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Regulation of Calpain-2 in Neurons: Implications for Synaptic Plasticity

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Abstract The family of calcium-dependent neutral proteases, calpains, was discovered more than 30 years ago, but their functional roles in the nervous system under physiological or pathological conditions still remain unclear. Although calpain was proposed to participate in synaptic plasticity and in learning and memory in the early 1980s, the precise mechanism regarding its activation, its target(s) and the functional consequences of its activation have remained controversial. A major issue has been the identification of roles of the two major calpain isoforms present in the brain, calpain-1 and calpain-2, and the calcium requirement for their activation, which exceeds levels that could be reached intracellularly under conditions leading to changes in synaptic efficacy. In this review, we discussed the features of calpains that make them ideally suited to link certain patterns of presynaptic activity to the structural modifications of dendritic spines that could underlie synaptic plasticity and learning and memory. We then summarize recent findings that provide critical answers to the various questions raised by the initial hypothesis, and that further support the idea that, in brain, calpain-2 plays critical roles in developmental and adult synaptic plasticity.

Keywords Calpain · Hippocampus · LTP · Estrogen · ERK · Growth cone · Plasticity · BDNF

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Calpain and Synaptic Plasticity: Problems and Questions

General Features of Brain Calpains

The role(s) of the intracellular proteases, calpains, in synaptic plasticity still remains a major question in neuroscience. Since its discovery about 30 years ago, numerous studies have been directed at understanding the role of this family of proteases in synaptic plasticity and in learning and memory. In 1984, Lynch and Baudry proposed a hypothesis postulating that calpain played a critical role in initiating the restructuring of dendritic spines following the induction of long-term potentiation (LTP) at glutamatergic synapses in CA1 field of the hippocampus [1]. In addition, calpain activation was proposed to participate in the increase in the number of synaptic glutamate receptors postulated to be responsible for the enhanced synaptic transmission that underlined potentiation. While this hypothesis has remained debated and only partially demonstrated, recent findings regarding calpain-2 regulation have identified a novel mechanism for calpain-2 activation in the central nervous system (CNS), thereby providing strong support for the critical role of calpain-2 in synaptic plasticity (see below).

Calpains constitute a family of cellular cysteine proteases that are activated by calcium in the cell cytosol at neutral pH [2–5]. As proteases, calpains are regulators of cellular processes by partially truncating their substrates, thereby producing post-translational protein modifications generally associated with modified functions [6, 7]. While the family of calpains has grown to 14 members since its discovery, two isoforms, calpain-1 and calpain-2 (also known as μ-calpain and m-calpain, respectively), are particularly abundant in the CNS [8]. In genetically engineered mice, knock-out of calpain-2 or calpain-4 (the small subunit common to calpain-1 and calpain-2) is embryonic lethal, while knock-out of calpain-1 is not, confirming the key roles of calpain-2 in cellular functions required for normal embryonic development [9]. Calpain-1 and calpain-2 isoforms as well as their endogenous inhibitor, calpastatin, are expressed in both neurons and glia, and, within neurons, are found in the soma, axons, synaptic terminals, and dendritic spines [10–14]. Recently, there has been some suggestion that calpain-1 is localized in mitochondria and that various isoforms of calpastatin are also targeted to various subcellular compartments [15–17]. Calpains are typically activated by elevation of intracellular calcium, either in response to activation of plasma membrane receptors and channels or by release of calcium from intracellular stores. Despite their structural similarities, calpain-1 and calpain-2 differ in the calcium concentration required for their activation. Calpain-1 is activated in the presence of micromolar (1–20 μM) calcium concentrations, while calpain-2 requires millimolar (0.250–0.750 mM) calcium concentrations [8, 18]. As intracellular calcium concentrations range from approximately 50–300 nM under most physiological conditions, the calcium requirement for calpain-2 activation has raised questions regarding the conditions under which calpain-2 could be activated [19].

A number of calpain substrates have been identified in neurons, including several subunits of α-amino-3-hydroxy-5-*methyl*-4-isoxazolepropionic acid (AMPA) and *N-methyl* D-aspartate (NMDA) receptors, cytoskeletal proteins such as spectrin and proteins involved in regulation of actin polymerization such as focal adhesion kinase (FAK), cortactin, and 14.3.3ς, postsynaptic density proteins such as postsynaptic density (PSD)-95 and glutamate receptor-interacting protein (GRIP), as well as enzymes, such as calmodulin-dependent protein kinase II (CaMKII) and PKC; most of these substrates are critical for synaptic plasticity, cytoskeletal organization, and synaptic function [20–25]. Several studies based on pharmacological inhibition of calpains have suggested a crucial role for calpains in many neuronal processes, such as excitability, neurotransmitter release, synaptic plasticity, signal transduction, vesicular trafficking, structural stabilization, and gene transcription [5, 26]. This diverse variety of calpain substrates has in fact made the task of determining the exact physiological role of calpains in synaptic transmission and plasticity difficult. As of now, there is no calpain-specific inhibitor, with the exception of calpastatin, the only known endogenous inhibitor. The use of calpastatin knock-out or overexpressing mutant mice has provided some insight regarding the roles of calpains in various physiological or pathological conditions [27, 28]. Furthermore, calpain-1 and calpain-2 also appear to share the same substrates, thus making it difficult to specify the respective roles of these two proteases in neuronal function.

Roles of Calpain in Synaptic Plasticity

As previously mentioned, calpains, by cleaving various proteins involved in the regulation of the cytoskeleton, play a critical role in the modification of synaptic structures, which has been proposed to underlie long-lasting changes in synaptic transmission and to form the structural substrate of learning and memory [29]. One of the first identified substrates of calpain was spectrin, a major structural component of the neuronal membrane cytoskeleton [30, 31]. Spectrin is a dimer consisting of α and ß-spectrin, with the former, α-spectrin (aka αII-spectrin), being a prototypical calpain substrate. Calpain cleaves α-spectrin at a single location between tyrosine1176 and glycine1177, creating two breakdown products with molecular weights of 150 and 145 kDa [32, 33]. These products are known as spectrin breakdown products (SBDP) and are widely used for both quantitative and spatial analysis of neuronal calpain activation [4, 34, 35]. Interestingly, tyrosine1176 phosphorylation protects spectrin from calpain-mediated truncation, a feature that is often found amongst calpain substrates [36, 37]. However, this is not a general rule, as for other substrates, such as cortactin, phosphorylation is actually required for calpain-mediated truncation [38].

The identification of spectrin as a calpain substrate was one of the initial elements used to propose that calpain activity was critical for LTP induction. LTP is accompanied by functional and morphological changes in synapses and specifically in dendritic spines and PSDs [39]. These LTP-associated cellular structural changes are thought to be calpain-dependent, as pharmacological studies have revealed that calpain inhibition by inhibitors or down-regulation with siRNA prevents LTP induction [40–42]. Conversely, rats or mice deficient in calpastatin exhibit enhanced LTP ([28, 43], and Saido, personal communication). Furthermore, both theta-burst stimulation and NMDA receptor activation resulted in calpain activation, as evidenced by the accumulation of selective spectrin breakdown products generated by calpain-mediated proteolysis [44, 45]. Beyond evidence for calpain proteolysis of

cytoskeletal proteins, our studies over several years have established that calpain directly regulates the structure of glutamate receptors by truncating the C-terminal domains of several polypeptidic chains constituting the receptors. In particular, calpain directly modifies the properties of AMPA receptors, including the GluR1 subunit [46–49] essential for LTP expression [20]. Interestingly, PSD-95 and GRIP are also calpain substrates, raising the possibility that truncation of the C-terminal domains of the glutamate receptor subunits, and of AMPA receptor-associated proteins, is involved in membrane targeting/anchoring of the receptors [21, 22]. Beyond this, we also found that tyrosine kinase-dependent phosphorylation mediated by the protoncogene fyn, significantly protected AMPA receptor GluR1 subunits from calpain-mediated truncation [50]. Thus, tyrosine phosphorylation is an important factor controlling AMPA receptor reorganization during synaptic plasticity by regulating calpain-mediated modifications of the interactions between AMPA receptor subunits and targeting/anchoring proteins.

More recently, mice lacking calpain-4 by conditional knock-out were found to exhibit impairment in LTP induction in field CA1 of the hippocampal slices [51], strongly supporting a critical role for calpain activation in LTP induction, although this study did not indicate which calpain isoform (calpain-1 or calpain-2) was implicated. The 1984 hypothesis strongly suggested that the synaptic remodeling that accompanied and possibly underlined LTP directly involved calpain-mediated proteolysis of spectrin. In many cell types, spectrin participates in the regulation of cytoskeletal structures by providing a meshwork of spectrin molecules associated with actin filaments and numerous proteins involved in their regulation and in anchoring microtubules [6, 7]. It has since been shown that spectrin, in conjunction with Beatty's protein, 4.1, a critical protein involved in cytoskeletal organization, interacts with several PSD proteins in postsynaptic structures, especially with AMPA and NMDA receptors [52]. Calpain-mediated spectrin truncation would therefore be expected to disrupt dendritic spine cytoskeleton and PSD organization, and possibly facilitate the insertion of AMPA receptors in the postsynaptic membrane to produce the potentiation of synaptic transmission. As discussed in the previous section, calpain has also been shown to truncate a number of glutamate receptor anchoring molecules, such as PSD-95 and GRIP [21, 22].

More recently, it has been proposed that LTP stabilization and memory consolidation may depend on the activation of the mitogen-activating protein kinase (MAPK) signaling pathway [53, 54]. The suprachiasmatic nucleus circadian oscillatory protein (SCOP) binds to the GTPase, K-Ras, and acts as a negative regulator of MAPK. Interestingly, SCOP is also a calpain substrate, as treatment of hippocampal neurons with a calpain inhibitor resulted in increased SCOP levels and decreased MAPK activity. Furthermore, in vivo studies showed that training for novel object recognition (novel object recognition behavioral assay) resulted in SCOP degradation, while overexpression of SCOP in hippocampus blocked memory for novel objects [55].

The Calpain Activation Paradox: Calpain-2 Calcium Requirement

Studies examining the specific roles of calpain-1 and calpain-2 in synaptic plasticity have been challenging. Eliminating both calpain-1 and calpain-2 activity by interfering with the expression of the small subunit, calpain-4 was embryonically lethal, while knocking out calpain-1 (*Capn1*$^{-/-}$) resulted in platelet dysfunction but otherwise normal mice survival. We examined the learning behavior in the calpain-1 knock-out mice (*Capn1*$^{-/-}$) and found no deficits in learning and memory and no deficit in LTP induction and magnitude [56]. While these results would indicate that calpain-1 is not likely involved in LTP and synaptic plasticity, yet the complexity of transgenic models still raises the question of whether other calpains could possibly compensate for the absence of calpain-1 activity during development and throughout the adult life of the animal. Evidently, the presence of calpain-2, which was normal in the *Capn1*$^{-/-}$ mice, might also have compensated for the absence of calpain-1 in these knock-out animals. In sum, the transgenic mice studies have only raised additional questions concerning the physiological function(s) of calpains. In particular, these results do not eliminate the possibility that calpain-1 plays some important role(s) in synaptic function as well as under pathological conditions. In this regard, recent results using viral-mediated down-regulation of calpain-1 or calpain-2 in adult rats have indicated that calpain-1 activation plays a critical role in ischemia-induced neurodegeneration in field CA1 of hippocampus [57].

The high concentration of calcium required for calpain-2 activation also raises questions regarding its participation in normal physiological functions. The Ca^{2+} concentrations required for calpain-2 proteolytic activity are much higher than the 50–300 nM Ca^{2+} concentrations that normally exist in living cells. Only the Ca^{2+} concentrations required by calpain-1 are in the physiological range. Phospholipids, such as phosphatidylinositol, were found to lower the Ca^{2+} concentration required for autolysis of calpain-1 [58]. Subsequent studies have shown that several phospholipids, such as phosphatidylinositol 4,5-bisphosphate, lower the Ca^{2+} concentration required for autolysis of either calpain-1 or calpain-2 by three- to sixfold [59, 60]. Yet, even in the presence of phospholipids, the Ca^{2+} concentrations required

for autolysis of calpain-2 and therefore activation are far above those found in living cells. Hence, calpain-1 would autolyze only in the presence of Ca^{2+} "spikes" of 1–10 μM that might occur in localized areas. It has been suggested that calpains interact with phospholipids at the plasma membrane (or other cellular membranes), perhaps in the area of a Ca^{2+} channel, and that this interaction would reduce the Ca^{2+} requirement for autolysis. Therefore, there are still a number of uncertainties as to whether phospholipid-facilitated autolysis could be the possible "activation" mechanism sought after for calpains, although as indicated earlier, autolysis and interaction with phospholipids likely have some as yet unknown roles in calpain function [61]. Clearly, more remains to be learned about how calpains function in different cells and about the signals that trigger calpain activation.

Novel Findings for Regulation of Brain Calpain

Assays for Calpain Activation

As previously described, calpain activation has traditionally been monitored by measuring the levels of SBDP. Recently, a more sensitive assay utilizing the fluorescence resonance energy transfer (FRET) kinetics of a synthetic cell permeable calpain substrate has provided both a reliable and quantitative measure of calpain activation and allowed to study spatio-temporal features of calpain activation in neurons in response to a wide range of stimuli [62].

ERK-Mediated Calpain-2 Activation in BDNF and EGF Signaling and in LTP

Recent studies in fibroblasts have revealed that calpain-2 can be activated independently of calcium; in particular, epidermal growth factor (EGF) activated calpain-2 by phosphorylation catalyzed by MAPK [63]. Utilizing both the SBDP assay and the FRET-based assay to monitor calpain activation in vitro, we have recently found that both EGF and brain-derived neurotrophic factor (BDNF), a critical modulator in synaptic plasticity, activated calpain-2 in neuronal cultures through extracellular regulated kinase (ERK)-mediated phosphorylation [64]. Immunoprecipitation experiments indicated that, after BDNF treatment of cultured neurons, calpain-2 but not calpain-1 was phosphorylated at a serine residue, an effect that was completely eliminated by an ERK inhibitor. Interestingly, EGF-mediated calpain activation was much faster than BDNF-mediated calpain activation. As it had previously been shown in fibroblasts that PKA-mediated calpain phosphorylation could inhibit calpain-2 activation [65], we postulated that the difference in time-course of activation between EGF and BDNF could be due to BDNF-mediated stimulation of PKA. In support of this hypothesis, we found that in the presence of a PKA inhibitor, the time-courses for calpain activation by EGF and BDNF were identical [63].

It is noteworthy that BDNF and EGF-mediated calpain activation was present not only in dendrites but also in dendritic spine-like structures in cultured hippocampal neurons. Taking into consideration the role of BDNF in LTP induction and its recently uncovered roles in regulation of the neuronal actin cytoskeleton [66, 67], these results implicate BDNF and calpain in facilitating cytoskeletal changes at the submembrane level. These results therefore suggest the possibility that BDNF, by stimulating the ERK pathway, produces calpain activation, which would further stimulate MAP kinase activity, possibly by degrading SCOP, and participate in dendritic spine reorganization that underlies stabilization of synaptic modifications involved in LTP consolidation and synaptic plasticity. Furthermore, we found that BDNF-mediated actin polymerization depended on calpain activation [63]. These results suggest that calpain-2 is rapidly activated in dendrites and dendritic spines by BDNF, as a result of ERK-mediated phosphorylation, and participates in cytoskeletal reorganization. These findings provide a new link between calpain-2 and synaptic plasticity but also shed new light on the paradox that calpain-2, although widely expressed in CNS, requires unphysiological calcium levels for activation. In addition, they reveal the existence of a critical link between several elements long proposed to participate in synaptic plasticity, learning and memory, and strengthen the hypothesis that calpain-2 plays a critical role in activity-dependent modifications of synaptic efficacy (Fig. 1).

ERK-Mediated Calpain Activation Also Participates in Estrogen-Induced Synaptic Plasticity

Estrogen, 17-ß-estradiol (E2), a steroid hormone, has also been shown to have pleiotropic effects in the nervous system [68]. In particular, E2 facilitates synaptic plasticity in part by enhancing NMDA receptor-mediated synaptic responses and by increasing spine density in hippocampus, which leads to increased LTP and facilitation of learning and memory [69, 70]. E2 has also been shown to activate ERK and stimulate actin polymerization in dendritic spines [71]. We recently showed that E2 rapidly activated calpain in cultured cortical and hippocampal neurons, especially in dendrites and dendritic spines [72]. E2-induced calpain activation was mediated through ERK, as it was completely blocked by MEK inhibitors. Activation of ERα and ERß receptors by specific agonists also stimulated calpain activity, in agreement with findings that both types of receptors are linked with ERK activation. E2 has been repeatedly shown to increase excitability in acute hippo-

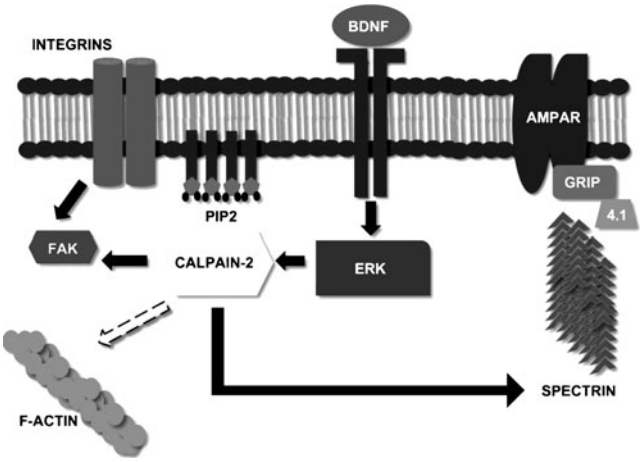

Fig. 1 Schematic representation of the links between BDNF, calpain, and synaptic modifications underlying long-term potentiation. Patterns of electrical activity producing LTP trigger the release of BDNF, resulting in activation of TrkB tyrosine kinase, followed by stimulation of ERK. ERK phosphorylates calpain-2, facilitating its activation possibly through binding to membrane-associated PIP2. Activated calpain can produce various modifications of synaptic structure and function by several mechanisms: (1) by truncating FAK, calpain can modify adhesion properties of dendritic spines, possibly influencing presynaptic terminals; (2) by regulating elements of the actin cytoskeleton, calpain can participate in the enlargement of dendritic spines; (3) by truncating spectrin and disrupting the receptor accumulation machinery, calpain can facilitate the insertion of AMPA receptors in postsynaptic densities

campal slices, and in particular to increase AMPA receptor-mediated synaptic responses, possibly through increased insertion of AMPA receptors in postsynaptic membranes [69, 71, 73]. We also found that E2-mediated increase in synaptic responses was prevented by calpain inhibition [71]. Moreover, E2 treatment of acute hippocampal slices resulted in increased actin polymerization and membrane levels of GluR1 but not GluR2/3 subunits of AMPA receptors; both effects were also blocked by calpain inhibition [71]. Thus, E2, by stimulating the ERK pathway, rapidly stimulates calpain-2 through phosphorylation, resulting in increased membrane levels of AMPA receptors and increased actin polymerization in dendritic spines. These effects could therefore be responsible for E2-mediated increase in neuronal excitability and facilitation of cognitive processes. Interestingly then, E2 like BDNF activates the same pathway that, following calpain activation, leads to facilitation of synaptic plasticity and of learning and memory; E2 and BDNF might thus belong to a family of synaptic modulators that play an important role in the regulation of synaptic plasticity [74]. It remained to be determined whether E2 acts in a direct manner or through the release of BDNF to modulate synaptic plasticity, since we have also shown that the neuroprotective effects of E2 against excitotoxicity are mediated by BDNF release [75]. As these results provided some resolution to the calcium activation paradox by indicating that ERK-mediated phosphorylation could lead to calpain-2 activation by various neuromodulators, they also suggested that calpain-2 could participate in a wider range of physiological functions in the nervous system.

Phosphorylation is Also Involved in Calpain-Mediated Regulation of Growth Cone

Recent findings indicate that calpain phosphorylation and activation participate in the regulation of axonal outgrowth. We previously showed that tumor suppressor p53, in its phosphorylated form (p-p53), played a critical role in growth cone regulation through a dual effect: (1) p-p53 interferes with Rho kinase (ROCK) signaling by inhibiting RhoA synthesis, and (2) p-p53 interferes with ROCK signaling by directly binding to ROCK [76, 77]. Semaphorin 3A is a negative regulator of growth cones and we evaluated the potential role of calpain activation in semaphorin 3A-induced growth cone collapse in cultured hippocampal neurons [78]. Semaphorin 3A treatment of cultured hippocampal or cortical neurons resulted in rapid growth cone collapse and truncation of p-p53; both effects were prevented by calpain inhibition with either calpain-2 specific siRNA or inhibitors. Furthermore, semaphorin 3A-mediated calpain activation and growth cone collapse were associated with calpain-2 phosphorylation and prevented by inhibition of ERK and p38 MAP kinase. Calpain treatment of membranes prepared from cortical neurons resulted in the partial truncation of p53, especially of p-p53. Thus, semaphorin 3A-induced growth cone collapse is mediated

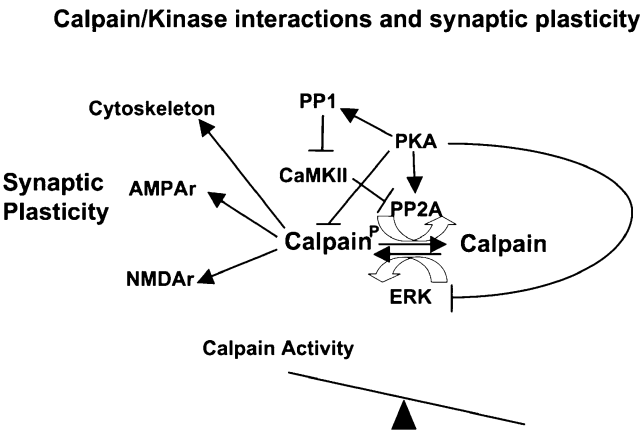

Fig. 2 Schematic representation of the regulation of calpain-2 by various kinases and phosphatases. Calpain-2 activity is regulated by complex interactions with kinases and phosphatases. Thus, ERK-mediated phosphorylation of calpain-2 facilitates while PKA-mediated phosphorylation inhibits calpain activation. In addition, PP2A dephosphorylates calpain-2 but is itself subjected to opposite regulation by PKA and CaMKII. As mentioned in the text, activated calpain can act on glutamate receptors and the cytoskeleton, thereby playing a critical role in synaptic plasticity

in part by calpain-2 activation, possibly through ERK-mediated phosphorylation, and the resulting truncation of p-p53, leading to ROCK activation and cytoskeletal reorganization. These findings demonstrate the existence of a new mechanism linking extrinsic and intrinsic signals to the regulation of axonal outgrowth. Calpain, and in particular calpain-2, is ideally suited to integrate these various signals, as a result of its dual regulation by ERK- and PKA-mediated phosphorylation, and, by truncating various proteins participating in the regulation of actin dynamics, to provide a critical switch for axonal outgrowth or retraction. In particular, the role of cAMP in axonal growth has been widely documented [79]. It is therefore tempting to propose that cAMP, by activating PKA and inhibiting calpain-2, protects p-p53 from degradation, thereby promoting axonal outgrowth. Interestingly, a role for calpain in adult axonal regeneration/degeneration has also been discussed [80, 81], suggesting that the function of calpain in regulation of axonal growth extends beyond the developmental period. This function of calpain in regulation of axonal growth is just another example of the pleiotropic nature of signaling cascades, which are used in a variety of physiological and pathological processes depending on their cellular and subcellular localization.

Summary and Conclusions

Critical new conceptual as well as technological advances have made it possible to test the hypothesis that calpain plays an important role in synaptic plasticity as well as in multiple forms of neuronal responses to physiological and pathological conditions. It is now clear that calpain-2 can be activated by MAP kinase-mediated phosphorylation and plays a critical role in cytoskeletal remodeling in a variety of cell types under physiological conditions. New tools have also been developed to study its pattern of activation. More recently, it has become widely acknowledged that calpain-2, by partially truncating a variety of cytoskeletal proteins, plays a critical role in the regulation of shape and motility in numerous cell types (see [6, 82] for recent reviews). A recent study indicated that calpain-2 functions as a molecular switch in Chinese hamster ovary cells to control cell spreading and retraction [83]. Interestingly, integrin activation also results in calpain activation and, depending on the state of phosphorylation of the integrin cytoplasmic domain, leads to either inhibition of RhoA and spreading or activation of RhoA and cell retraction. It is therefore tempting to envision a similar type of molecular switch in dendritic spines and to equate spreading to potentiation and retraction to depression. The dual regulation of calpain by ERK and PKA suggests that complex interactions between calpain-mediated proteolysis and kinase-mediated phosphorylation play critical roles in many processes in which calpain has been implicated (Fig. 2).

The precise role of this ubiquitous protease in synaptic plasticity has been an enigma for over 20 years. Since its discovery in synapses more than 25 years ago, many new substrates have been identified, with many of them localized in synapses and in axons and axon terminals. New techniques have been developed to study its function and its regulation, and in particular to address the “calpain paradox”. Further research is needed to fully understand how calpain activation can initiate actin depolymerization during growth cone collapse, while in adult neurons it stimulates pathways responsible for actin polymerization, intracellular receptor trafficking, and cellular migration. Clearly, a better understanding regarding the properties of calpains and the roles of this family of proteases in numerous physiological and pathological processes will provide important information to answer fundamental questions related to not only normal brain functions but also numerous diseases involving calcium and other second messenger system deregulation.

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