1989). Inhibition of calpain by a synthetic oligopeptide corresponding to an exon of the human calpastatin gene. *J. Biol. Chem.* 264, 18866-18869.
- Manev, H., Favaron, M., Siman, R., Guidotti, A., and Costa, E. (1991). Glutamate neurotoxicity is independent of calpain I inhibition in primary cultures of cerebellar granule cells. *J. Neurochem.* 57, 1288-1295.
- Marcantonio, J. M., and Duncan, G. (1991). Calcium-induced degradation of the lens cytoskeleton. *Biochem. Soc. Trans.* 19, 1148-1150.
- March, K. L., Wilensky, R. L., Roeske, R. W., and Hathaway, D. R. (1993). Effects of thiol protease inhibitors on cell cycle and proliferation of vascular smooth muscle cells in culture. *Circ. Res.* 72, 413-423.

- Mata, M., Kupina, N., and Fink, D. J. (1991). Calpain II in rat peripheral nerve. *Brain Res.* 564, 328–331.
- Matsueda, R., Umeyama, H., Puri, R. N., Bradford, H. N., and Colman, R. W. (1990). Potent affinity labeling peptide inhibitors of calpain. *Chem. Lett.* 191–194.
- Matsumura, Y., Kusuoka, H., Inoue, M., Hori, M., and Kamada, T. (1993). Protective effect of the protease inhibitor leupeptin against myocardial stunning. *J. Cardiovasc. Pharmacol.* 22, 135–142.
- Mattson, M. P., Barger, S. W., Cheng, B., Lieberburg, I., Smith-Swintosky, V. L., and Rydel, R. E. (1993). B-amyloid precursor protein metabolites and loss of neuronal Ca^{2+} homeostasis in Alzheimer's disease. *Trends Neurosci.* 16, 109–114.
- McBride, W., Lange, R. A., and Hillis, L. D. (1988). Restenosis after successful coronary angioplasty. *N. Engl. J. Med.* 318, 1734–1737.
- McConkey, D. J., Hartzell, P., Nicotera, P., and Orrenius, S. (1989). Calcium activated DNA fragmentation kills immature thymocytes. *FASEB J.* 3, 1843–1849.
- McConkey, D. J., Chow, S. C., Orrenius, S., and Jondal, M. (1990). NK cell-induced cytotoxicity is dependent on a Ca^{2+} increase in the target. *FASEB J.* 4, 2661–2664.
- McGowan, E. B., Becker, E., and Detwiler, T. C. (1989). Inhibition of calpain in intact platelets by the thiol protease inhibitor E-64d. *Biochem. Biophys. Res. Commun.* 158, 432–435.
- Mehdi, S., Angelastro, M. R., Wiseman, J. S., and Bey, P. (1988). Inhibition of the proteolysis of rat erythrocyte membrane proteins by a synthetic inhibitor of calpain. *Biochem. Biophys. Res. Commun.* 157, 1117–1123.
- Meldrum, B., and Garthwaite, J. (1990). Excitatory amino acid neurotoxicity and neurodegenerative disease. *Trends Pharmacol. Sci.* 11, 379–387.
- Minami, N., Tani, E., Maeda, Y., Tamaura, I., and Fukami, M. (1992). Effects of inhibitors of protein kinase C and calpain in experimental delayed cerebral vasospasm. *J. Neurosurg.* 76, 111–118.
- Molinari, M., Maki, M., and Carafoli, E. (1995). Purification of μ -calpain by a novel affinity chromatography approach. New insights into the mechanism of the interaction of the protease with targets. *J. Biol. Chem.* 270, 14576–14581.
- Murachi, T. (1989). Intracellular regulatory system involving calpain and calpastatin. *Biochem. Int.* 18, 263–294.
- Nixon, R. A., Saito, K. I., Gryspan, F., Griffin, W. P., Katayama, S., Honda, T., Mohan, P. S., Shea, T. B., and Beermann, M. (1994). Calcium-activated neutral proteinase (calpain) system in aging and Alzheimer's disease. *Ann. NY Acad. Sci.* 747, 77–91.
- Ogura, K., Maeda, M., Nagai, M., Tanaka, T., Nomoto, K., and Murachi, T. (1985). Purification and structure of a novel cysteine proteinase inhibitor, strepin P-1. *Agric. Biol. Chem.* 49, 799–805.
- O'Halloran, t., Beckerle, M. C., and Burrige, K. (1985). Identification of talin as a major cytoplasmic protein implicated in platelet activation. *Nature* 317, 449–451.
- Olney, J. W., Labruyere, J., Wang, G., Wozniak, D. F., Price, M. T., and Sesma, M. A. (1991). NMDA antagonist neurotoxicity: Mechanism and prevention. *Science* 254, 1515–1518.
- Omura, S., Tanaka, H., Shimoi, K., and Liu, J. R. (1993). Pyrrole propionic acid amide (cystamidine A) manufacture with *Streptomyces*, and its use as calpain inhibitor. Eur. Pat. Appl. EP 569122 A1.
- Onji, T., Takagi, M., and Shibata, N. (1987). Calpain abolishes the effect of filamin on the actomyosin system in platelets. *Biochim. Biophys. Acta* 912, 283–286.
- Parkes, C., Kembhavi, A. A., and Barrett, A. J. (1985). Calpain inhibition by peptide epoxides. *Biochem. J.* 230, 509–516.
- Peet, N. P., Burkhart, J. P., Angelastro, M. R., Giroux, E. L., Mehdi, S., Bey, P., Kolb, M., Neises, B., and Schirlin, D. (1990). Synthesis of peptidyl fluoromethyl ketones and peptidyl α -keto esters as inhibitors of porcine pancreatic elastase, human neutrophil elastase, and rat and human neutrophil cathepsin G. *J. Med. Chem.* 33, 394–399.

- Persson, H., and Karlsson, J.-O. (1991). Calpain activity in a subcellular fraction enriched in partially degraded CNS myelin fragments compared with myelin. *Neurosci. Lett.* 130, 81-84.
- Pliura, D. H., Bonaventura, B. J., Smith, R. A., Coles, P. J., and Krantz, A. (1992). Comparative behavior of calpain and cathepsin B toward peptidyl acyloxymethyl ketones, sulfonium methyl ketones and other potential inhibitors of cysteine proteinases. *Biochem. J.* 288, 759-762.
- Postmantur, R., Hayes, R. L., Dixon, E., and Taft, W. C. (1994). Neurofilament 68 and neurofilament 200 protein levels decrease after traumatic brain injury. *J. Neurotrauma* 11, 533-545.
- Posner, A., Raser, K. J., Hajimohammadreza, I., Yuen, P.-w., and Wang, K. K. W. (1995). Aurintricarboxylic acid is an inhibitor of calpain I and II. *Biochem. Mol. Biol. Int.* 36, 291-299.
- Puri, R. N., Zhou, F. X., Bradford, H., Hu, C. J., Colman, R. F., and Colman, R. W. (1989). Thrombin-induced platelet aggregation involves an indirect proteolytic cleavage of aggregin by calpain. *Arch. Biochem. Biophys.* 271, 346-358.
- Puri, R. N., Hu, C. J., Matsueda, R., Umeyama, H., and Colman, R. W. (1992). Aggregation of washed platelets by plasminogen and plasminogen activators is mediated by plasmin and is inhibited by a synthetic peptide disulfide. *Thromb. Res.* 65, 533-547.
- Puri, R. N., Matsueda, R., Umeyama, H., Bradford, H. N., and Colman, R. W. (1993a). Modulation of thrombin-induced platelet aggregation by inhibition of calpain by a synthetic peptide derived from the thiol-protease inhibitory sequence of kininogens and S-(3-nitro-2-pyridinesulfonyl)-cysteine. *Eur. J. Biochem.* 214, 233-241.
- Puri, R. N., Matsueda, R., Umeyama, H., and Colman, R. W. (1993b). Specificity of the sequence in Phe-Gln-Val-Val-Cys (-3-nitro-2 pyridinesulfonyl)-Gly NH₂—A selective inhibitor of thrombin-induced platelet aggregation. *Thromb. Res.* 72, 183-191.
- Rami, A., and Kriegelstein, J. (1993). Protective effects of calpain inhibitors against neuronal damage caused by cytotoxic hypoxia *in vitro* and ischemia *in vivo*. *Brain Res.* 609, 67-70.
- Rasnick, D. (1985). Synthesis of peptide fluoromethyl ketones and the inhibition of human cathepsin B. *Anal. Biochem.* 149, 461-465.
- Richard, I., Broux, R. O., Allamand, V., Fougereousse, F., Chiannikulchai, N., Bourg, N., Brenguier, L., Devaud, C., Pasturaud, P., Roudaut, C., *et al.* (1995). Mutation in the proteolytic enzyme calpain 3 cause limb-girdle muscular dystrophy type 2A. *Cell* 81, 27-40.
- Saido, T. C., Suzuki, H., Yamazaki, H., Tanoue, K., and Suzuki, K. (1993). *In situ* capture of mu-calpain activation in platelets. *J. Biol. Chem.* 268, 7422-7426.
- Saido, T. C., Sorimachi, H., and Suzuki, K. (1994). Calpain: New perspectives in molecular diversity and physiological-pathological involvement. *FASEB J.* 8, 814-822.
- Saito, K.-I., Elce, J. S., Hamos, J. E., and Nixon, R. A. (1993). Widespread activation of calcium-activated neutral proteinase (calpain) in the brain in Alzheimer disease: A potential molecular basis for neuronal degeneration. *Proc. Natl. Acad. Sci. USA* 90, 2628-2632.
- Saito, M., Kawaguchi, N., Hashimoto, M., Kodama, T., Higuchi, N., Tanaka, T., Nomoto, K., and Murachi, T. (1987). Purification and structure of novel cysteine proteinase inhibitors, staccopins P1 and P2, from *Staphylococcus tanabeensis*. *Agric. Biol. Chem.* 51, 861-868.
- Sakai, K., Akanuma, H., Imahori, K., and Kawashima, S. (1987). A unique specificity of a calcium activated neutral protease indicated in histone hydrolysis. *J. Biochem.* 101, 911-918.
- Sakon, M., Kambayashi, J., Ohno, H., and Kosaki, G. (1981). Two forms of Ca⁺⁺ activated neutral protease in platelets. *Thromb. Res.* 24, 207-214.
- Salvesen, G., Parkes, C., Abrahamson, M., Grubb, A., and Barrett, A. J. (1986). Human low-Mr kininogen contains three copies of a cystatin sequence that are divergent in structure and in inhibitory activity for cysteine proteinases. *Biochem. J.* 234, 429-434.

- Sarin, A., Adams, D. H., and Henkart, P. A. (1993). Protease inhibitors selectively block T cell receptor-triggered programmed cell death in murine T cell hybridoma and activated peripheral T cells. *J. Exp. Med.* 178, 1693–1700.
- Sarin, A., Clerici, M., Blatt, S. P., Hendrix, C. W., Shearer, G. M., and Henkart, P. A. (1994). Inhibition of activation-induced programmed cell death and restoration of defective immune response of HIV+ donors by cysteine protease inhibitors. *J. Immunol.* 153, 862–872.
- Sasaki, M., Taniguchi, K., Suzuki, K., and Imahori, K. (1983). Human plasma 1- and 2-thiol proteinase inhibitors strongly inhibit Ca^{2+} -activated neutral protease from muscle. *Biochem. Biophys. Res. Commun.* 110, 256–261.
- Sasaki, T., Kikuchi, T., Yumoto, N., Yoshimura, N., and Murachi, T. (1984). Comparative specificity and kinetic studies on porcine calpain I and calpain II with naturally occurring peptides and synthetic fluorogenic substrates. *J. Biol. Chem.* 259, 12489–12494.
- Sasaki, T., Kikuchi, T., Fukui, I., and Murachi, T. (1986). Inactivation of calpain I and calpain II by specificity-oriented tripeptidyl chloromethyl ketones. *J. Biochem.* 99, 173–179.
- Sasaki, T., Kishi, M., Saito, M., Tanaka, T., Higuchi, N., Kominami, E., Katunuma, N., and Murachi, T. (1990). Inhibitory effect of di- and tripeptidyl aldehydes on calpains and cathepsins. *J. Enz. Inhib.* 3, 195–201.
- Schlaepfer, W. W., and Micko, S. (1979). Calcium-dependent alternations of neurofilament proteins of rat peripheral nerve. *J. Neurochem.* 32, 244–249.
- Schlaepfer, W. W., Lee, C., Lee, V. M., and Zimmerman, U. J. (1985). An immunoblot study of neurofilament degradation *in situ* and during calcium-activated proteolysis. *J. Neurochem.* 44, 502–509.
- Schmaier, A. H., Bradford, H., Silver, L. D., Farber, A., Scott, C. F., Schutsky, D., and Colman, R. W. (1986). High molecular weight kininogen is an inhibitor of platelet calpain. *J. Clin. Invest.* 77, 1565–1573.
- Schröder, E., Phillips, C., Garman, E., Harlos, K., and Crawford, C. (1993). X-ray crystallographic structure of a papain-leupeptin complex. *FEBS Lett.* 315, 38–42.
- Seubert, P., Lee, K., and Lynch, G. (1989). Ischemia triggers NMDA receptor linked cytoskeletal proteolysis in hippocampus. *Brain Res.* 492, 366–370.
- Shaw, E. (1990). Cysteine proteinases and their selective inactivation. *Adv. Enzymol.* 63, 271–347.
- Shearer, T. R., David, L. L., and Anderson, R. S. (1987). Selenite cataract. *Curr. Eye Res.* 6, 289–300.
- Shearer, T. R., Azuma, M., David, L. L., and Murachi, T. (1991). Amelioration of cataracts and proteolysis in cultured lenses by cysteine protease inhibitor E64. *Ophthalmol. Vis. Sci.* 32, 533–540.
- Siman, R., and Noszek, J. C. (1988). Excitatory amino acids activate calpain I and induce structural protein breakdown *in vivo*. *Neuron* 1, 279–287.
- Siman, R., Card, J. P., and Davis, L. G. (1990). Proteolytic processing of beta amyloid precursor by calpain. I. *J. Neurosci.* 10, 2400–2411.
- Sorimachi, H., Imajoh, O. S., Emori, Y., Kawasaki, H., Ohno, S., Minami, Y., and Suzuki, K. (1989). Molecular cloning of a novel mammalian calcium dependent protease distinct from both m- and mu-types. Specific expression of the mRNA in skeletal muscle. *J. Biol. Chem.* 264, 20106–20111.
- Sorimachi, H., Ishiura, S., and Suzuki, K. (1993). A novel tissue specific calpain species expressed predominantly in the stomach comprises two alternative splicing products with and without Ca^{2+} binding domain. *J. Biol. Chem.* 268, 19476–19482.
- Spencer, M. J., Croall, D. E., and Tidbal, J. G. (1995). Calpains are activated in necrotic fiber from mdx dystrophic mice. *J. Biol. Chem.* 270, 10909–10914.
- Squier, M. K. T., Miller, A. C. K., Malkinson, A. M., and Cohen, J. J. (1994). Calpain activation in apoptosis. *J. Cell. Physiol.* 159, 229–237.

- Stein, R. L., Strimpler, A. M., Edwards, P. D., Lewis, J. J., Mauger, R. C., Schwartz, J. A., Stein, M. M., Trainor, D. A., Wildonger, R. A., and Zottola, M. A. (1987). Mechanism of slow-binding inhibition of human leukocyte elastase by trifluoromethyl ketones. *Biochemistry* 26, 2682-2689.
- Stone, S. R., Rennex, D., Wikstrom, P., Shaw, E., and Hofsteenge, J. (1992). Peptidyl diazomethanes. A novel mechanism of interaction with prolyl endopeptidase. *Biochem. J.* 283, 871-876.
- Suda, H., Aoyagi, T., Hamada, M., Takeuchi, T., and Umezawa, H. (1972). Antipain, a new protease inhibitor isolated from actinomycetes. *J. Antibiot.* 25, 263-266.
- Sugita, H., Ishiura, S., Suzuki, K., and Imahori, K. (1980). Inhibition of epoxide derivatives on chicken calcium-activated neutral protease (CANP) *in vitro* and *in vivo*. *J. Biochem.* 87, 339-341.
- Suzuki, K. (1983). Reaction of calcium-activated neutral protease (CANP) with an epoxysuccinyl derivative (E64c) and iodoacetic acid. *J. Biochem.* 93, 1305-1312.
- Suzuki, K. (1987). Calcium activated neutral protease: Domain structure and activity regulation. *Trends Biochem. Sci.* 12, 103-105.
- Suzuki, K., Tsuji, S., Kubota, S., Kimura, Y., and Imahori, K. (1981a). Limited autolysis of Ca^{2+} -activated neutral protease (CANP) changes its sensitivity to Ca^{2+} ions. *J. Biochem.* 90, 275-278.
- Suzuki, K., Tsuji, S., and Ishiura, S. (1981b). Effect of calcium ion on the inhibition of calcium-activated neutral protease by leupeptin, antipain and epoxysuccinate derivatives. *FEBS Lett.* 136, 119-122.
- Suzuki, K., Shimizu, K., Hamamoto, T., Nakagawa, Y., Hamakubo, T., and Yamamuro, T. (1990). Biochemical demonstration of calpains and calpastatin in osteoarthritic synovial fluid. *Arthritis Rheum.* 33, 728-732.
- Suzuki, K., Shimizu, K., Hamamoto, T., Nakagawa, Y., Murachi, T., and Yamamuro, T. (1992). Characterization of proteoglycan degradation by calpain. *Biochem. J.* 285, 857-862.
- Szomor, Z., Shimizu, K., Fujimori, Y., Yamamoto, S., and Yamauro, T. (1995). Appearance of calpain correlates with arthritis and cartilage destruction in collagen induced arthritic knee joints of mice. *Ann. Rheum. Dis.* 54, 477-483.
- Taft, W. C., Yang, K., Dixon, C. E., and Hayes, R. L. (1992). Microtubule-associated protein 2 level in hippocampus following traumatic brain injury. *J. Neurotrauma* 9, 281-290.
- Takano, E., Kitahara, A., Sasaki, T., Kannagi, R., and Murachi, T. (1986). Two different molecular species of pig calpastatin. Structural and functional relationship between 107 kDa and 68 kDa molecules. *Biochem. J.* 235, 97-102.
- Takano, E., Ma, H., Yang, H. Q., Maki, M., and Hatanaka, M. (1995). Preference of calcium-dependent interactions between calmodulin-like domains of calpain and calpastatin subdomains. *FEBS Lett.* 362, 93-97.
- Takano, H., Kawaguchi, H., Iizuka, K., and Yasuda, H. (1991). Platelet aggregation induced by platelet-activating factor is suppressed by cystine protease inhibitor. *Biochem. Med. Metab. Biol.* 45, 41-47.
- Tallant, E. A., Brumley, L. M., and Wallace, R. W. (1988). Activation of a calmodulin-dependent phosphatase by a Ca^{2+} -dependent protease. *Biochemistry* 27, 2205-2211.
- Tamai, M., Yokoo, C., Murata, M., Oguma, K., Sota, K., Sato, E., and Kanaoka, Y. (1987). An efficient synthetic method for ethyl (+)-(2S,3S)-3-[(S)-3-methyl-1-(3-methylbutyloxy)-2-oxiranecarboxylate (EST), a new inhibitor of cysteine proteinases. *Chem. Pharm. Bull.* 35, 1098-1104.
- Toda, G., Matsushita, S., Kuramoto, K., Oda, S., Ezaki, H., Hattori, A., and Kawashima, S. (1989). Calcium-activated neutral protease inhibitor (E64c) and reperfusion for experimental myocardial infarction. *Jpn. Heart J.* 30, 375-386.
- Tolnai, S., and Korecky, B. (1986). Calcium-dependent proteolysis and its inhibition in the ischemic rat myocardium. *Can. J. Cardiol.* 2, 42-47.

- Towatari, T., Tanaka, K., Yoshikawa, D., and Kataunuma, M. (1978). Purification and properties of a new cathepsin from rat liver. *J. Biochem.* **84**, 659–672.
- Toyo-Oka, T., Shimizu, T., and Masaki, T. (1978). Inhibition of proteolytic activity of calcium activated neutral protease by leupeptin and antipain. *Biochem. Biophys. Res. Commun.* **82**, 484–491.
- Tsujinaka, T., Kajiwar, Y., Kambayashi, J., Sakon, M., Higuchi, N., Tanaka, T., and Mori, T. (1988). Synthesis of a new cell penetrating calpain inhibitor (calpeptin). *Biochem. Biophys. Res. Commun.* **153**, 1201–1208.
- Turner, P. R., Westwood, T., Regen, C. M., and Steinhardt, R. A. (1988). Increased protein degradation results from elevated free calcium levels found in muscle from mdx mice. *Nature* **335**, 735–738.
- Turner, P. R., Schultz, R., Ganguly, B., and Steinhardt, R. A. (1993). Proteolysis results in altered leak channel kinetics and elevated free calcium in mdx muscle. *J. Membr. Biol.* **133**, 243–251.
- Valentino, K., Newcomb, R., Gadbois, T., Singh, T., Bowersox, S., Bitner, S., Justice, B. A., Tamashiro, D., Hoffman, B. B., Ciaranello, R., Milianich, G., and Ramachandran, J. (1993). A selective N-type calcium channel antagonist protects against neuronal loss after global cerebral ischemia. *Proc. Natl. Acad. Sci. USA* **90**, 7894–7897.
- van der Westhuyzen, D., Matsumoto, K., and Etlinger, J. D. (1981). Easily releasable myofilaments from skeletal and cardiac muscles maintained *in vitro*. *J. Biol. Chem.* **256**, 11791–11797.
- Van Heyningen, R. (1959). Formation of polyols by the lens of the rat with 'sugar' cataract. *Nature* **184**, 194–195.
- Wagner, B. M., Smith, R. A., Coles, P. J., Copp, L. J., Ernest, M. J., and Krantz, A. (1994). *In vivo* inhibition of cathepsin B by peptidyl (acyloxy)methyl ketones. *J. Med. Chem.* **37**, 1833–1840.
- Wang, K. K. W. (1990). Developing selective inhibitors of calpain. *Trends Pharmacol. Sci.* **11**, 139–142.
- Wang, K. K. W., Villalobo, A., and Roufogalis, B. D. (1989). Calmodulin binding proteins as calpain substrates. *Biochem. J.* **262**, 693–706.
- Wang, K. K. W., and Yuen, P.-w. (1994). Calpain inhibition: An overview of its therapeutic potential. *Trends Pharmacol. Sci.* **15**, 412–419.
- Wang, K. K. W., Nath, R., Posner, A., Raser, K. J., Buroker-Kilgore, M., Ye, Q., Takano, E., Hatanaka, M., Maki, M., Marcoux, F. W., Caner, H., Collins, J. L., Fergus, A., Lee, K. S., Lunney, E. A., Hays, S. J., and Yuen, P.-w. (1996). Alpha-mercaptoacrylic acid derivatives are selective non-peptide and calcium-binding domain-targeting calpain inhibitors. *Proc. Natl. Acad. Sci.* In press.
- Watt, F., and Molloy, P. L. (1993). Specific cleavage of transcription factors by the thiol protease, m-calpain. *Nucleic Acids Res.* **21**, 5092–5100.
- Waxman, S. G. (1993). Demyelination in spinal cord injury and multiple sclerosis: What can we do to enhance functional recovery? *J. Neurotrauma* **9** (Suppl 1), S105–S117.
- Wilcox, D., and Mason, R. W. (1992). Inhibition of cysteine proteinases in lysosomes and whole cells. *Biochem. J.* **285**, 495–502.
- Wilensky, R. L., and March, K. L., and Hathaway, D. R. (1991). Restenosis in an atherosclerotic rabbit model is reduced by a thiol protease inhibitor. *J. Am. Coll. Cardiol.* **17**, 268A.
- Yamamoto, S., Shimizu, K., Shimizu, K., Suzuki, K., Nakagawa, Y., and Yamamuro, T. (1992). Calcium-dependent cysteine proteinase (calpain) in human arthritic synovial joints. *Arthritis Rheum.* **35**, 1309–1317.
- Yang, H. Q., Ma, H., Takano, E., Hatanaka, M., and Maki, M. (1994). Analysis of calcium-dependent interaction between amino-terminal conserved region of calpastatin functional domain and calmodulin-like domain of μ -calpain large subunit. *J. Biol. Chem.* **269**, 18977–18984.

- Yano, Y., Shiba, E., Kambayashi, J., Sakon, M., Kawasaki, T., Fujitani, K., Kang, J., and Mori, T. (1993). The effects of calpeptin (a calpain specific inhibitor) on agonist-induced microparticle formation from the platelet plasma membrane. *Thromb. Res.* 71, 385-396.
- Yoshida, H., Murachi, T., and Tsukahara, I. (1984). Limited proteolysis of bovine lens alpha-crystallin by calpain, a Ca^{2+} -dependent cysteine proteinase, isolated from the same tissue. *Biochim. Biophys. Acta* 798, 252-259.
- Yoshida, H., Murachi, T., and Tsukare, I. (1985a). Distribution of calpain I, calpain II, and calpastatin in bovine lens. *Invest. Ophthalmol. Vis. Sci.* 26, 953-956.
- Yoshida, H., Murachi, T., and Tsukahara, I. (1985b). Age-related changes of calpain II and alpha-crystallin in the lens of hereditary cataract (Nakano) mouse. *Curr. Eye Res.* 4, 983-988.
- Yoshida, K.-I., Sorimachi, Y., Fujiwara, M., and Hironaka, K. (1995). Calpain is implicated in rat myocardial injury after ischemia or reperfusion. *Jpn. Circ. J.* 59, 40-48.
- Zimmerman, U.-J. P., and Schlaepfer, W. W. (1984). Calcium-activated neutral protease (CANP) in brain and other tissues. *Prog. Neurobiol.* 23, 63-78.
- Zumbrunn, A., Stone, S., and Shaw, E. (1988). Synthesis and properties of Cbz-Phe Arg-CHN₂ (benzyloxycarbonylphenylalanylarginyldiazomethane) as a proteinase inhibitor. *Biochem. J.* 250, 621-623.