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Review

Calpain-calcineurin Signaling in the Pathogenesis of Calcium-dependent Disorder

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Intracellular calcium is a powerful secondary messenger that affects a number of calcium sensors, including calpain, a Ca^{2+}-dependent cysteine protease, and calcineurin, a Ca^{2+}/calmodulin-dependent protein phosphatase. Maintenance of low basal levels of intracellular calcium allows for the tightly regulated physiological activation of these proteins, which is crucial to a wide variety of cellular processes, such as fertilization, proliferation, development, learning, and memory. Deregulation of calpain and calcineurin has been implicated in the pathogenesis of several disorders, including hypertension, heart disease, diabetes, cerebral ischemia, and Alzheimer's disease. Recent studies have demonstrated an interplay between calpain and calcineurin, in which calpain can directly regulate calcineurin activity through proteolysis in glutamate-stimulated neurons in culture and *in vivo*. The calpain-mediated proteolytic cleavage of calcineurin increases phosphatase activity, which promotes caspase-mediated neuronal cell death. Thus, the activation of the calpain-calcineurin pathway could contribute to calcium-dependent disorders, especially those associated with Alzheimer's disease and myocardial hypertrophy. Here, we focus briefly on recent advances in revealing the structural and functional properties of these 2 calcium-activated proteins, as well as on the interplay between the 2, in an effort to understand how calpain-calcineurin signaling may relate to the pathogenesis of calcium-dependent disorders.

Key words: calpain, calcineurin, calcium, proteolysis, neurodegeneration

Calpain and calcineurin are 2 important effectors of the intracellular actions of calcium. Calpain is a Ca^{2+}-activated neutral cysteine protease that catalyzes limited proteolysis of substrate proteins. Calcineurin is a widely distributed class of protein phosphateses and belongs to the protein phosphatase 2B family of Ca^{2+}/calmodulin-dependent serine/threonine protein phosphatases [1, 2]. Activation of these 2 proteins by calcium levels in the physiological range has widespread effects, from the direct effects of proteolysis and dephosphorylation of target proteins, to the indirect modulation of diverse downstream signaling pathways. This regulated cleavage by calpain and dephosphorylation by calcineurin is critical in a variety of cell processes, including cell embryonic development, proliferation, differentiation, migration, cell cycle progression, meiosis, and mitosis [3–12]. Deregulation of calpain and calcineurin, caused by a disruption of calcium

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homeostasis, is critically involved in the pathogenesis of several important calcium-dependent diseases, such as hypertension, heart disease, diabetes, and Alzheimer's disease. Moreover, under certain pathological conditions, calpain and calcineurin may interact, and this interaction may play a role in the pathogenesis of many calcium-dependent disorders. Here we provide an overview of Ca^{2+}-dependent activation of calpain and calcineurin at the molecular and cellular levels and discuss the potential interplay between the 2 in the pathogenesis of calcium-dependent disorders.

General Properties of the Calpain Family

Calpains function as cytoplasmic cysteine proteinases, regulatory enzymes transducing intracellular Ca^{2+} signals into the controlled proteolysis of their substrates. Because of the presence of numerous downstream targets in a variety of signaling pathways, calpains are speculated to play important roles in cytoskeletal remodeling, cell differentiation, apoptosis, necrosis, embryonic development, and long-term potentiation in the central nervous system. The overactivation of calpain is connected to a number of diseases, including muscular dystrophy, cardiac and cerebral ischemia, traumatic brain injury, platelet aggregation, restenosis, neurodegenerative diseases, rheumatoid arthritis, and cataracts, making calpain an attractive drug target [13–18].

Calpains are intracellular nonlysosomal Ca^{2+}-regulated cysteine proteases ubiquitously found in animal tissues [19]. Based on human sequence homology, 14 human genes have been identified as members of the calpain large catalytic 80 kDa family, and 2 human genes for the small regulatory 30 kDa family [20, 21] (Table 1). The large catalytic subunit of calpains consists of 2 groups, typical and atypical, containing nine and 6 members, respectively. Calpains 1, 2, 3a, 3b, 8, 9, 11, 12, and 13 are typical calpains characterized by a C-terminal Ca^{2+}-binding domain that includes an EF-hand motif. The small optic lobe homology, including calpains 5, 6, 7, 8b, 10a, and 15, are atypical calpains; they lack EF-hand motifs and contain additional domains different from those of typical calpains. Among the typical

Table 1 The General information of calpain family

	Common Name	Gene	Used Name	Distribution	Species	Amino Acid Residues
Typical Calpain	Calpain 1	*Capn1*	μ-Calpain	Ubiquitous	Mammalian	714
	Calpain 2	*Capn2*	m-Calpain	Ubiquitous	Mammalian	700
	Calpain 3a	*Capn3*	nCL-1, p94	Skeletal muscle	Mammalian	821
	Calpain 3b	*Capn3*	Lp82	Lens	Mammalian	821
	Calpain 8a	*Capn8*	nCL-2	Stomach	Mammalian	703
	Calpain 9	*Capn9*	nCL-4	Digestive tracts	Mammalian	690
	Calpain 11	*Capn11*		Testis	Mammalian	702
	Calpain 12	*Capn12*		Hair follicle	Mammalian	720
	Calpain13a Calpain 13b	*Capn13*		Ubiquitous	Mammalian	423
Atypical Calpain	Calpain 5	*Capn5*	nCL-3, htra3	Ubiquitous	Mammalian	640
	Calpain 6	*Capn6*	CANPX	Placenta, Embryonic muscle	Mammalian	641
	Calpain 7	*Capn7*	palBH	Ubiquitous	Mammalian	813
	Calpain 8b	*Capn8*	nCL-2	Stomach	Mammalian	703
	Calpain 10	*Capn10*		Ubiquitous	Mammalian	672
	Calpain 15	*Capn15*	SOLH	Ubiquitous	Mammalian	1,086
Small Calpain	Small Subunit I	*Capn4 (SOL)*		Ubiquitous	Mammalian	268
	Small Subunit II	*Capn14*			Mammalian	248

calpains, μ- and m-calpains (also called calpain I and calpain II, respectively) are the most characterized heterodimeric members. They are encoded by genes *CAPN1* and *CAPN2* in mammalians. Based on amino acid sequence comparisons, the large subunit of μ-calpain and that of m-calpains are each comprised of up to 4 distinct domains (I-IV; Fig. 1). The N-terminal region contains residues 1–19 and is a single α-helix; it can interact with domain VI of the small subunits and may be important for stability. Domain II is known to carry residues Cys105, His262, and the Asn286 triad, which are responsible for calpain catalytic activity. It is structurally similar to the catalytic domains of other cysteine proteases, such as papain, caspases, and cathepsins B, L, and S. This domain is composed of 2 subdomains – IIa (residues 20–210) and IIb (residues 211–355) – and a substrate binding cleft. Subdomain IIa includes the catalytic Cys105, while subdomain IIb contains the His262 and Asn286 residues of the catalytic triad [22, 23]. Domain III (residues 356–514) can bind $Ca^{2+}$and consists of eight β-strands arranged in a β-sandwich configuration similar to those of C2 domains, a stretch of approximately 130 amino acids that binds phospholipids in a Ca^{2+}-dependent manner found in phospholipase C, protein kinase C, and so on [24, 25]. Domain IV (residues 531–700), at the C-terminal end of the large subunit, is a Ca^{2+}-binding domain structurally containing 5 sets of EF-hand similar to those found in calmodulin [26–28]. In addition, there is a long, exposed linker region spanning through amino acid residues 516–530 between domains III and IV. The small 30 kDa regulatory subunit contains 2 domains. Domain V (residues 1–101), the N-terminal region of the small subunit, is a hydrophobic domain rich in glycine and may function as a membrane anchor. Domain VI (residues 102–268), the C-terminal end of the small subunit, is a Ca^{2+}-binding region similar to domain IV of the large subunit [29–30]. The large catalytic subunit associates with the small regulatory subunit through the extreme C-terminal fifth EF-hand motif in IV and VI to form a heterodimeric calpain [29, 31–33].

Ca^{2+}-dependent Calpain Activation

An understanding of the molecular-level details of calpain activation is crucial for comprehending the functional properties of this protease and its characterization of pathophysiological significance in many diseases. X-ray structural analyses have revealed that there are at least 3 different Ca^{2+}-binding sites

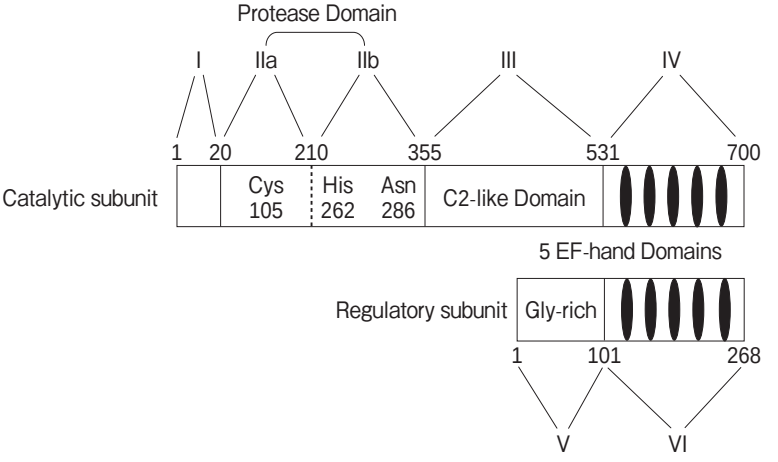

Fig. 1 Schematic representation of domain architecture of the classical calpains. The catalytic subunit possesses domains I-IV. Domain I contains residues 1–19 and interacts with domain VI of the small subunits. Domain II contains residues 20–355 and is divided into 2 subdomains, IIa and IIb, which carry residues of Cys105, His262, and the Asn286 triad responsible for calpain catalytic activity. Domain III contains residues 356–514, which harbors the C2 area that binds phospholipids. Domain IV contains residues 515–700 and is the C-terminal end of the large subunit. It consists of 5 consecutive EF-hand motifs. The regulatory subunit contains domain V, which is a highly flexible, glycine-rich region, and domain VI, which is a Ca^{2+} binding region, similar to domain IV of the catalytic subunit.

in m-calpain: the two EF-hands (calmodulin-like domains IV and VI), the cysteine catalytic region (domain II), and the acidic loop (C2-like domain III) [23, 30, 34]. In the absence of Ca^{2+}, the 2 subdomains of the catalytic subunit, IIa and IIb, are separated by a deep crevice, thus maintaining the active site in a state in which the catalytic triad residues are under a structural conformation that does not allow for substrate hydrolysis (Fig. 2). In this conformation, subdomain IIa is restrained by a circular arrangement of domain I, the N-terminal anchor peptide, binding to domain IV. Subdomain IIb is bound by an acidic loop in domain III [35–37]. When calcium binds to these domains, a major conformational change occurs that ultimately produces a competent active site in the cysteine protease region. Studies have suggested that calpains undergo a Ca^{2+}-dependent two-stage activation [37, Fig. 2]. At the first stage, the binding of calcium to domain III and two EF-hand regions results in an auto-cleavage of domain I, eliminating the N-terminal link between the large 80 kDa catalytic subunit and the small 30 kDa regulatory subunit. This calcium binding would allow movement within domain II, in which subdomain IIb turns over towards subdomain IIa, thereby forming an active site [34, 38–47]. At the second stage of activation, the binding of calcium directly to the cysteine residue causes a shift in the conformation where subdomains IIa and IIb reposition the catalytic site cleft to a spatial arrangement favorable for substrate hydrolysis. This proposed two-stage Ca^{2+}-dependent process is a general activation mechanism for calpain superfamily members. The activation mechanism for the nonheterodimeric calpains that do not contain small subunits and those lacking EF-hand or C2-like domains in the large subunit could be alterative. Unlike the cysteine catalytic site (domain II) present in all members of the calpain superfamily, the flanking domains – domains III, IV, and VI – are varied in atypical calpains [48]. These nonheterodimeric calpains could be directly activated by the cooperative binding of Ca^{2+} to domain II without the first stage of activation.

Inhibition of Calpain Activities

Because calpain irreversibly cleaves numerous signaling and structural proteins, with widespread impact on cell functioning and viability, the protease activity is highly controlled *in vivo* by multiple mechanisms, including phosphorylation and an endogenous inhibitor, calpastatin [49, 50]. Calpastatin is an interacting partner of calpain that is capable of inhibiting calpain activity. The binding of calpastatin to calpain is a Ca^{2+}-dependent event and is reversible [51, 52]. Studies have suggested that binding of calpastatin to calpain occurs before calpain can initiate proteolytic activity, as the Ca^{2+}concentration required for calpastatin binding to calpain is less than the Ca^{2+}concentration required for the half-maximal proteolytic activity of μ- and m-calpains [52, 53]. Although calpastatin is the only known inhibitor

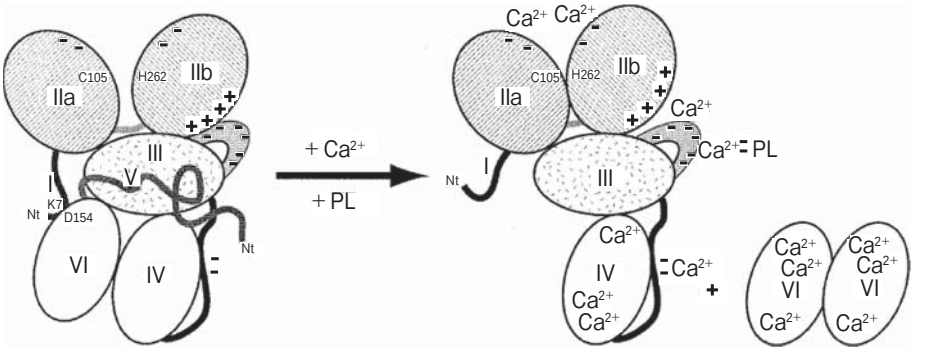

Fig. 2 Calcium-dependent calpain activation. Fig. 2 was adapted from [21] and represents the activation mechanism of calpain by Ca^{2+}. A shows activation in the absence of Ca^{2+}; the 2 subdomains of the catalytic subunit, IIa and IIb, are separated by a deep crevice. B shows the binding of Ca^{2+} and phospholipids (PL) to calpain, initiating a series of structural movements that result in IIa and IIb close together to form a functional catalytic site.

with absolute specificity for both μ- and m-calpains, it possesses a large molecular mass, making it cell-impermeable, and thus has limited therapeutic use.

Calpastatin has 4 repeating, marginally homologous (23–36%) inhibitory domains (I-IV), each having approximately 140 amino acid residues, and an N-terminal domain L that has no inhibitory activity (Fig. 3) [54–57]. Each individual domain consists of 3 subdomains, A, B, and C, with subdomain B playing a central role in calpain inhibition [58–60]. A 27-residue peptide (CS), containing most of subdomain B from domain I of human calpastatin, is a potent and specific inhibitor of calpain *in vitro* but has little ability to translocate across the cell membrane. However, fusion of this CS peptide to a protein transduction domain, an 11 poly-arginine peptide (11R), allows it to be cell-permeable and effectively inhibits calpain activity [61, 62–64]. Studies have compared *in vitro* inhibitory ability between 11R-CS and the natural peptide CS on calpain auto-cleavage in cultured hippocampal neurons, and found that the IC_{50} values are 0.48 μM for CS and 0.51 μM for 11R-CS [62]. Application of the 11R-CS to hippocampal cultures at a concentration of 50 μM substantially protects neurons from 500 μM glutamate-induced excitotoxicity [63].

Phosphorylation is another way to control calpain acitivity. Calpain has several phosphorylation sites. One of them is phosphorylated by protein kinase A (PKA), which negatively regulates calpain activity. It has been reported that domain III of the human m-calpain large subunit is directly phosphorylated *in vitro* at Ser369 or Thr370 by PKA [65, 66]. Ser369/Thr370 is located in the interface region between domains III and IV, and phosphorylation of these sites presumably leads to contact between these domains, which can prevent the formation of the calpain active cleft. The biological role of PKA-mediated phosphorylation of m-calpain has been determined in living cells. In NR6WT mouse fibroblasts, phosphorylation of m-calpain by PKA at Ser 369 or Thr370 decreases epidermal growth factor (EGF)-induced activation of m-calpain and inhibits fibroblast migration [65].

In addition, inhibitors derived from natural sources or produced synthetically have been developed and proven to be effective against calpain activity. For example, the representative peptidyl epoxysuccinate inhibitors are trans-epoxysuccinyl-L-leucylamido-4-guanidino-butane (E64) and its derivative, E64d; peptidyl aldehyde inhibitors include leupeptin, calpain inhibitor I, calpain inhibitor II, calpeptin, and MDL28170. These inhibitors inactivate calpain reversibly or irreversibly by forming a covalent bond with the active site thiolate or interacting with the Ca^{2+}-binding domain of the calpain large subunit [67]; they show specificity for calpains over other cysteine proteases and low cell membrane permeability [68].

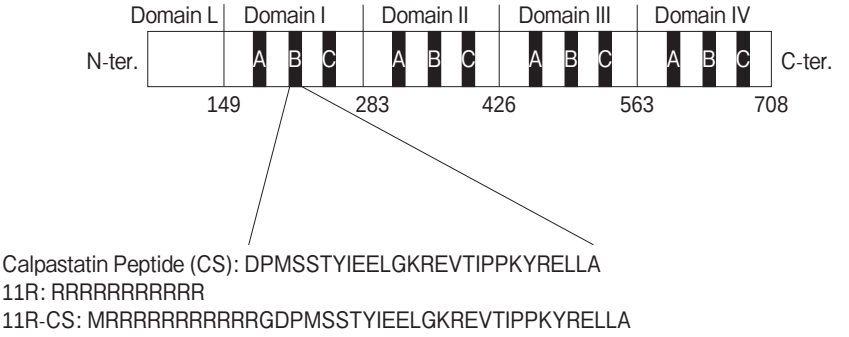

Fig. 3 Schematic diagram showing the domain structure of human calpastatin, calpastatin peptide (CS), [11] arginine (11R), and 11R-fused CS. Calpastatin is comprised of an N-terminal domain L and four repeated domains, each of which contains 140 amino acid residues. A, B, and C regions are subdomains having significant sequence homology within each domain. Subdomain B contains a highly conserved sequence that has been implicated in calpain inhibition. CS is a 27-residue oligopeptide encoded by exon 1B of human calpastatin. 11R is an effective protein transduction domain including 11 poly-arginine peptides.

General Properties of Calcineurin

Calcineurin is a heterodimer consisting of a catalytic subunit (calcineurin A) with a molecular mass of about 57–59 kDa and a regulatory calcium-binding subunit (calcineurin B) with a molecular mass of 19 kDa [69]. These subunits are tightly associated and can be dissociated only by the use of denaturants [70]. Calcineurin is ubiquitously distributed in eukaryotes and widely distributed in the brain, with high levels in the hippocampus and caudate putamen [69, 71–76]. Immunohistochemistry and *in situ* hybridization have shown the presence of calcineurin A in cell bodies, postsynaptic densities (PSDs), dendrites, axons, and spines. Within the cell, approximately half of the calcineurin population is in the cytosol, and the other half is associated with the plasma membrane [69]. Calcineurin is largely absent from glia and interneurons in the hippocampus [77].

Calcineurin has intrinsic Ca^{2+}-binding properties [71, 78]. Structural and functional analyses suggest that calcineurin B contains four "EF"-hand, Ca^{2+}-binding sites, a myristoylated-binding domain, and an affinity for calcineurin A [79–81]. Mammalians have 3 isoforms of calcineurin A (α, β, and γ, also called $\alpha1$, $\alpha2$, and $\alpha3$) and 2 of calcineurin B, B1 and B2 [82–85]. Expression of calcineurin Aγ and B2 is restricted to the testis, while calcineurin Aα, Aβ, and B1 are expressed in a wide spatiotemporal distribution [86].

The active site of calcineurin is located on the A subunit (Fig. 4). The catalytic subunit calcineurin A (521 residues) contains a phosphatase domain (residues 1–328), a calcineurin B-binding helical domain (residues 348–368), a calmodulin binding region (residues 390–414), and an autoinhibitory loop (residues 468–490). The gene for mammalian calcineurin B encodes a protein of 170 amino acids containing four Ca^{2+}-binding EF-hand motifs [87] (Fig. 4). Calcineurin B consists of two Ca^{2+}-binding domains,

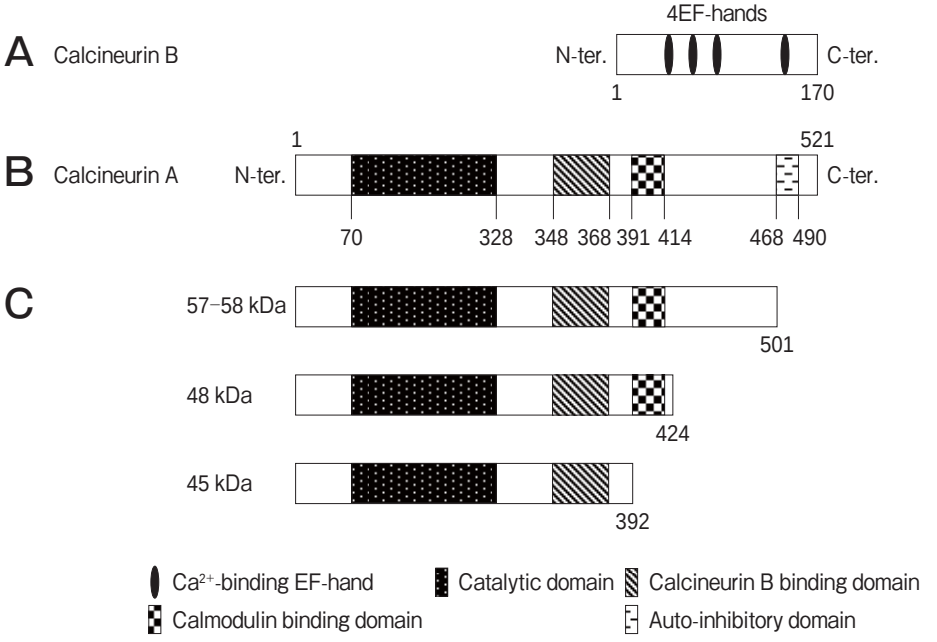

Fig. 4 Schematic representation of structure of calcineurin subunits and calpain-dependent truncation of calcineurin A. **A,** Calcineurin B protein, the regulatory subunit, encoded by the mammalian calcineurin B gene. It has four Ca^{2+}-binding EF-hand motifs; **B,** Calcineurin A, the catalytic subunit. The linear peptide sequence of the calcineurin A subunit is shown, indicating the catalytic domain (residues 70–328), the calcineurin B binding domain (residues 348–362), the calmodulin binding domain (residues 391–414), and the autoinhibitory domain (residues 468–490); **C,** Three calpain-dependent truncated forms of calcineurin A *in vitro*. Analysis by MALDI-TOF has shown that the N-terminal remaining fragments are 1–392 residues, 1–424 residues, and 1–501 residues, which correspond to 45, 48, and 57–58 kDa truncated calcineurin A.

domain 1 (residues 1–84) and domain 2 (residues 86–169), which are arranged linearly along its binding domain in calcineurin A. Each domain contains two Ca^{2+}-binding EF-hand motifs that are similar to those of calmodulin.

Ca^{2+}-dependent and Calpain-dependent Activation of Calcineurin

As a serine/threonine protein phosphatase, calcineurin acts as an effector of Ca^{2+} signaling by regulating the phosphorylation state of proteins and participates in a number of cellular processes, including immune system responses [1, 69, 88–92], cardiac hypertrophy [93–101], neuronal and muscle development [102, 103], the second messenger cAMP pathway [89, 104], Na/K ion transportation in nephron [105], and cell cycle regression in lower eukaryotes [106].

Full activation of the phosphatase activity requires both the binding of Ca^{2+} to calcineurin B and Ca^{2+}-dependent binding of calmodulin to calcineurin A [1, 2, 69, 109]. In the inactive state, the autoinhibitory domain sterically blocks the active site. When the calcium concentration increases, calcium and calmodulin bind to their binding sites on heterodimeric calcineurin and trigger a conformational shift, resulting in the release of the autoinhibitory domain from the catalytic active site. The proposed Ca^{2+}/calmodulin-triggered activation of calcineurin takes place during physiological conditions and is reversible (Fig. 5).

In addition to the conventional activation pathway, studies have suggested that calcineurin activation is also protease-dependent. Irreversible proteolytic activation of calcineurin occurs *in vitro* and *in vivo*. Proteases such as calpain, trypsin, and chymotrypsin C have been reported to cleave calcineurin A *in vitro* [63, 110–113]. This proteolytic truncation of calcineurin A is site-limited, as only the carboxy-terminus of the molecule containing the calmodulin-binding domain and the autoinhibitory domain can be readily cleaved by proteases [112, 114]. The NH_2-terminal two-thirds of the molecule, which comprise the phosphatase catalytic domain and calcineurin B-binding domain, are resistant to proteolysis [112]. Proteolytic modification removes the regulatory domain of calcineurin A and changes the phos-

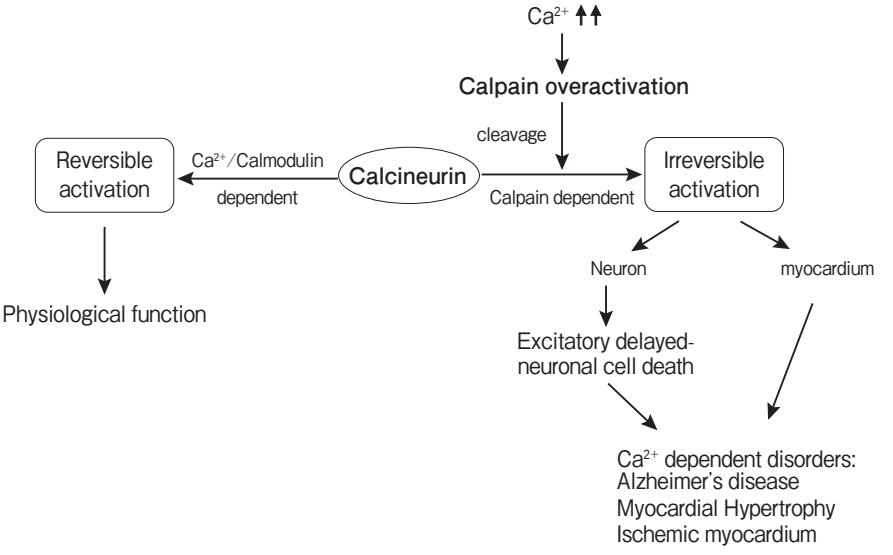

Fig. 5 Schematic representation of calpain-calcineurin signaling in the pathogenesis of calcium-dependent disorder. Under physiological conditions, calcineurin activity is regulated by Ca^{2+}/calmodulin in a reversible manner. Under some pathological conditions, such as Ca^{2+}-mediated disorders, calcineurin activity is increased by overactivated calpain. Calpain-mediated irreversible activation of calcineurin is correlated with the major pathology, the number of neurofibrillary tangles in human Alzheimer's disease brains, and the myocardial hypertrophy in human.

phatase to its constitutively active form, which no longer requires calcium and calmodulin for activation [112, 115].

Calpain and calcineurin are both Ca^{2+}-regulated proteins in the brain, in which the calcineurin-mediated signaling pathway is regulated by calpain. Increased calpain activity is able to cleave cain/cabin 1, an endogenous calcineurin inhibitor [116]. When cleaved, cain/cabin 1 can no longer inhibit calcineurin. Evidence from Jurkat cells has shown that cleavage of cain/cabin 1 by calpain is a necessary step in calcineurin-mediated cell death. In addition, calcineurin A has been shown to be a specific substrate of calpain in neuronal cultures and in mouse hippocampus [63]. The calpain-mediated post-translational modification made the protein phosphatase constitutively active. Mass spectrometry analysis by MALDI-TOF has identified several cleavage sites in calcineurin A after *in vitro* cleavage by calpain. The N-terminal remaining fragments are 1–392 residues, 1–424 residues, and 1–501 residues, which correspond to 45, 48, and 57 kDa truncated forms of calcineurin A. The calpain-cleaved 45 kDa form of calcineurin A does not include the calmodulin-binding domain or the autoinhibitory domain, while the 48 kDa truncated form contains the cleaved C-terminal region of the calmodulin-binding domain. Both products lack the autoinhibitory domain. The 57 kDa truncated calcineurin A is a result of cleavage at the C-terminal side of the autoinhibitory domain, and includes the calcineurin B-binding, calmodulin-binding, and autoinhibitory domains. Studies have shown that the calpain-cleaved 48 kDa and 45 kDa truncations of calcineurin A have full enzyme activity and thus are constitutively active forms. In transfected HEK cells, these two N-terminal truncated forms can initiate calcineurin-mediated NFAT (nuclear factor of activated T-cell) gene transcription. In cultured hippocampal neurons, overexpressing an adenoviral-based 48 kDa truncation of calcineurin A induces caspase activation and neuronal cell death. Moreover, calpain activation and the production of 45–48 kDa truncation of calcineurin A is associated with glutamate-induced neuroexcitotoxicity in cultures of hippocampal neurons and kainate-induced neuroexcitotoxicity in mouse hippocampus.

Calcineurin Inhibitors

Calcineurin activity can be inhibited by its autoinhibitory peptide, which is a 26-residue peptide that interacts with the catalytic domain of the A subunit. This peptide blocks calcineurin activity with an IC_{50} of 5 μM but lacks the ability to permeate the cell membrane [114]. It has been shown that fusing this peptide with 11R, the protein transduction domain that is used to introduce the calpain inhibitory peptide CS through the cell membrane, also allows the autoinhibitory peptide to go through the cell membrane [117]. Application of the 11R autoinhibitory peptide into cultured neurons efficiently inhibits the phosphatase activity of calcineurin, calcineurin-dependent NFAT nuclear translocation, and NFAT-dependent promoter activity *in vivo*. Applying the peptide at a 50 μM concentration provides neuroprotection on glutamate-induced excitatory cell death involving a calcineurin-mediated mechanism.

Based on the 11R transduction domain, a high-affinity calcineurin-binding peptide has been developed by the fusion of this peptide with VIVIT, the calcineurin docking motif of NFAT [118, 119]. The 11R-VIVIT interferes selectively with the interaction between calcineurin and its substrate NFAT, blocking activation and expression of NFAT-dependent cytokine genes without affecting the expression of other cytokines that require calcineurin but not NFAT. The substrate-selective inhibitory peptide has an advantage over other calcineurin inhibitors in target specificity, which indiscriminately blocks all signaling downstream of the phosphatase. Data have shown that this 11R-VIVIT peptide provides immunosuppression for fully mismatched islet allografts in mice without affecting insulin secretion [119]. A more recent study has demonstrated that this peptide is capable of preventing the development of pressure-overload cardiac hypertrophy in a rat model. This specific NFAT inhibitor peptide can decrease the ratio of rat heart weight to body weight, the size of cardiac myocytes, and the serum brain natriuretic peptide and atrial natriuretic peptide levels during the pressure-overload hypertrophic response [120].

Immunosuppressant drugs cyclosporine A (CsA) and FK506 have long been known as specific potent inhibitors of calcineurin [121]. They are fungal-

derived compounds that require binding to their cognate intracellular immunophilins (cyclophilin A for cyclosporine A and FKBP12 for FK506) prior to inhibiting calcineurin activity. The cyclosporine A/cyclophilin A or FK506/FKBP12 complex binds to a variety of sites in calcineurin, including the N-terminus of the calcineurin B binding helix, the calcineurin B-subunit, and the catalytic domain of calcineurin [121].

In addition to synthetic and natural inhibitors, calcineurin protein phosphatase activity is also known to be potentially inhibited by a number of endogenous cellular proteins, such as protein kinase A anchoring protein (AKAP79), cain/cabin 1, calcineurin homologous protein (CHP), and the calcipressin family of proteins [122–128]. In rat hippocampal neurons, calcineurin and the regulatory subunit of protein kinase A colocalize via AKAP79, which contains a domain homologous to FKBP that is predicted to be a calcineurin binding domain [122]. Cain/cabin 1 is a 2220-residue phosphoprotein that inhibits calcinuerin phosphatase activity in a noncompetitive fashion [123]. In cells, the overexpression of CHP inhibits calcineurin phosphatase activity by 50% and presents in a dose-dependent manner. As the major member of the calcipressin family, calcipressin 1, also known as Down Syndrome Critical Region 1 (DSCR1), is expressed in diverse cell types and tissues, including heart/cardiac muscle, striate muscle, brain/neuronal cells, and T-cells [127, 129–135]. Calcipressin 1 binds to calcineurin at or near the active site and negatively regulates calcineurin phosphatase activity. Its biological roles include protection against calcium-mediated oxidative stress, cardiac hypertrophy, VEGF-mediated signaling during angiogenesis, and the formation of aggresomes in Alzheimer's disease [132, 136–141].

Calpain-calcineurin Signaling in Calcium-dependent Disorders

Calpain-calcineurin signaling in Alzheimer's disease. Alzheimer's disease is a progressive and irreversible neurodegenerative disorder characterized by cognitive, memory, and behavioral impairments [142, 143]. The disease process involves the degeneration of synapses and neurons, particularly in the hippocampus and neocortex. The histological hallmarks of these brain regions of patients with Alzheimer's include extracellular deposits of β-amyloid in neuritic plaques, intracellular neurofibrillary tangles consisting of abnormally hyperphosphorylated aggregates of the microtubule-associated protein tau, and selective neuronal loss. Although the molecular pathogenesis of Alzheimer's disease is not fully understood, dysregulation of calcium homeostasis is believed to play an important role in neurodegeneration. Evidence has shown that the disturbance of calcium homeostasis causes widespread activation of calpain in the brain in Alzheimer's disease; an abnormal increase in calpain activity could be a potential molecular basis for neuronal degeneration [144, 145]. In Alzheimer's disease, the ratio of activated calpain I to its latent precursor isoform in the neocortex is threefold than that in normal individuals. In surviving cells, persistent calpain activation in the brain in Alzheimer's disease strongly correlates with neurofibrillary pathology and with the extent of decline in levels of secreted amyloid precursor protein in the brain [144, 145]. Moreover, researchers have observed that the overactivation of calpain I in the brain in Alzheimer's disease contributes to proteolytically activated calcineurin, and that the calpain-mediated activation of calcineurin is correlated with major brain pathology and the number of neurofibrillary tangles (NFTs) in human Alzheimer's brains [146]. Analysis by mass spectrometry has indicated that in the brain with Alzheimer's disease, calpain I cleaved off C-terminal 20 amino acids from 60 kDa full-length to 57 kDa truncated calcineurin A at lysine 501, a position C-terminal to the autoinhibitory domain. Similar to the wild type, the 57 kDa truncated calcineurin A still requires Ca^{2+}/calmodulin for its phosphatase activity, but this phosphatase activity is remarkably activated upon truncation. Calpain I-mediated truncation and activation of calcineurin are correlated with the numbers of NFTs but not with that of β-amyloid plaques. This finding revealed a critical role of dysregulated calpain-calcineurin signaling resulting from the disturbance of calcium homeostasis in neurofibrillary degeneration in Alzheimer's disease (Fig. 5).

Calpain-calcineurin signaling in myocardial hypertrophy and ischemic myocardium. While the hypertrophic response is initially a compensatory mechanism that augments cardiac output, sustained

hypertrophy can lead to dilated cardiomyopathy, heart failure, and sudden death. The calcineurin-mediated transcriptional pathway is crucially involved in the pathogenesis of cardiac hypertrophy [93, 147–149]. A variety of hypertrophic stimuli, such as angiotensin II, phenylephrine, and endothelin-1, lead to an elevation of intracellular Ca^{2+} and subsequent activation of calcineurin, which leads to dephosphorylation of the nuclear transcription factor NF-ATc (nuclear factor of activated T-cells), resulting in the induction of genes typical of cardiac hypertrophy. Calpain-induced activation of calcineurin has recently been observed in hypertrophied myocardium both *in vitro* and *in vivo* [150]. In an animal model of myocardial hypertrophy, stimulation of rat cardiomyocytes with angiotensin II for 24 h causes a significant increase in calpain activity and calpain-mediated proteolysis of calcineurin A. Proteolysis of calcineurin A by calpain in angiotensin II-stimulated cardiomyocytes produces a 48 kDa N-terminal fragment (residues 1–424), which lacks the autoinhibitory domain and matches exactly the N-terminal truncation of calcineurin A found in *in vitro* digestion by m-calpain [63]. Without the autoinhibitory domain, the truncated calcineurin A is constitutively nuclear and active, even after removal of the hypertrophic stimulus. The 48 kDa N-terminal truncated form of calcineurin A has been found *in vivo* in human hypertrophied myocardium [150]. In addition to myocardial hypertrophy, studies have shown that during ischemia and reperfusion, there is increased influx of Ca^{2+} into the cells, which can activate u-calpain and m-calpain [151, 152]. Rat heart tissues that experienced 30 min ischemia followed by 30 min reperfusion displayed increased calpain activity and m-calpain-mediated degradation of full-length calcineurin A. In that model, calpain-mediated cleavage created a 46 kDa truncated calcineurin A and caused increased calcineurin phosphotase activity in general [153]. This suggests that calpain-calcineurin signaling might be a critical contributor to the pathogenesis of rat ischemic myocardium (Fig. 5).

Concluding Remarks

Calpain-mediated limited proteolysis has emerged as a key post-translational mechanism that regulates a large number of intracellular proteins. Tight regulation of calpain activity could potentially control substrate function, which may be crucial to cellular pathophysiological processes of some Ca^{2+}-dependent disorders (Fig. 5). Recent substantial evidence has demonstrated that calpain-calcineurin signaling is potentially associated with several Ca^{2+}-dependent disorders, including Alzheimer's disease and cardiac hypertrophy, providing a better understanding of the pathogenesis of these diseases. Abnormal calpain activity can lead to cleavage of calcineurin, resulting in calcineurin phosphatase overactivation, which could initiate mitochondrial dysfunction and further the mitochondria-dependent cell death pathway [154–157]. In addition, calpain-mediated cleavage of calcineurin activates the protein phosphatase, resulting in cardiac hypertrophy. Inhibition of calcineurin activ-

Table 2 Cell membrane permeable 11R fusion peptides and their properties

Peptide name	Inhibitory Target	Role of the peptide	Reference
Eleven Arginine-Calpastatin Peptide (11R-CS)	Calpains	Inhibition of calpain auto-cleavage (IC_{50}, 0.51 μM), calpain-mediated cleavage of calcineurin (50 μM), and glutamate-induced neuroexcitotoxicity (50 μM)	62, 63, 64
11R-auto-inhibitory Peptide	Calcineurin	Inhibition of alcineurin-dependent NFAT nuclear translocation, NFAT-dependent promoter activity, and glutamate-induced neuro-excitotoxicity (50 μM)	117
11R-VIVIT	the NFAT-calcineurin interaction	Inhibition of activation and expression of NFAT-dependent cytokine genes, immunosuppression of fully mismatched islet allografts in mice, prevention of rat development of pressure-overload cardiac hypertrophy.	119, 120
11R-VEET	NA	A control peptide of 11R-VIVIT	119, 120

ity in transgenic mice expressing activated calcineurin by administration of the immunosuppressant CyA blocks the development of hypertrophy [158–162]. Thus, calpain inhibitors, which block calpain-dependent calcineurin activation, may merit investigation as potential therapeutics for certain forms of heart and neurodegenerative disease. Further biochemical and physiological experiments will be necessary to establish their role, both *in vitro* and *in vivo*, in the inhibition of the calpain-calcineurin pathway, using 11R-fused member-permeable peptide inhibitors (Table 2) in those Ca^{2+}-related diseases.

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