Molecular Neurobiology

ISSN 0893–7648/06/33(3): 215–236/$30.00
ISSN (Online) 1559–1182

Calpain and Synaptic Function

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Abstract

Proteolysis by calpain is a unique posttranslational modification that can change integrity, localization, and activity of endogenous proteins. Two ubiquitous calpains, μ-calpain and m-calpain, are highly expressed in the central nervous system, and calpain substrates such as membrane receptors, postsynaptic density proteins, kinases, and phosphatases are localized to the synaptic compartments of neurons. By selective cleavage of synaptically localized molecules, calpains may play pivotal roles in the regulation of synaptic processes not only in physiological states but also during various pathological conditions. Activation of calpains during sustained synaptic activity is crucial for Ca^{2+}-dependent neuronal functions, such as neurotransmitter release, synaptic plasticity, vesicular trafficking, and structural stabilization. Overactivation of calpain following dysregulation of Ca^{2+} homeostasis can lead to neuronal damage in response to events such as epilepsy, stroke, and brain trauma. Calpain may also provide a neuroprotective effect from axotomy and some forms of glutamate receptor overactivation. This article focuses on recent findings on the role of calpain-mediated proteolytic processes in potentially regulating synaptic substrates in physiological and pathophysiological events in the nervous system.

Index Entries: Calpain; calcium; NMDA receptor; proteolysis; synapse.

Introduction

Calpains, a unique family of proteases, have various functions in cells and organisms, including roles in cell division, neoplasia, and cardiac function. In the nervous system, calpains are crucial for neuronal functions such as learning and memory and mechanisms of neuronal death. In 1964, Guroff *(1,2)* discovered a Ca^{2+}-dependent neutral proteinase in rat brain, which was identified as calpain in 1968. In 1984, the complementary DNA was cloned for the large subunit of calpain *(3,4)*. Based on the human genome sequence *(5)*, at least 14 homologs of the calpain large subunit belong to the calpain superfamily; these homologs are classified as ubiquitous or tissue-specific based

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Received October 31, 2005; Accepted February 20, 2006.

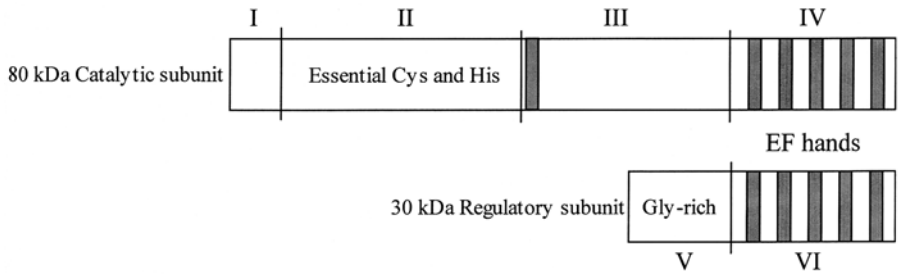

Fig. 1. Domain structure of the large and small subunits of calpain. The six EF-hand motifs in the 80-kDa subunit and the five EF-hand motifs in the 30-kDa subunit are shown as vertical bars. The 80-kDa large subunit of calpain consists of four domains (I, II, III, IV), and the 30-kDa regulatory subunit contains two domains (V, VI). Domain I is the region of autolysis. The Cys- and His-containing region in domain II catalyzes protease activity. Domain III is involved in binding Ca^{2+} and phospholipids. The EF-hands in domains IV and VI are Ca^{2+} binding sites. Domain V is a glycine-rich domain. Two EF-hand regions, domains IV and VI, associate to form the heterodimeric calpain.

on their expression patterns *(1,6)*. μ-calpain and m-calpain (also called calpain I and calpain II, respectively), the major calpain isoforms, are widely expressed and are similar in substrate specificity but differ in their sensitivity to Ca^{2+} in vitro. m-calpain requires 0.4 to 0.8 m*M* calcium for half-maximal proteolytic activity in vitro, whereas μ-calpain requires 3 to 50 μ*M* calcium for activity *(7)*. These isoforms are the only ones known to be expressed in neuronal tissue.

The two major forms of calpain have overlapping structural features. They contain distinct large 80-kDa catalytic subunits and a common small 30-kDa regulatory subunit (Fig. 1). The large subunits of μ-calpain and m-calpain are organized into four domains (I, II, III, and IV) and are encoded by the *CAPN1* and *CAPN2* genes, respectively *(7)*. Domain I is the N-terminal α-helix domain, which contains the site where autolytic cleavage occurs. This region is important for regulating the activity and dissociation of the two subunits. Domain II contains the essential cysteine and histidine residues involved in catalytic activity and interacts with both substrates and the inhibitory region of calpastatin, an endogenous inhibitor of calpain *(1,8–10)*. The function of domain III is unknown, but it may be involved in binding Ca^{2+} and phospholipids and in regulating calpain activity by its participation in critical electrostatic interactions *(11–13)*. Domain IV, at the C-terminal end of the large subunit, has five sets of sequences that predict the presence of an EF-hand helix–loop–helix Ca^{2+}-binding motif. This region of calpain is marginally homologous to calmodulin and is involved in dimer formation *(14)*.

The 30-kDa regulatory subunit contains two domains. Domain V, the N-terminal region of the small subunit, is a glycine-rich, hydrophobic domain, and may function as a membrane anchor. Domain VI, the C-terminal end of the small subunit, is a Ca^{2+}-binding region, similarly to domain IV of the large subunit *(12,13)*. The Ca^{2+}-binding domains of the catalytic and regulatory subunits associate to form heterodimeric calpain. In genetically engineered mice, absence of both μ- and m-calpain activity created by interfering with expression of the regulatory subunit is lethal at embryonic day 11.5 *(15,16)*, confirming the key role of calpains in essential cellular functions and development.

Calpains are typically activated by an elevation of intracellular Ca^{2+}, either in response to activation of plasma membrane receptors and channels or by release of calcium from intracellular stores. They can also be activated by pathological increases in intracellular calcium. Calpain activity is directly regulated by calpastatin, a specific endogenous inhibitor of the proteolytic activity of both μ- and m-forms of

calpain in mammalian cells *(17–20)*. Calpastatin blocks the substrate binding site in a use-dependent manner. In adult rat brain, calpastatin messenger RNA is expressed throughout the brain, with especially high levels in cranial nerve nuclei and the brain stem *(21)*.

Although calpain and calpastatin are both found in the soluble fraction of the cell, they may have different intracellular localizations *(22)*. Additionally, although the exact subcellular localization of calpain and calpastatin has not yet been fully defined in neurons, calpastatin is localized in structures close to nuclear invaginations in pheochromocytoma (PC12) and neuroblastoma (LAN-5) cells, whereas calpain is located diffusely in the cytosol. The inhibitory action of calpastatin on calpain activity requires an increase in intracellular calcium *(23–25)*. Following an elevation of intracellular calcium, calpastatin diffuses into the cytosol and colocalizes with calpain, thereby modulating calpain activity. Calpastatin is phosphorylated by cyclic adenosine monophosphate-dependent protein kinase in a manner that changes the specificity of calpastatin for the major isoforms of calpain, increasing its ability to inhibit m-calpain but decreasing inhibition of μ-calpain *(26)*. This regulation suggests a mechanism for controlling calpain activity through phosphorylation of this endogenous inhibitor. Additionally, posttranslational modifications (such as phosphorylation) of substrates commonly alter the susceptibility of substrates to calpain-mediated proteolysis *(7,27–30)*, allowing the effects of calpain in general and on specific substrates to be regulated by second messenger systems.

Calpains participate in various intracellular signaling pathways mediated by Ca^{2+} through limited proteolysis of target proteins, thereby irreversibly altering the function of these proteins or facilitating further degradation. In the nervous system, the two major forms of calpain are widely expressed and are found in both the soma and synaptic terminals of neurons. Calpain substrates in neurons include synaptic proteins such as membrane receptors, cytoskeletal proteins, postsynaptic density proteins, and intracellular mediators that are critical for synaptic function *(31–41)*. Pharmacological approaches based on pharmacological inhibition of all calpains suggest that calpains participate in many neuronal processes, such as excitability, neurotransmitter release, synaptic plasticity, signal transduction, vesicular trafficking, structural stabilization, and gene transcription *(42–47)*. However, generally, the role of calpains in physiological properties has not been closely associated with their ability to cleave specific substrates.

Neurotoxic insults (such as those causing excitotoxicity) may also lead to increases in intracellular calcium levels that activate calpain *(7,33,34,40,41)*. Such uncontrolled calpain activity mediates the degradation of many cellular proteins in the course of neuronal death and may contribute to the pathophysiology of neurological disorders such as ischemia, spinal cord injury, trauma, Alzheimer's disease, Huntington's disease, Parkinson's disease, and aging *(48–55)*. However, calpain can also protect against acute Ca^{2+}-mediated neuronal injury in other situations, including some acute excitotoxic lesions (*56–59)*.

This article concentrates on the manner by which calpain-mediated proteolysis modulates a series of individual proteins at the synapse. It also discusses the potential physiological significance of calpain-mediated proteolysis in synaptic transmission and neuroplasticity. Finally, it highlights the concept that calpain-mediated synaptic modification may constructively regulate brain function as well as the sometimes conflicting role of calpain in Ca^{2+}-mediated neuronal disorders.

Regulation of Synaptic Receptors by Calpain

Calpain-Mediated Regulation of N*-Methyl-*D*-Aspartate Receptors*

The *N*-methyl-D-aspartate (NMDA) receptor, a subtype of glutamate receptor, is a ligand- and voltage-gated, calcium-permeable ion chan-

nel that generates excitatory postsynaptic currents at glutamatergic synapses and increases intracellular calcium levels in neurons *(60)*. NMDA receptor-mediated signals are critical for neuroplasticity, synaptic homeostasis, and many pathophysiological events *(61–66)*. Functional NMDA receptors are heteromeric proteins that likely contain two NR1 subunits and two NR2 subunits (selected from among four NR2 subtypes—NR2A–NR2D). Different NR2 subunits, which are developmentally and spatially regulated, confer distinct pharmacological and kinetic properties to the receptor. Each subunit of the NMDA receptor has an extracellular N-terminal region, three membrane-spanning domains, one intramembrane loop, and an intracellular C-terminal region *(67)*. The C-terminal tail interacts with intracellular signal molecules, such as anchoring proteins, cytoskeletal elements, phosphatases, and kinases. The major mediator of NMDA receptor-mediated signaling is intracellular calcium, the major means by which calpain is physiologically activated in neurons.

Although NMDA receptor stimulation leads to calpain activation, the C-termini of three NR2 subunits (NR2A, NR2B, and NR2C) are targeted by calpain proteolysis, which may change NMDA receptor levels and activity at synapses *(29,33,40,41,67–71)*. In homogenates from human embryonic kidney (HEK) 293t cells transfected with NR1and NR2A (termed NR1/2A), in vitro digestion with purified calpain I creates two stable breakdown products of recombinant NR2A, most likely by cleaving before amino acids 1279 and 1330. Both of these sites are located in the final 200 amino acids of NR2A. However, cleavage at these sites does not directly inactivate the receptor. Truncated NR1/2A receptors retain the ability to bind [^{125}I]MK801, a labeled NMDA receptor channel-blocking agent that marks functional NMDA receptors. When NMDA receptors truncated to amino acid 1279 or 1330 are co-expressed with NR1 in HEK 293 cells, the receptor acts similarly to wild-type receptors in assays of agonist-stimulated increases in intracellular calcium and ^{45}Ca uptake. Additionally, basic electrophysiological properties are similar between wild-type receptors and receptors that contain NR2A subunits truncated to the sites of calpain cleavage.

The NR2A subunit is also a selective substrate for calpain in cell culture expression systems *in situ (34)*. In transfected cells, cleavage of NR2A by activated calpain is found in both the intracellular fraction and the plasma membrane fraction *(72)*. However, NR2A that is not co-assembled with NR1 does not leave the endoplasmic reticulum (ER) and is not a substrate for calpain *in situ*, suggesting that cleavage of NR2A requires either a particular structural motif created by interaction with NR1 or that NR2A must leave the ER to be accessible to calpain.

Conversely to in vitro studies, calpain-mediated cleavage of NR2A does not produce stable N-terminal fragments *in situ (34)*, suggesting that other degradation mechanisms in living cells, such as proteosomal processes, can rapidly further degrade NR2A fragments produced by calpain. Because the fragment of NR2A is not stable in HEK cells, proteolysis of NR2A by activated calpain functionally limits NMDA receptor activity in this system *(34)*. During 100-μ*M* glutamate and glycine stimulation for 10 min of NR1/2A transfected HEK 293t cells, inhibition of calpain-mediated cleavage of NR2A significantly increases the levels of [^{125}I]-MK801 binding, the size of NMDA receptor-mediated intracellular calcium responses, and the amount of agonist-induced ^{45}Ca uptake. Interestingly, although NR1 does not appear to be a direct calpain substrate in HEK cells, NR1 that is co-assembled with NR2A is destroyed in a calpain-dependent manner, suggesting that calpain-mediated cleavage of the C-terminus of NR2A can act as a mechanism for destruction of the combined NR1/2A complex *(72)*. Furthermore, although the products of NR2A do not appear to be stable in this system, the sites of calpain-mediated cleavage appear to the same as the sites in vitro, because removal of the sites identified in vitro (by use of already truncated receptors) blocks the effects of calpain inhibition on NR2A levels in transfected cells.

In neurons, cleavage of NR2A by calpain is less consistent. Calpain-mediated cleavage of NR2A cannot readily be detected in hippocampal neurons at ages greater than 18 d in vitro *(73)*. However, NR2A can be cleaved in younger neurons from both hippocampal and cortical cultures (14–16 d in vitro) *(41,72)*. The reason for this developmental dependence may be the binding of NR2A to postsynaptic density protein (PSD)-95. PSD-95 is a major membrane-associated guanylate kinase (MAGUK) that binds to the C-terminal cytoplasmic tail of NR2A in the PSD in mature neurons. Developmental increases in synaptic PSD-95 correlate with the increase in NR2A expression in neuronal development *(74)*. Disruption of the PSD-95–NR2A interaction in neurons and transfected cells by blocking palmitoylation of PSD-95 or by removing the PSD-95 binding site from NR2A allows NR2A to be a substrate for calpain *(67,72,75,76)*. Additionally, synapse-associated protein (SAP)-102, a MAGUK protein containing PDZ domains similar to those of PSD-95 but lacking the palmitoylation motifs and the ability to cluster *(77,78)*, does not block calpain-mediated cleavage of NR2A. These studies suggest that protection of NR2A from cleavage by calpain by PSD-95 requires not only the PDZ-domain-mediated association of these proteins but also palmitoylation-dependent clustering of PSD-95 and NR2A or other PSD-95-specific associations.

NR2B is also a substrate for calpain in vitro and in vivo *(33,34,41,72,73)*. NR2B can be digested in vitro by purified calpain I to three fragments with the molecular masses of approx 140, 130, and 120 kDa *(33,34)*. Among the three products, only the smallest (120 kDa) is stably created in hippocampal neurons in which calpain is activated by NMDA receptor stimulation *(41,72,73)*. In rats subjected to transient bilateral carotid occlusion, calpain activity increases in hippocampal regions 24 to 48 h later in association with increases in the levels of a 115- to 120-kDa low-molecular-weight form of NR2B, matching the calpain-generated cleavage product *in situ (73)*. Calpain-mediated cleavage of NR2B has also been found in models of status epilepticus *(79)*. In hippocampal neurons in culture, the 115- to 120-kDa form of the NR2B subunit is created rapidly (within 5 min of receptor activation) and remains on the cell surface *(73)*. Based on the sites of cleavage and its presence on the cell surface, the fragment of NR2B generated in neurons should be part of an active NMDA receptor. However, the truncated NR2B, which lacks most of the C-terminal cytoplasmic region, should not associate with intracellular signaling molecules such as protein kinases, phosphatases, and scaffolding proteins. It is not known whether the calpain-mediated NR2B fragment is indeed active in neurons. Additionally, the physiological and pathophysiological importance of its proteolytic conversion remains unknown.

The fact that NR2B is readily cleaved by calpain in mature cultured neurons and NR2A remains whole most likely reflects the features of the postsynaptic MAGUK protein with which the subunits are associated. NR2B generally is associated with SAP-102, which does not protect NR2 subunits from degradation, whereas NR2A is associated with PSD-95, which blocks cleavage of NR2 subunits *(72,74,80)*. Alternatively, posttranslational modification of the C-terminus of NR2B may facilitate cleavage of NR2B.

Calpain-Mediated Regulation of Other Glutamate Receptors

The AMPA receptor mediates the majority of fast excitatory neurotransmission in the mammalian brain. These receptors are heterotetrameric ionotropic glutamate receptors composed of subunits designated GluR1–4. The subunits GluR1, GluR2, and GluR3 are likely to be targets of calpain *(36,38,81)*. As for NMDA receptors, the cleavage of the GluR1 subunit by calpain occurs in the C-terminal domain and truncates such subunits to an apparent molecular mass of about 98 kDa (compared with 105 kDa for the full-length species). In vitro, calpain-mediated proteolysis

of GluR1–3 appears to remove AMPA receptors from isolated postsynaptic densities (Table 1). In rat brain sections, calpain activation by freeze–thaw cycles decreases GluR1 immunoreactivity in dendritic fields and increases immunoreactivity in cell bodies, suggesting that calpain may not only degrade AMPA receptors but may also expose regions of the protein that are not generally accessible in postsynaptic densities. Brief exposure of organotypic hippocampal cultures to NMDA promotes the formation of lower molecular weight species of GluR1, which is consistent with cleavage by calpain. In cultured rat hippocampal slices, CX614, an AMPA receptor modulator that slows receptor desensitization and de-activation, can activate calpain through sustained AMPA receptor activation with subsequent increases in intracellular calcium, leading to a loss of total GluR1–3 proteins *(82)*. These data suggest that calpain activation can promote turnover of synaptic AMPA receptors.

Calpain-Mediated Regulation of L-Type Calcium Channels

L-Type calcium channels mediate longlasting calcium currents in response to membrane depolarization and play a key role in various neuronal functions such as membrane excitability, neurotransmitter release, protein phosphorylation, gene expression, and synaptic plasticity *(35)*. Brain L-type Ca^{2+} channels contain three different subunits, including α_1 subunits as their pore-forming subunit. α_{1C} subunits are clustered on neuronal cell bodies and proximal dendrites—a pattern that is typical for synaptic proteins—in both the hippocampus and cerebral cortex *(35)*. Calpain is capable of converting full-length α_{1C} subunits to a truncated form in vitro as well as in hippocampal neurons in culture *(83)*. This cleavage removes 250 to 300 amino acid residues from the C-terminus of the α_{1C} subunit, which may change the electrophysiological properties of voltage-dependent Ca^{2+} channels *(84,85)*. Removal of 300 to 470 amino acid residues from the C-terminus of α_{1C} subunits by treatment with proteases or by expression of complementary DNAs encoding truncated forms of α_{1C} subunits (modifications similar to those observed with calpain cleavage) enhances activation of Ca^{2+} channels in response to depolarization. Therefore, it is possible that calpain-mediated truncation of the α_{1C} subunits augments the activity of L-type channels in the postsynaptic membrane (Table 1).

Calpain-Mediated Regulation of the Na^+/Ca^{2+} Exchanger

Calpain can also modulate the major plasma membrane Ca^{2+} extruding system of neuronal membranes, the Na^+/Ca^{2+} exchanger (NCX; ref. *39*). NCX ejects Ca^{2+} across the plasma membrane and has both pre- and postsynaptic functions *(86)*. Among the three subtypes of NCXs, NCX1 and NCX3 are calpain substrates *(39)*. NCX3 is cleaved to a doublet of approx 58 to 60 kDa by calpain in both cultured neurons exposed to excitotoxic insults and ischemic rat brain. Calpain-mediated degradation of NCX1 has only been detected in ischemic rat brain. NCX2 is resistant to calpain-mediated proteolysis. Calpain-mediated cleavage of NCX inactivates its calcium extrusion activity, leading to a delayed increase in intracellular Ca^{2+} following excitotoxic insults (Table 1; ref. *39*).

Calpain-Mediated Regulation of the Inositol 1,4,5-Triphosphate Receptor/Ca^{2+} Channel

The inositol 1,4,5-triphosphate (IP3) receptor, an intracellular membrane protein that controls release of Ca^{2+} from intracellular stores, plays an important role in controlling amplitudes of excitatory postsynaptic currents in postsynaptic hippocampal CA1 neurons. This intracellular receptor also mediates accumulation of extracellular calcium through the process termed intracellular-calcium-release-activated uptake, which is believed to be mediated by transient receptor potential channels. Intracellular Ca^{2+} increases, either in response to opening plasma membrane channels or

Table 1
General Effect of Calpain on Individual Synaptic Proteins

Synaptic substrates	Attributed function(s)	Reference
Synaptic receptors		
NR2A,NR2B	Downregulation of NMDA receptor levels?	*41*
NR2C	Unclear	
GluR1, GluR2, GluR3	Removal of surface AMPA receptors	*36, 38, 81*
L-Type calcium channel	Activate	*83–85*
Na^+/Ca^{2+} exchanger	Inactivate	*39*
IP3 receptor/Ca^{2+} channel	Activate	*87, 88*
Synaptic architecture proteins		
αII-spectrin, MAP2	Changes in synaptic integrity and stability	*94, 96, 98, 100, 104, 106–110*
Postsynaptic density proteins		
PSD-95, SAP97, GRIP1	Change stabilization of synaptic receptors	*37, 72, 82*
Calmodulin-dependent proteins and related kinases		
CaM kinase IIα	Activate	*119*
nNOS	Inactivate	*119*
Calcineurin A	Activate	*40*
PKC	Activate	*135–139*
GAP-43	Prevent CaM binding	*144*
Neuron-specific activators of Cdk5		
P35 and P39	Deregulate Cdk5 activity	*145–149*

CaM kinase, calmodulin-dependent protein kinase; Cdk5, cyclin-dependent kinase 5; GAP-43, growth-associated protein 43; GluR, gamate receptor; GRIP, glutamate receptor-interacting protein; MAP2, microtubule-associated protein 2; NR, *N*-methyl-D-asparate receptor; NOS, neuronal nitric oxide syntheses; PKC, protein kinase C; PSD95, postsynaptic density protein 95; SAP97, synopse-associated protein 97.

release of calcium from intracellular stores, can induce calpain-mediated degradation of the IP3 receptor *(87,88)*. Calpain digests the IP3 receptor in vitro and in vivo into major stable fragments with approximate molecular masses of 130 and 95 kDa, both of which are derived from the C-terminus of the protein. This region contains the Ca^{2+} channel domain of the IP3 receptor. Because the N-terminal peptide domain of the IP3 receptor (which is released from the Ca^{2+} channel domain by cleavage with calpain) can gate the transient receptor potential channel in the absence of the IP3 receptor C-terminal channel domain *(89,90)*, the calpain-generated N-terminal fragment of IP3 receptor could activate endogenous store-operated Ca^{2+} channels directly and lead to secondary calcium entry by intracellular-calcium-release-activated uptake.

Regulation of Other Synaptic Proteins by Calpain (Table 1)

Modulation of Synaptic Architecture

Spectrin isoform αII, known as brain spectrin or α-fodrin, is the major structural component of the neuronal membrane cytoskeleton and is particularly abundant in axons and presynaptic terminals *(91–93)*. Spectrin is also the prototypical substrate for calpain *(1,94–96)*. Calpain cleaves αII-spectrin predominately at a single location between Tyr1176 and Gly1177, creating two products of nearly equal electrophoretic mobility (150 and 145 kDa); these are calpain-signature spectrin breakdown products *(94,96–98)*. Antibodies to these fragments are available and useful for immunological detection of calpain activation. Because

αII-spectrin is anchored to the plasma membrane by an ankyrin-like protein and binds to actin, calmodulin, and microtubules, cleavage of spectrin by calpain can alter the dynamic organization of membrane domains and membrane trafficking events (Table 1; ref. *93*). αII-spectrin is a calpain substrate in the hippocampus, cerebellum, and probably many other brain regions *(99–102)*. Proteolysis of αII-spectrin by overactivated calpain is an early event in ischemic injury in the rodent forebrain *(103)*.

The microtubule-associated protein-2 is associated with the cytoskeleton in the soma and dendrites of neurons *(104,105)*. As a crosslinker of the dendritic cytoskeleton, microtubule-associated protein-2 stabilizes intracellular scaffolding. It is highly sensitive to cleavage by calpain during excitotoxic conditions. Pharmacological stimulation of rat hippocampal neurons by systemic or intraventricular administration of kainate or NMDA induces μ-calpain-mediated loss of microtubule-associated protein-2 *(100)*. Calpain activation significantly decreases microtubule-associated protein-2 levels in rat models of focal cerebral ischemia and in spinal cord injury *(104,106–110)*. Proteolysis of microtubule-associated protein-2 by calpain is also observed in the cell culture model of oxygen-glucose deprivation and in a model of delayed ischemic neuronal death that uses the gerbil CA1 hippocampus *(111,112)*.

Cleavage of Structural Postsynaptic Density Proteins

Although PSD-95 can modulate the cleavage of other calpain substrates (such as NR2A) in neurons, PSD-95 is a substrate of calpain in vitro *(37,72)*. Incubation of rat forebrain synaptic membranes with purified μ-calpain degrades full-length PSD-95 and generates at least two relatively stable products with molecular masses of approx 50 and 36 kDa, respectively. Calpain-mediated cleavage of PSD-95 occurs in calcium-treated rat brain sections and in NMDA-treated hippocampal slices *(37)*. However, brief NMDA treatment can also lead to reductions of PSD-95 levels in some models through ubiquitination of PSD-95 and subsequent proteasomal degradation. Therefore, the role of calpain-mediated cleavage of PSD-95 in turnover of PSD-95 in vivo may be complex *(113,114)*.

Calpain also targets two other postsynaptic proteins: SAP-97 and glutamate receptor interacting protein (GRIP1; ref. *82*). Activation of the AMPA receptor by CX614 induces calpain-mediated degradation of both SAP-97 and GRIP1 in cultured rat hippocampal slices *(82)*. Because SAP-97 and GRIP1 anchor and stabilize AMPA receptors at the synapse *(115–118)*, degradation of these two postsynaptic proteins by calpain activation could lead to loss of stability of AMPA receptors in postsynaptic membranes, in conjunction with direct cleavage of AMPA receptors by calpain *(82)*.

Calpain-Mediated Modulation of Ca^{2+}/Calmodulin-Dependent Proteins and Related Kinases

Type IIα calmodulin-dependent protein kinase (CaMKIIα) and neuronal nitric oxide synthase (nNOS), two important calmodulin-dependent enzymes in neurons, are degraded by calpain both in vitro and in vivo *(119)*. CaMKIIα, which is abundant in neuronal postsynaptic membranes, phosphorylates various substrates, including NMDA receptors, AMPA receptors, and calcium channels *(120,121)*. Calpain cleaves the full-length CaMKIIα (54 kDa) to a catalytically active kinase fragment of 35 kDa in vitro *(119)*. This fragment possesses the entire catalytic domain but has lost its calmodulin-binding auto-inhibitory domains (refs. *122* and *123*; Figs. 2 and 3; Table 2). The same pattern of calpain-mediated breakdown of CaMKIIα has been detected in cerebrocortical cultures exposed to various neurotoxins, including calcium ionophores, maintotoxin, okadaic acid, and kainate, and in rat cerebral hemispheres that undergo NMDA-receptor-mediated excitotoxic events *(119)*.

nNOS catalyzes production of NO, a retrograde second messenger between neurons and

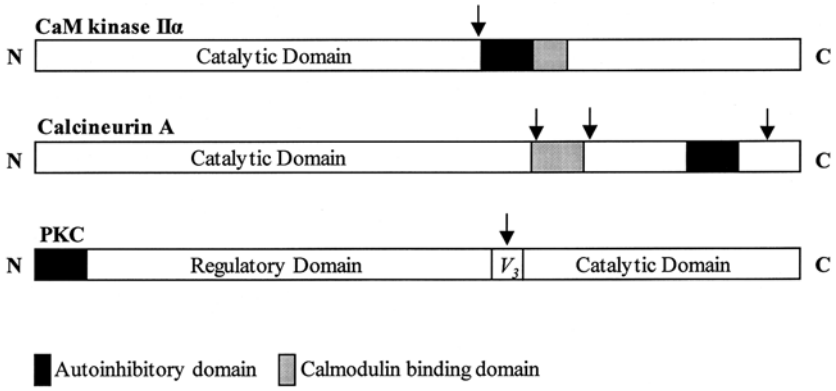

Fig. 2. Calpain-mediated cleavage regions in three Ca^{2+}-dependent synaptic enzymes. The arrows point to the site or areas of calpain-mediated proteolysis in three synaptic enzymes. Calpain removes auto-inhibitory domains from the catalytic domains.

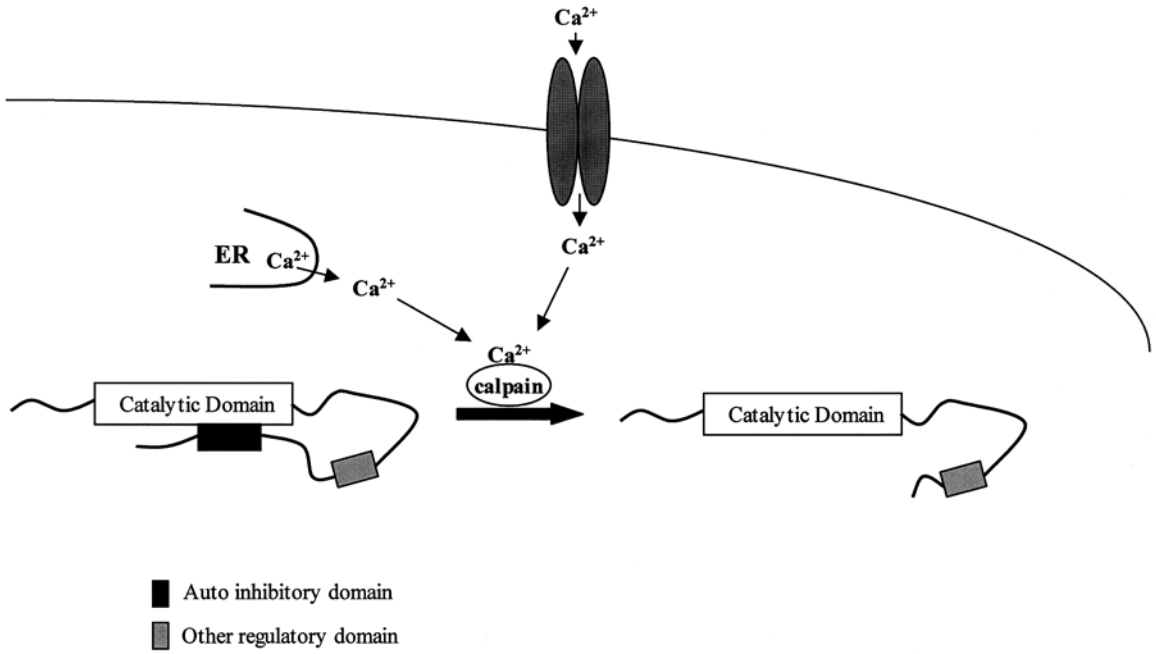

Fig. 3. Schematic representation of calpain-dependent activation of three synaptic Ca^{2+}-dependent enzymes: CaM kinase II, calcineurin A, and PKC. In the basal inactive state, the auto-inhibitory domains of all enzymes interact with their catalytic domains to block substrate (ATP) or regulator (calmodulin, Ca^{2+}, diacylglycerol) binding sites. Calpain-mediated cleavage releases the auto-inhibitory domains from catalytic active sites and irreversibly activates these enzymes.

a mediator of glutamate-generated excitotoxicity *(124–126)*. Calpain creates three fragments from nNOS in vitro that have molecular masses of approx 140, 130, and 65 kDa, respectively *(119)*. This fragmentation pattern of nNOS is also found under toxic conditions in neurotoxin-challenged cultures and NMDA-treated rat cerebral hemispheres *(119)*. Con-

Table 2
Some Known Calpain Cleavage Position(s) and Major Product(s)

Synaptic protein	Cleavage position(s)	Fragment(s) (kDa)	Reference
NR2A	Phe1279; Ser1330	140; 130	*33*
NR2B	C-terminal	140; 130; 115–120	*33*
NR2C	C-terminal	110; 100	*33*
GluR1	Asn833; Arg837	98	*36*
L-Type calcium channel	C-terminal (removal of last 250–300 amino acids)	Unclear	*83–85*
NCX3	Lys370; Asn504 Arg510; Val512	58–60	*39*
IP3 receptor	Unclear	130; 95	*87, 88*
αII-spectrin	Gly1230; Tyr1176	145; 150	*94, 96, 98*
CaM kinase II	Unclear	35	*119*
nNOS	Unclear	140; 130; 65	*119*
Calcineurin A	Arg392; Lys424 Lys501	45; 48; 57	*40*
PKC	Variable region (V_3)	36; 45–49	*137*
P35	Phe98	25	*150*
P39	Gly115	29	*152*

versely to cleavage of CaMKIIα, calpain-mediated cleavage inactivates nNOS. The rapid proteolytic degradation of nNOS may provide a mechanism for control of the level of NO biosynthesis through nNOS catabolism *(127–130)*.

Calcineurin A, the only known Ca^{2+}/calmodulin-dependent protein phosphatase in the brain, is critical in modulating NMDA receptor activity and synaptic transmission *(131,132)*. Although calcineurin is usually a calmodulin-activated phosphatase, under conditions such as overstimulation of NMDA receptor, calcineurin A can become constitutively active by calpain-mediated proteolysis (ref. *40*; Fig. 3). Glutamate and kainate-generated activation of calpain results in proteolysis of calcineurin A (60 kDa) at three sites, generating fragments with molecular masses of 45, 48, and 57 kDa, respectively (Fig. 2; Table 2). Calcineurin A has a calmodulin-binding domain and an auto-inhibitory domain in the C-terminal region. The calpain-generated 45- and 48-kDa fragments of calcineurin A result from cleavage at and after the C-terminal calmodulin-binding domain, respectively. Both products lack the auto-inhibitory domain, and the 45-kDa fragment also lacks the calcium/calmodulin regulation site. These truncated forms are active because they can initiate calcineurin-mediated-nuclear factor of activated T-cells gene transcription. In hippocampal cultures, calpain-mediated truncation of calcineurin A secondarily increases caspase activation, leading to neuronal cell death. Proteolysis of calcineurin A by calpain has also been detected in the hippocampus of mice treated with kainate *(40)*. The calpain-mediated 57-kDa fragment of calcineurin A, truncated at lysine 501 within the C-terminal auto-inhibitory domain, has been found in brains from individuals with Alzheimer's disease *(133)*. This particular truncation results in a twofold increase in calcineurin phosphatase activity in such brains. Therefore, calpain-mediated activation of calcineurin A may contribute to neurodegeneration or synaptic dysregulation in Alzheimer's disease.

Calpain also acts on regulatory proteins not associated with calmodulin. As noted for calcineurin and CaMKIIα, protein kinase C (PKC) is cleaved by calpain in vitro to produce a catalytically active fragment (Fig. 3; refs. *134–137*).

Calpain converts each of the three 82-kDa isoforms of PKC (α, β, and γ) to two major fragments: a 45- to 49-kDa catalytic fragment and a 36-kDa regulatory fragment (Fig. 2; Table 2). Activation of PKC usually requires binding of 1,2-diacylglycerol and calcium. All subspecies of PKC have a common structure containing four conserved (C_1–C_4) and five variable (V_1–V_5) regions *(138)*. The calpain-mediated cleavage sites in PKC are located in the third variable region (V_3) of each PKC isoform, a region that connects the regulatory and protein kinase domains. Similarly to calcineurin A, calpain-mediated proteolysis of PKC provides an alternative mechanism for activation of PKC by removing the inhibitory regulatory domain and, therefore, the need for coupling to 1, 2-diacylglycerol generation and IP3-receptor-mediated intracellular Ca^{2+} release (refs. *135–137*; Fig. 3). The removal of these domains by proteolysis has been specifically implicated in the maintenance phase of some forms of long-term potentiation (LTP; refs. *139* and *140*).

Calpain also modulates proteins on the presynaptic side of synapses. Growth-associated protein (GAP)-43 (neuromodulin), a presynaptic protein implicated in neurotransmitter release and signal transduction *(141–144)*, is cleaved by calpain in two sites: after the 5th and after the 41st amino acid residues *(144)*. When the cleavage occurs after Ser-41, proteolysis of GAP-43 removes calmodulin binding to GAP-43 and Ser-41 phosphorylation. Therefore, proteolysis leads to retention of GAP-43-mediated enzymatic properties but separation from the calmodulin-binding regulatory domain.

Calpain-Mediated Cleavage of the Neuron-Specific Activator of Cyclin-Dependent Kinase 5

Cyclin-dependent kinase 5 (Cdk5), a kinase active predominantly in postmitotic neurons, is involved in neuronal migration during development, neurite outgrowth, synaptic plasticity, postsynaptic signaling of some neurotransmitters, the regulation of the cytoskeleton, and vesicle trafficking *(145–149)*. This kinase is activated by binding of a series of activator proteins. Activated calpain cleaves the Cdk5 activators p35 and p39 in their N-terminal domains, generating C-terminal-truncated products termed p25 and p29. Production of p25 and p29 causes prolonged activation and aberrant localization of Cdk5. Calpain-mediated proteolysis of p25 in the nervous system also has been described in ischemic brains, in patients with Alzheimer's disease, and in a mouse model of familial amyotrophic lateral sclerosis *(42,150–152)*.

Calpain Activation in Synaptic Transmission and Plasticity

Is there any evidence for a direct role of calpain in synaptic modification? Activity-dependent changes in synaptic transmission arise from a large number of mechanisms known collectively as synaptic plasticity, including LTP and long-term depression. Based on pharmacological inhibitors and bioactive reagents, calpain is activated during synaptic transmission, and activation may be required for LTP formation in some situations *(45,153–161)*. Inhibition of calpain activity by leupeptin, a relatively selective calpain inhibitor, reduces the degree of LTP that is induced in the CA1 field of hippocampal slices *(45,155,156)*. Extracellular application of *N*-acetyl-Leu-Leu-norleucinal and *N*-acetyl-Leu-Leu-methioninal, two highly potent but incompletely selective synthetic calpain inhibitors, blocks LTP in both the Schaffer collateral-CA1 synaptic zone of the rat hippocampal slice and the perforant path-stimulated dentate granule cells in the intact hippocampus *(157)*. Reduction of μ-calpain expression by transfection of antisense oligonucleotides in cultured hippocampal slices decreases the incidence and magnitude of LTP that is induced by a Θ-burst stimulation paradigm without affecting baseline synaptic responses *(159)*. Collectively, the evidence from several different systems suggests that calpain activation is involved in at least some forms of LTP generation and, therefore, is essential for at least some types of synaptic

transmission and neuroplasticity. However, the direct contribution of calpain activation to spatial learning and memory has not yet been studied in animal models. Additionally, the exact substrates of calpain (from the many possible substrates mentioned earlier) involved in LTP have not been fully defined.

Calpain in Synaptic Function: What Is Its Overall Role?

Similarly to the proteins noted earlier, calpain clearly degrades or modifies a large number of synaptic proteins, but the conceptual role of calpain in synaptic events is not entirely clear. Based on the diversity of its synaptic substrates, calpain could modulate synaptic environments under both physiological and pathological conditions. Calpain regulates a diverse group of proteins (including Ca^{2+} channels, pumps, Ca^{2+}-effector enzymes, and scaffold proteins) that couple changes in cytosolic Ca^{2+} with a wide variety of physiological responses *(36,38,81,162)*. Calcium flowing through the NMDA receptor channel, the principal receptor that controls activity-dependent plasticity, activates sensor proteins inside the PSD. Calpain and three Ca^{2+}-dependent enzymes (CaMKIIα, PKC, and calcineurin A) are activated immediately after opening of the NMDA receptor channel. Consequently, activated calpain would control NMDA receptor function and Ca^{2+}-dependent biochemical pathways mediated by all four enzymes at the synapse. The scaffold proteins PSD-95, microtubule-associated protein-2, SAP-97, and GRIP1 are all anchored to NMDA receptors and AMPA receptors at the cytosolic face of the postsynaptic membrane and contribute to the construction of an accurate spatial environment for synaptic signaling. Together with the regulation of synaptic Ca^{2+} channels and Ca^{2+}-dependent specific signaling enzymes, proteolysis of synaptic scaffolding proteins might position all Ca^{2+} signaling sensors in a particular spatial order, thereby holding receptors near their appropriate effectors and protein kinases or phosphatases near their appropriate targets. Such events could determine the precise kinetics of individual cascades, thus modifying synaptic strength. Although calpain-mediated changes are functionally diverse, it appears that they are coordinated in the number of receptors in the postsynaptic membrane, the activity of synaptic enzymes, and the size of PSD. It is important to understand how the extensive and divergent biochemical processes that control synaptic strength are orchestrated and the role that calpain-mediated proteolysis plays in facilitating changes in synaptic function.

Before such events can be understood, the exact events controlling calpain activation at the synapse must be identified. Although calpain activation clearly occurs during synaptic transmission, most paradigms examining specific substrates of calpain use paradigms that also contain an excitotoxic component (*154*). Additionally, although calcium is required for calpain activation, the specific sources of the increase in intracellular calcium required for calpain activation are not well defined. Activation of the NMDA receptor is preferentially associated with calpain activation—particularly activation of NR2A-containing receptors *(73)*. However, other conditions in which calcium is mobilized via AMPA (in association with blockade of desensitization or de-activation) and IP3 receptors may activate calpain *(82,87)*. Indeed, the association of calpain activation with NMDA receptors may reflect the relatively prolonged intracellular calcium response noted with activation of the NMDA receptor. Because assays of calpain activity are usually biochemical, the association with NMDA receptor activation could represent a feature of the assay limitations rather than a physiological requirement.

Calpain Activity in Ca^{2+}-Mediated Neurological Disorders

Calpain has long been implicated in a wide range of pathological states, including stroke, epilepsy, traumatic nerve injury, neurodegenerative disorders, and aging. Unregulated calpain activity created by dysregulation of

neuronal calcium homeostasis is a potentially unifying mechanism for the significance of calpain in many paradigms *(53,163–166)*.

However, in different disorders, calpain may have more specific targets. Dysfunction of Cdk5 by calpain cleavage of its activators may be involved in the biochemical mechanisms of Alzheimer's disease. Cdk5 closely interacts with early neurofibrillary tangles, a pathological hallmark of Alzheimer's disease. These structures are composed mainly of hyperphosphorylated and aggregated τ, another well-characterized calpain substrate *(42,167–170)*. The cleavage of the activators of Cdk5 facilitates τ phosphorylation, potentially promoting aggregation of these structures. In Huntington's disease, calpain-generated N-terminal fragments of huntingtin undergo nuclear translocation, potentially leading to various aberrant regulatory events *(54,171–173)*. In Parkinson's disease, α-synuclein, the major protein of Lewy bodies, is cleaved by calpain in vitro and *in situ (174–176)*, but the significance of this event is unclear. Calpain activation also mediates the loss of nigral dopamine neurons in the *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of Parkinson's disease, but the exact substrates of calpain in this model also are unknown *(55)*.

In most acute neuronal disorders, NMDA-receptor-mediated excitotoxicity has been proposed to be a common pathological manifestation *(40,52,65,177,178)*. As a downstream effector of NMDA receptor activation, calpain is activated rapidly, and cleavage of intracellular proteins by calpain is believed to be one of the destructive forces within the cell *(7,40,42, 166,178,179)*. Pharmacological inhibition of calpain reduces proteolysis and enhances neuronal survival in many, although not all, models of excitotoxicity *(40,180–186)*.

Despite a wide consensus that overactivation of calpain is a causative mechanism in neurodegeneration, activation of calpain may also be a component of neuroprotection or recovery from injury in other situations. Activation of calpain is necessary for recovery from dendritic injury induced by glutamate receptor activation, ionomycin stimulation, or axotomy *(56–59)*. In cultured *Aplysia* neurons, dendritic injury primes localized calpain activity, which is required for restoration of dendritic structure. Calpain may also facilitate turnover of NMDA receptors, potentially allowing calpain to have a neuroprotective effect in NMDA-mediated excitotoxicity *(34,41,73)*. Consequently, understanding the substrates of calpain in specific situations and the alterations in bioactivity of these substrates is necessary to understand the overall role of calpain in different pathophysiological scenarios.

Conclusion

Modification of synaptic function frequently depends on an initial Ca^{2+} signal. As a Ca^{2+}-activated enzyme, calpain may modulate the function of a variety of its substrates in synapses and translate the Ca^{2+} signaling into enduring changes. These posttranslational modifications by calpain may be a fundamental mechanism for modulating neuronal connectivity and synaptic function, although definitive evidence for such a mechanism is incomplete (Fig. 4). In response to Ca^{2+} signaling, calpain proteolyzes cytoskeletal proteins and PSD proteins, thus changing synaptic stability and organization that is essential for receptor association and function at synapse. On the other hand, calpain-mediated cleavage of plasma membrane and ER receptors can directly alter modulators of intracellular Ca^{2+} concentrations and, therefore, differentially regulate the activities of intracellular mediators that control synaptic physiology. Finally, by virtue of its direct interaction with cytosolic and nuclear mediators, calpain modulates the activation of downstream molecules, thereby changing synaptic function. Particular areas that require further investigation are the specific roles of calpain-mediated cleavage of individual proteins in distinct synaptic compartments and the exact pre- and postsynaptic changes that are mediated by calpain-generated posttranslational mechanisms. Determin-

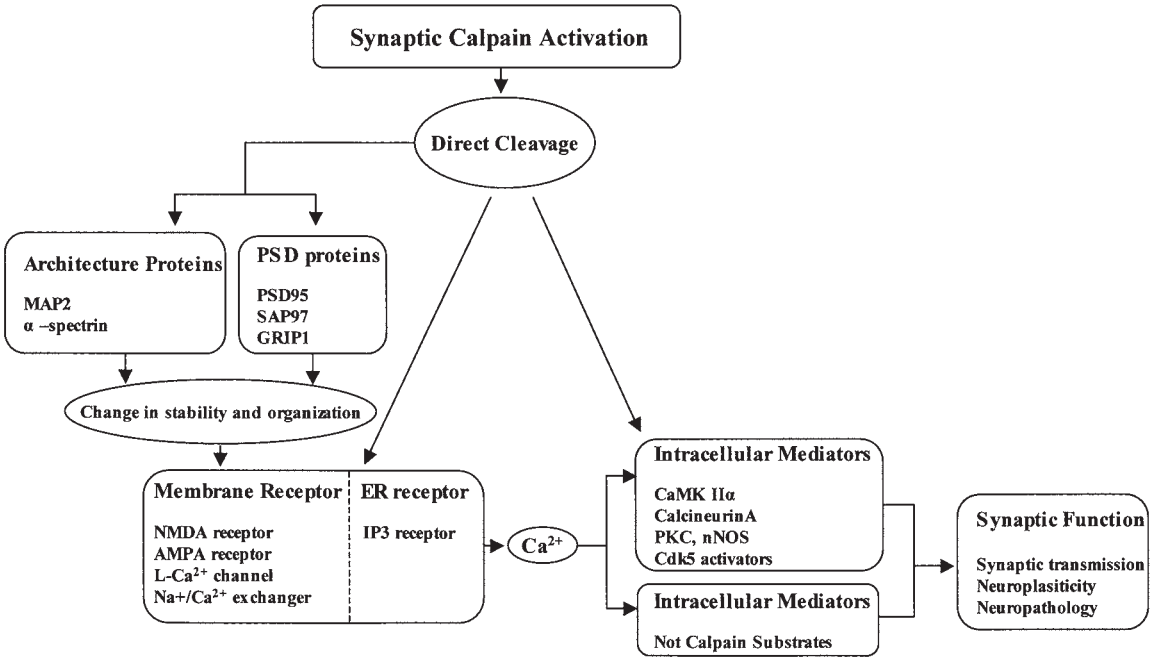

Fig. 4. Overview of the effects of calpain on synaptic function. Calpain activation proteolyses architecture and PSD proteins, leading to change in synaptic stability and organization. Calpain-mediated cleavage of plasma membrane and ER receptor directly alters intracellular Ca^{2+} levels and regulates the activities of intracellular mediators. Calpain directly alters cytosolic and nuclear mediators inducing these molecules to become active or inactive. Therefore, calpain targets substrates at different levels and could play a basic regulatory role in synaptic function.

ing the precise roles of calpain-mediated modification in synapses is important for understanding both synaptic physiology and progressive synaptic neuronal pathologies.

Acknowledgment

This work was supported by NS45986 to D. R. Lynch, the Mental Retardation Research Center of the Children's Hospital of Philadelphia (P30-26979), and donations to the Trisomy Program of the Children's Hospital of Philadelphia. We would like to thank Elisa Waxman and Isabelle Baconguis for reading the manuscript.

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