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SPOTLIGHT REVIEW

Regulation and physiological roles of the calpain system in muscular disorders

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Abstract Calpains, a family of Ca^{2+}-dependent cytosolic cysteine proteases, can modulate their substrates' structure and function through limited proteolytic activity. In the human genome, there are 15 calpain genes. The most-studied calpains, referred to as conventional calpains, are ubiquitous. While genetic studies in mice have improved our understanding about the conventional calpains' physiological functions, especially those essential for mammalian life as in embryogenesis, many reports have pointed to overactivated conventional calpains as an exacerbating factor in pathophysiological conditions such as cardiovascular diseases and muscular dystrophies. For treatment of these diseases, calpain inhibitors have always been considered as drug targets. Recent studies have introduced another aspect of calpains that calpain activity is required to protect the heart and skeletal muscle against stress. This review summarizes the functions and regulation of calpains, focusing on the relevance of calpains to cardiovascular disease.

Keywords Calpain • Intracellular proteolysis • Sarcomere • Transgenic mice • Calcium

This article is part of the Review Focus on: The Calpain Family in the Cardiovascular System

1. Introduction

Calpains[1–4] (Clan CA, family C02; EC 3.4.22.17) are defined by their amino acid (aa) sequence similar to the calpain-like Cys protease motif CysPc, which is registered as cd00044 in the National Center for Biotechnology Information's conserved domain database. Using this criterion, the database identifies calpain homologues in a wide range of living organisms. The physiological relevance of calpains has been elusive, partly because each calpain species is fairly specific in its function as a critical regulator for cellular functions. On the other hand, calpains are often described as aggravating factors in various pathophysiological phenomena;[5–11] therefore, inhibiting calpains is an established research interest. Although little has been elucidated about when and how calpain is activated, fortunately, a new perspective emerges. Improved genetic techniques have revealed cause-and-effect relationships between calpain deficiencies and dysfunctions of tissues and organs.[12] These calpain-deficiency diseases, which should generally be called calpainopathies, provide clear evidence of the physiological importance of calpains.

Although calpains have frequently been characterized as deleterious degradative proteases in pathogenic conditions including cardiovascular diseases, calpains are actually processing rather than degradative proteases. Calpains differ from other major intracellular proteolytic components such as proteasomes[13] and lysosomal proteases functioning in autophagy;[14] these systems eliminate and recycle their substrates by degradation. Calpains act by proteolytic processing, as in the activation of conventional protein kinase C (PKC) (see Section 5.2.1). Calpains are unique in that they directly recognize substrates, whereas proteasomes and autophagy rely on other systems—ubiquitylation and autophagosome formation, respectively—to tag their substrates (*Figure 1*).

After a brief overview of the molecules that comprise the calpain system, we will review calpains' activation mechanisms and physiological and pathophysiological roles, focusing particularly on their relevance in cardiac and skeletal muscle tissues.

2. The calpain system

Foundational calpain studies have focused on the mammalian μ- and m-calpains,[15] which are thus called the conventional calpains; all other calpains are referred to as unconventional, and their structure

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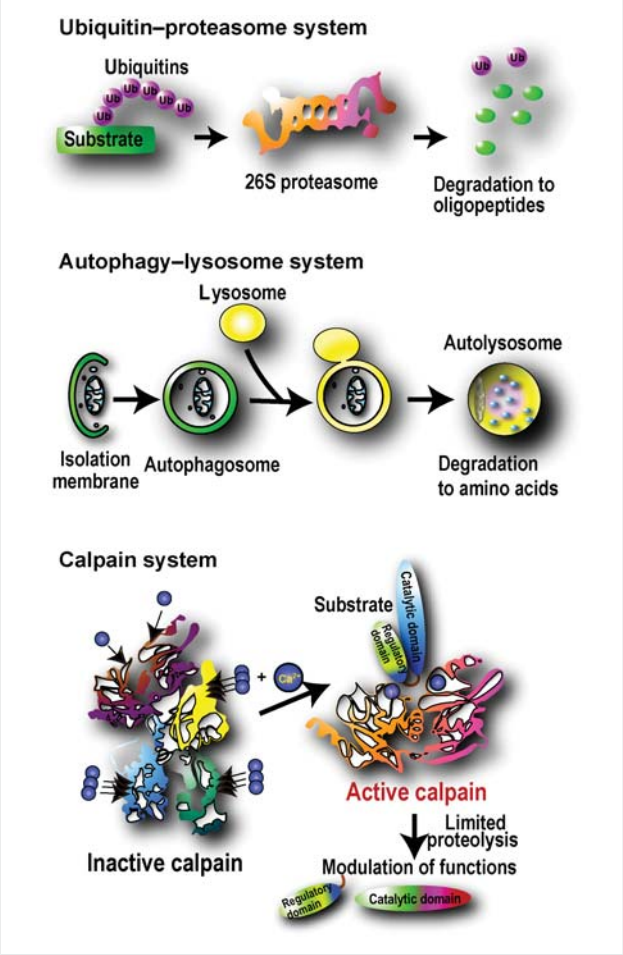

Figure 1 Major intracellular proteolytic systems. The ubiquitin–proteasome system degrades and eliminates specific substrate proteins with an ubiquitin-tagging system consisting of >1000 ubiquitin ligases. The autophagy-lysosome system primarily degrades non-specific cell components, including proteins and microorganisms, contained by isolation membranes. The caspase system (not shown) is a major intracellular proteolytic system with primarily apoptotic functions.[114] In contrast, calpains primarily elicit proteolytic processing, rather than degradation, to modulate or modify substrate activity, specificity, longevity, localization, and structure.

is often described relative to that of the conventional calpains (*Figure 2*).

2.1 Unifying the nomenclature for calpains and their domains

A unified calpain nomenclature was recently proposed based on a web-based discussion among calpain researchers, aiming to overcome hindered development of calpain studies due to complex naming.[4] This nomenclature defines mammalian calpain gene products as CAPN1, CAPN2, and so on; this follows the gene product nomenclature defined by the Human Genome Organization Gene Nomenclature Committee. To avoid confusion, this review refers to calpains by their proposed name followed by their previously common name, where applicable, in square brackets (e.g. CAPN1[μCL] or CAPN2[mCL]; μCL and mCL denote μ- and m-calpain larger catalytic subunits, respectively). Accordingly, conventional calpains are heterodimers of CAPN1[μCL] or CAPN2[mCL] and CAPNS1[30 K], and are called CAPN1/S1[μ-calpain] and CAPN2/S1[m-calpain] (CAPN1/S1 is short for CAPN1/CAPNS1).

CAPN1[μCL] and CAPN2[mCL] are divided into four regions/domains: the N-terminal anchor helix; the CysPc protease domain, which is divided into two protease core domains (PC1 and PC2);[16,17] a C2 domain-like (C2L) domain; and a penta-EF-hand (PEF(L)) domain.[18] CAPNS1[30K], the smaller regulatory subunit, contains an N-terminal Gly-rich (GR) domain and a PEF(S) domain (*Figure 2*).

2.2 Calpain homologues

Although other papain superfamily proteases (clan CA) have weak local similarities to the CysPc domain, they are clearly differentiated from calpains by their low aa sequence similarity. Accordingly, the human genome has 15 calpain genes, and other mammals have nearly the same number. Calpain genes exist in almost all eukaryotes and a few bacteria,[1] and these non-mammalian calpains are also enormously interesting scientific subjects. In this review, we focus on mammalian calpains, which are classified by two criteria: structure and distribution.[1,4,19]

2.2.1 Classical and non-classical calpains

The domain structure of CAPN1[μCL] and CAPN2[mCL], in which CysPc is followed by C2L and PEF domains (*Figure 2*), is classical by definition. Accordingly, non-classical calpains are missing C2L and/or PEF domains.[20] Both classical and non-classical calpains may have additional domains. The nine human classical calpains—CAPN1–3, 8, 9, and 11–14[21]—share strong sequence similarities, which, however, does not necessarily indicate functional or biochemical similarities. For example, of all the human classical calpains, only CAPN1[μCL] and CAPN2[mCL] form heterodimers with CAPNS1[30 K] *in vivo*.

Non-classical calpains are divided into several subfamilies. The PalBH subfamily is the most evolutionarily conserved, being found from humans (CAPN7[PalBH]) to fungi (PalB) and yeast (Rim13[Cpl1]), but not in plants.[22] The PalB subfamily has two tandem C2L/C2 domains following the CysPc domain, and may have up to two microtubule interaction and trafficking (MIT) motifs at the N-terminus. The SOL subfamily is also evolutionarily conserved, with orthologues in almost all animal species, including humans (CAPN15[SOLH]), drosophila, and green algae. Its domain structure is characterized by several Zn^{2+}-finger motifs and a specific SOL-homology (SOH) domain at the CysPc N- and C-termini, respectively (*Figure 2*).

2.2.2 Ubiquitous and tissue-specific calpains

Six human calpain genes are tissue-specific; the others, including conventional calpains, are ubiquitous. Human tissue-specific calpains are CAPN3[p94] in skeletal muscle,[23] CAPN6 in the placenta and embryonic muscles,[24] CAPN8[nCL-2] and CAPN9[nCL-4] in the gastrointestinal tract,[20] CAPN11 in the testis,[25] and CAPN12 in hair follicles.[26] It is widely assumed that ubiquitous calpains have basic roles in the cell, whereas tissue-specific calpains are involved in specific cell functions. Accordingly, defects in ubiquitous calpains can be lethal, as seen in *Capn2*$^{-/-}$ and *Capns1*$^{-/-}$ mice,[27–30] whereas defects in tissue-specific calpains may cause tissue-specific phenotypes, such as the muscular dystrophy caused by *CAPN3* mutations.[31] In conditions such as cardiomyopathy, muscular dystrophies, or traumatic ischaemia, conventional calpain overactivation has been

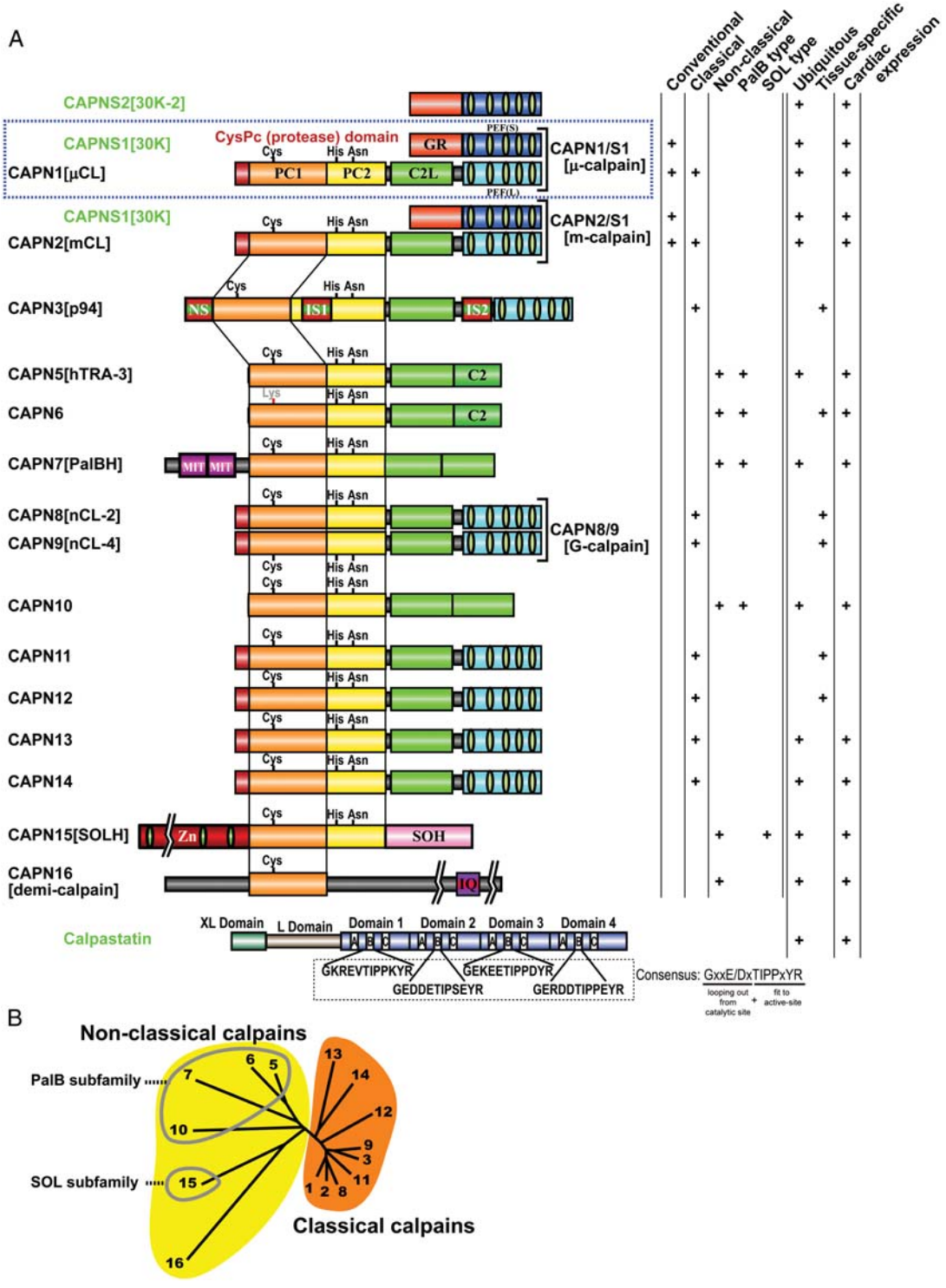

Figure 2 Human calpains and their regulatory molecules. (*A*) Schematic structures. In this review, human calpains are presented in a proposed name (previous name, if any) format. Calpain structural classification and tissue distribution, particularly regarding the heart, is indicated in the right-hand columns. Calpain enzyme complexes (tertiary structures) that have been elucidated *in vivo* are shown along with their enzyme names (single brackets). Bottom: the domain structure of the longest calpastatin isoform. The four repetitive inhibitory units are labelled as domains 1–4; in each of these, the *A–C* regions are important for calpastatin's inhibitory activity. The consensus aa sequence in the B-region, which directly interacts with the calpain active site, is shown. Exons encoding the XL and L domains are subject to alternative splicing. Symbols: PC1 and PC2, protease core domains 1 and 2 in the calpain protease (CysPc) domain; C2L, C2-domain-like domain; PEF(L) and PEF(S), penta-EF-hand domains in the larger (L) and smaller (S) subunits, respectively; GR, glycine-rich hydrophobic domain; NS/IS1/IS2, CAPN3[p94]-characteristic sequences; MIT, microtubule interacting and trafficking motif; C2, C2 domain; Zn, Zn-finger motif domain; SOH, SOL-homology domain; IQ, a motif that interacts with calmodulin. (*B*) Human calpain (represented by its number) phylogenetic tree, drawn using the neighbour-joining and bootstrap method after aligning all sequences.[4] Non-classical calpains are divided into further subfamilies.

identified as an aggravating factor, probably because the intracellular Ca^{2+} homeostasis is compromised.[32] In such cases, attenuating symptoms by specifically inhibiting conventional calpains is a major objective.[33] In contrast, functional loss of a tissue-specific calpain can perturb the tissues in which it is expressed, as with muscular dystrophy and stress-induced gastric ulcers.[31,34–36] Such systems might enable the identification of biological events in which calpain is important.

3. Calpain system regulatory components

The calpain system has two essential regulatory components, CAPNS1[30 K] and calpastatin. Notably, however, the effect of these molecules, whether positive or negative, is limited to a few calpains, and mostly, to the conventional calpains. Other molecules may exist that govern other calpains or the calpain activity in specific tissues.

3.1 The conventional calpain smaller regulatory subunit, CAPNS1[30 K]

The PEF(S) domain of CAPNS1[30 K] is significantly similar to PEF(L), and the interaction between the fifth EF-hand motifs of CAPNS1[30 K] and CAPN1[μCL] or CAPN2[mCL] forms a heterodimer, resulting in the conventional calpains. The CAPNS1[30 K] GR domain contains hydrophobic Gly-clusters, most of which are autolysed as conventional calpains are activated. Three-dimensional structural analysis shows this domain to have a very soft structure.

CAPNS1[30 K] is an important chaperone-like component for conventional calpains. Without CAPNS1[30 K], during *in vitro* renaturation CAPN2[mCL] is very slow to become active, if it does at all.[37] Consistent with this, both CAPN1[μCL] and CAPN2[mCL] are almost completely down-regulated in *Capns1*$^{-/-}$ mice, resulting in embryonic lethality; these mice rarely survive past E11.5.[30,38] Thus, CAPNS1[30 K] is absolutely required for the stability of both conventional calpain catalytic subunits. So far, CAPNS1[30 K] has only been shown to be necessary for CAPN1[μCL] and CAPN2[mCL]. Although there is one paralogue, CAPNS2[30K-2], in the human genome, its regulatory effect on calpains is unknown.[39] Recent studies showing *Capns1* involvement in osteoblasts and chondrocytes[29,40] suggest that CAPNSs may have as-yet-unknown functions.

3.2 Calpastatin: the one and only specific endogenous calpain inhibitor

Calpastatin is the only known endogenous-specific inhibitor of the conventional calpains.[41] Among calpain homologues so far examined, calpastatin also inhibits CAPN8[nCL-2][42] and CAPN9[nCL-4],[43] but not CAPN3[p94],[44] *in vitro*. Calpastatin is effectively proteolysed by CAPN3[p94], implying that CAPN3[p94] helps regulate conventional calpains in skeletal muscle.[44] One calpastatin molecule contains four inhibitor units (*Figure 2*); each unit inhibits one calpain molecule with variable efficiency.[45–47] Oligopeptides as short as 20 aa derived from these inhibitory units can inhibit calpain, although with reduced efficacy. Calpastatins have poor primary sequence conservation between species, despite their high specificity even to different species' conventional calpains,

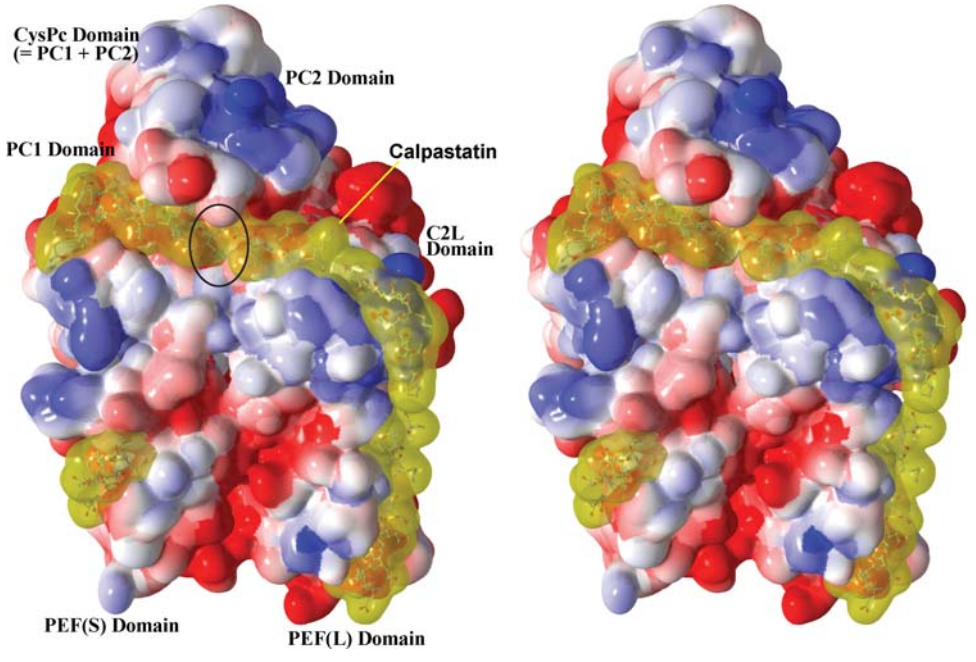

Figure 3 Inactive and active CAPN2/S1[m-calpain] 3D structures. Surface-type schematic 3D structures (cross-eyed view) of active (Ca^{2+}- and calpastatin-bound) CAPN2/S1[m-calpain], using the PDB data 3BOW.[48] Oligopeptides, represented by the yellow surface + ball-and-stick inside, indicate calpastatin tightly bound to CAPN2/S1[m-calpain]; due to its soft structure, some parts of calpastatin are not visible. The active protease domain (CysPc) is formed by the fusion of the PC1 and PC2 subdomains upon the binding of one Ca^{2+} to each subdomain. Though not visible here, the active site exists deep inside at the site circled in black. None of the 10 Ca^{2+} is visible here.

which are highly conserved: humans and rat CAPN1[μCL] are 89% identical, whereas calpastatins are only 66% identical. The 3D structure of CAPN2/S1[m-calpain] co-crystallized with a calpastatin fragment and Ca^{2+} revealed that calpastatin's intrinsically unstructured property enables it to bind calpain tightly (*Figure 3*) while looping several adjacent aa residues out from the active site to protect itself from proteolysis.[48,49]

4. Calpain activation and regulation

4.1 Insights into the structure of CysPc

The first calpain primary structure to be determined was chicken CAPN11[μ/mCL];[50] 15 years later, the Ca^{2+}-free 3D structure of CAPN2/S1[m-calpain] was solved.[16,17] This revealed that the CysPc domain is split into two halves, keeping the active-site residues and the potential substrate-binding cleft in non-functional conformations. Thus, the CysPc domain is characterized as two protease core (PC1/2) domains within one protease domain.[4]

Next, 3D structures of the CAPN1[μCL] and CAPN2[mCL] Ca^{2+}-bound CysPc domains brought three major findings. Firstly, two unique Ca^{2+}-binding sites (CBS-1 and -2) exist in the PC1 and PC2 domains, respectively.[51,52] Secondly, upon binding Ca^{2+}, the PC1 and PC2 domains move towards each other to form the active site. Thirdly, the active-site cleft is somewhat deeper and narrower than those of other papain-like Cys proteases,[53] so the substrates must be in an extended conformation to fit the cleft; this partly explains why calpains preferentially proteolyse inter-domain unstructured regions. Determination of the whole 3D structure of active CAPN2/S1[m-calpain] co-crystallized with calpastatin and Ca^{2+} confirmed the above activation mechanism[48,49] (*Figure 3*).

4.2 Other mechanisms of calpain activation

In mammalian conventional calpains, CAPNS1[30K] must form a heterodimer with CAPN1[μCL] or CAPN2[mCL] to regulate the calpain activity. What, then, is known about the regulation of other calpains?

CAPN3[p94], which is specific to skeletal muscles, has a unique modification within CysPc. Without any subunit, CAPN3[p94] autodegrades very rapidly under physiological conditions; this process depends on the specific insertion sequences IS1 and IS2, located within PC2 and in the linker region between the C2L and PEF domains, respectively[54] (*Figure 2*). This autolytic activity was recently shown to be Na^{+}-dependent in the absence of Ca^{2+}, making this the first example of an intracellular Na^{+}-dependent enzyme.[55] These properties are unique to CAPN3[p94], differentiating it from any other calpains. Some *CAPN3* and *Capn3* stage-specific alternative splice variants, lacking IS1, IS2, or both, show decreased autolytic activity[56] and result in myopathy when overexpressed in muscles.[57] Taken together, these findings suggest that additional aspects of CAPN3[p94] contribute to its CysPc domain regulation, and support CAPN3[p94]'s function under conditions specific to skeletal muscles.

CAPN8[nCL-2] and CAPN9[nCL-4] form the heterodimer CAPN8/9[G-calpain] *in vivo*; heterodimer formation is essential for the stable existence of CAPN8/9[G-calpain], which is the first example of a hybrid heterodimer of two distinct calpain catalytic subunits.[36] Another example regulating calpain activation is that CAPN7[PalBH] interacts with the ESCRT-III protein IST1 (increased sodium tolerance-1) via its MIT motifs, enhancing its autolytic activity.[58]

4.3 Calpain activation mechanism *in vivo*: the Ca^{2+} requirement

One of the classical calpain research questions is how the conventional calpains are activated in the cytosol, since their activation *in vitro* requires a high Ca^{2+}concentration (at least tens of μM) that is seldom available *in vivo*. One explanation is that the vicinity of plasma and endosomal membranes is a favourable niche for calpain activation; *in vitro* experiments have shown that phospholipids, a major component of plasma membranes, lower the Ca^{2+} concentration required to activate calpain.[59–61] Another possibility is that a very small number of calpain molecules, localized to a small region with a high local Ca^{2+} concentration, is sufficient to fulfill calpain's functions.

On the other hand, Ca^{2+}/Na^{+} concentrations change dynamically at the neuromuscular junction (NMJ). Indeed, calpain was activated at the NMJ in muscle cells from patients with slow-channel myasthenic syndrome,[62] in which Ca^{2+} overload occurs at the NMJ due to mutations in the genes encoding nicotinic acetylcholine receptor subunits. In a mouse model of this syndrome, the transgenic (Tg) overexpression of calpastatin ameliorates the symptoms and neuromuscular transmission. These suggest that under the normal condition, calpains should be tightly regulated to elicit proper functions at the NMJ; further studies will clarify which calpains, conventional and/or muscle-specific calpains, are critical to the phenomenon.

4.4 Substrate specificity of calpains

Another classical research question is how calpains' substrate specificities are defined. The substrate specificities of the two conventional calpain species, CAPN1/S1[μ-calpain] and CAPN2/S1[m-calpain], are almost indistinguishable.[63] Some preferences for calpain substrate sequences have been suggested, but a clear rule like that for caspases and trypsins is still elusive. To find such a rule, two major approaches were taken: a recursive method, comparing substrate cleavage-site sequences published thus far, and a deductive method using short oligopeptide libraries.[64–66] Intriguingly, these methods drew somewhat different conclusions; the former studies found T[W > P][L > T > V][K > Y > R]|SPP for the preferred P4-P3-P2-P1-|-P1′-P2′-P3′ sequence (|, cleavage site),[64] whereas the latter found F[F > L > P][L > V][L = F]|[M > A > R]E[R > K].[66] This difference may indicate that suboptimal sequences make better conventional calpain substrates because of their reduced reaction rates, a possible advantage for precise modulation by calpain-mediated proteolysis.[66]

In a more recent approach, a prediction tool was constructed using bioinformatics, that is, to analyse experimental data by a machine learning process.[67] This assists our understanding of calpain-mediated proteolysis by predicting where the cleavage site, if any, is. This allows efficient speculation about possible functions mediated by calpain substrates. A draft version can be found at http://calpain.org.[67]

5. Calpain function in the heart and skeletal muscles

The importance of proteolytic systems for maintaining cellular function is increasingly recognized; calpains are no exception. The

impact of calpain function depends on the particular substrates and conditions investigated,[68–74] as illustrated by the calpain activity in skeletal and cardiac muscles.

5.1 Skeletal muscle homeostasis and CAPN3[p94]

The first tissue-specific calpain, the skeletal muscle-specific CAPN3[p94], was identified in 1989.[23] Although CAPN3[p94] is a classical calpain, it contains three additional regions: NS (located at the N-terminus), IS1, and IS2. These give CAPN3[p94] a rapid autolytic activity and specific binding to connectin/titin, an elastic filamentous muscle protein of >3000 kDa. In 1995, *CAPN3* mutations were discovered to be responsible for limb-girdle muscular dystrophy type 2A (LGMD2A).[31] Accordingly, *Capn3*$^{-/-}$ mice have an LGMD2A-like phenotype.[34,35] So far, *CAPN3* mutations and LGMD2A have the only clearly demonstrated cause-and-effect

Table 1 Calpains involved in cardiovascular diseases

Types of disorders	+/−[a]	Model system (Animal)	Calpain[b]	Inhibition or activation of calpain(s)	Substrates	Ref.
Cardiac hypertrophy and cardiomyocyte loss	–	Right ventricular pressure overload (feline)	1 and/or 2	ZLNal[c]	(gelsolin?)	89
Cardiac contractile dysfunction	–	Right ventricular pressure overload (swine)	1 and/or 2	ZVFal[d]	—	90
Cardiac infarction and DNA damage	–	Coronary artery occlusion/ reperfusion (rat)	1 and/or 2	ALLNal[e]	—	91
Cardiac infarction and apoptosis	–	Ischaemia/reperfusion (rabbit and rat)	1 and/or 2	ALLNal, ZVFal	Bid	92
Cardiac contractile dysfunction	–	Ischaemia/reperfusion (rat)	1 and/or 2	Leupeptin	SR proteins	93
Cardiac infarction and contractile dysfunction	–	Coronary artery occlusion/ reperfusion (swine)	1 and/or 2	A-705253	—	115
Cardiac infarction, dysfunction, and apoptosis	–	Ischaemia/reperfusion (mouse)	1	Conditional CAPN1 Tg[f], conditional PKCα fragment Tg[f], calpastatin Tg[f], *Capn1*$^{-/-}$	PKCα	107
Myocardial hypertrophy/ fibrosis (associated with type 1 diabetes)	–	Ove26 Tg[g] and streptozotocin-injection (mouse)	1 and/or 2	Cardiac-specific *Capns1*$^{-/-}$	—	94
Hyperglycaemia	–	Streptozotocin injection (rat)	1	ZLLal[h], PD150606	—	95
Hyperglycaemia with hypoinsulinaemia	–	Zucker diabetic fatty rat	1	Anti-sense nucleotide, ZLLal	—	96
Hypertension, cardiovascular hypertrophy, and perivascular inflammation	–	Angiotensin-II infusion (mouse)	1 and/or 2	Calpastatin Tg[i]	Spectrin	97
Atherosclerosis and abdominal aortic aneurysms	–	*Ldlr*$^{-/-}$ and angiotensin-II infusion (mouse)	1	BDA-410	Spectrin-1	98
Atherosclerosis	–	*Ldlr*$^{-/-}$, *Apoe*$^{-/-}$ (mouse)	2	ALLMal[j], ZLNal, siRNA	VE-cadherin	99
Lethality	–	CAPN1 or CAPN2 Tg over-expression (mouse)	1 and 2	CAPN1 Tg, CAPN2 Tg[k]		105
Lethality with cardiomyocyte necrosis	–	CAPN1 Tg over-expression (mouse)	1	CAPN1 Tg[f]	Desmin, PKCα	105
(No phenotype)	NA	CAPN2 Tg over-expression (mouse)	2	CAPN2 Tg[f]		105
Dilated cardiomyopathy and atrial arrhythmias	+	Calpastatin Tg over-expression (mouse)	1	Calpastatin Tg[k]		105
Cardiomyopathy, cardiac dysfunction, and plasma membrane damage	+	Transverse aortic constriction, β-adrenergic stress (mouse)	1 and/or 2	Cardiac-specific *Capns1*$^{-/-}$		110

[a] + or − indicates calpain(s) play a roles as an ameliorating or aggravating factor, respectively. NA, not applicable.
[b] '1' and '2' stand for CAPN1/S1[μ-calpain] and CAPN2/S1[m-calpain], respectively.
[c] Benzyloxycarbonyl-Leu-Norleucinal, also called calpeptin.
[d] Benzyloxycarbonyl-Val-phenylalaninal, also called calpain inhibitor III or MDL-28170.
[e] Acetyl-Leu-Leu-Norleucinal, also called calpain inhibitor I.
[f] tetracycline-suppressible ('tet-off') *Myh6* promoter-driven conditional Tg mice.
[g] FVB(Cg)-Tg(ins2-CALM)26OveTg(Cryaa-Tag)1Ove/PneJ Tg.
[h] Benzyloxycarbonyl-Leu-leucinal.
[i] Cytomegalovirus immediate-early enhancer/promoter-driven conventional Tg mice.
[j] Acetyl-Leu-Leu-methioninal, also called calpain inhibitor II.
[k] *Myh6* promoter-driven conventional Tg mice.

relationship between a calpain gene mutation and human disease; thus, LGMD2A is also called calpainopathy.

Studies using CAPN3[p94] knock-in (*Capn3*$^{CS/CS}$) mice, which have a structurally intact but protease-inactive CAPN3[p94]:C129S mutant, showed that compromised CAPN3[p94] protease activity is primarily responsible for LGMD2A, and that Ca^{2+} release from the sarcoplasmic reticulum (SR) is reduced in *Capn3*$^{-/-}$, but not *Capn3*$^{CS/CS}$, muscles.[75–77] Thus, two independent activities of CAPN3[p94], proteolytic and non-proteolytic, contribute to its physiological functions.[76]

In *Capn3*$^{CS/CS}$ mice analysed under exercise conditions, the adaptive up-regulation of heat-shock proteins and of muscle ankyrin-repeat protein-2 (MARP2, also called Ankrd2), a muscle-specific transcriptional regulator, is compromised.[75,78] Altogether, it is hypothesized that pathogenic *CAPN3* mutations disrupt this calpain's ability to control multiple homeostatic mechanisms in skeletal muscles, resulting in LGMD2A.

CAPN3[p94] is expressed at much lower levels in the heart than in skeletal muscle, to the point of being undetectable.[79] However, the proteins it interacts with, such as connectin/titin, MARP2,[80] and MARP1 [also called Ankrd1, or cardiac ankyrin-repeat protein (CARP)],[81] exist in both cardiac and skeletal muscles. This implies that another protease in the heart, most probably conventional calpains, may undertake the role played by CAPN3[p94] in skeletal muscle. In this context, it is intriguing that gene mutations causing small deletions of the human connectin/titin C-terminus, which contains a CAPN3[p94]-binding site, down-regulate CAPN3[p94] and produce early-onset myopathy with fast progressive dilated cardiomyopathy, leading to death.[82] It was recently shown that myospryn/C5orf10/CMYA5 (cardiomyopathy associated 5), which is a filamentous protein of ~450 kDa with fibronectin type III motifs and a SPRY domain at its C-terminus, interacts with both CAPN3[p94] and the connectin/titin C-terminus.[83]

CAPN3[p94] binds not only to the connectin/titin C-terminus, but also to its N2A region, where binding sites for MARP1, MARP2, and MARP3 [also called Ankrd23, or diabetes-related ankyrin-repeat protein (DARP)] are located.[84] CAPN3[p94] and MARP2 accumulate at the N2A region under stress, suggesting that the connectin/titin N2A region is a molecular base for sensing physical muscle cell stress at the sarcomere level.[75,84–86] Notably, CAPN1[μCL] is also reported to localize to the N2A region.[87] Although expressed at very low levels in normal skeletal muscle, MARP1 is also a CAPN3[p94] substrate.[81] Intriguingly, mutations in MARP1 are found in some cases of dilated cardiomyopathy.[88]

5.2 Calpains in cardiovascular diseases

It was recently reported that calpain activity is necessary for cardiac cell function (*Table 1* and *Figure 4*). However, as mentioned earlier, increased conventional calpain activity has often been reported as an aggravating factor in cardiovascular diseases and other pathophysiological conditions. Some of candidate calpain substrates related to cardiovascular system are shown in *Table 2*.

5.2.1 Is calpain activity undesirable in the heart?

Many conditions that damage the cardiovascular system appear to be improved by inhibiting calpains. Contractile dysfunction after acute pressure overload, which was reported to be associated with calpain activation, is ameliorated by inhibiting calpains.[89,90] Calpain activation, apoptosis, and the proteolysis of several substrates, including Bid and the SR proteins, have been reported in cardiac ischaemia or infarction.[91–93] Again, inhibiting calpain activity ameliorates these pathological conditions. Diabetes-associated cardiovascular complications often result in morbidity and mortality. Heart-specific *Capns1* disruption mitigated the myocardial hypertrophy and fibrosis in type 1 diabetes model mice.[94] In other words, knocking down both CAPN1/S1[μ-calpain] and CAPN2/S1[m-calpain] reduced the cardiac hypertrophy and fibrosis in these mice. Accordingly, inhibition of calpains by inhibitors and/or antisense nucleotides improved hyperglycaemia in streptozotocin-injected rats[95] and Zucker diabetic fatty rats,[96] animal models for type 1 and 2 diabetes, respectively.

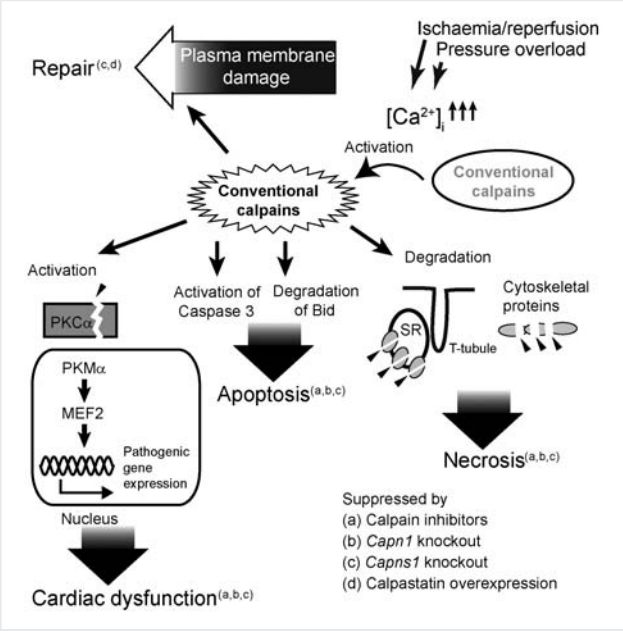

Figure 4 Multiple involvements in cardiovascular diseases. Schematic of calpains involved in cardiovascular diseases; details of each report are given in *Table 1*.

There are other examples where calpain inhibition reduces disease severity. Chronic infusion of angiotensin-II and/or low-density lipoprotein receptor-deficiency [*Ldlr*$^{-/-}$] in mice cause cardiovascular disorder such as hypertension and atherosclerosis. Tg overexpression of calpastatin in mice suppressed left ventricle and media hypertrophy, perivascular inflammation and fibrosis associated with angiotensin-II-induced hypertension.[97] Atherosclerosis and abdominal aortic aneurysms induced by angiotensin-II infusion in *Ldlr*$^{-/-}$ mice were also attenuated by administration of the calpain inhibitor.[98] CAPN2/S1[m-calpain], but not CAPN1/S1[μ-calpain], is overactivated in the atherosclerosis of endothelial cells (ECs). Miyazaki *et al.*[99] reported that both the CAPN2[mCL] level and proteolysis of vascular endothelial cadherin (VE-cadherin) are increased in aortic ECs from human patients suffering from atherosclerotic lesions, and from *Ldlr*$^{-/-}$ mice; CAPN2[mCL] siRNA and calpain inhibitors prevented this disorder's progression. Although there is no report on relationship between acute coronary syndrome (ACS) and calpain activity, these findings (exacerbation of atherosclerosis[99] as well as angiotensin-II-induced vascular inflammation[97] and abdominal aortic aneurysms[98] by calpain activity) link calpain activity to ACS, if not directly. As mentioned above, VE-cadherin is one of targets of CAPN2/

Table 2 Modulation of muscle-related proteins by calpain-mediated proteolysis

Substrate	Accession No.	Cleavage site(s) after	Calpain(s)[a]	Effect of proteolysis	Ref.[b]
Annexin I	NP_000691	26	1	Enhancement of Ca^{2+} sensitivity	116
Ezrin	NP_062230	467	1	Liberation from apical membrane	117
Glutamate receptor, ionotropic, NMDA 2A	NP_036705	1278, 1329	1	Dissociation from PSD-95	118
Insulin-like growth factor-binding protein 4 (IGFBP4)	NP_001543	23, 107, 143, 159	1	Reduction in IGF avidity	119
Interleukin 1α (IL-1α)	NP_000566	118	1	Maturation and secretion	120
Ras homologue gene family, member A (RhoA)	NP_001655	180	1	Dominant negative (inhibition of integrin-induced stress fibre assembly) effect	121
Spectrin β	NP_001020029	2058	1	Membrane skeleton reorganization?	122
Talin 1	NP_035732	433	1	Redistribution of the talin functional domain	123
Transient receptor potential canonical 6 (TRPC6)	NP_038866	16	1	Down-regulation	124
Troponin T2, cardiac (TnTc)	NP_035749	71	1	Altered affinities to TnI and tropomyosin	125
BH3 interacting domain death agonist (BID)	NP_001187	70	2	Induction of apoptosis	126
Calcineurin	NP_058737	421, 422, 423, 425	2	Activation	127
Caspase 9	NP_001220	115, 330	2	Inactivation	128
ErbB-1, epidermal growth factor receptor (EGFR)	NP_005219	683, 733, 1030, 1059, etc.	2	Down-regulation	129
NFκB inhibitor α (IκBα)	NP_065390	50	2	Activation of NFκB	130
Integrins β1, 2, 3, 7	NP_002202, etc.	771, 777, etc.	2	Dissociation from cytoskeleton	131
Phospholipase C β1	NP_777242	880	2	Loss of Gαq interaction	132
Vimentin	NP_035831	18, 20, 32, etc.	2	Turnover	133
BCL2-associated X protein (BAX)	NP_620116	28	1 or 2	Pro-apoptotic effect	134
Caspases 3, 7, 9	NP_116786, NP_001218, NP_001220	7; 36; 115, 120, 143, etc.	1 or 2	Activation	135,136
Filamin A	NP_001447	1761	1 or 2	Change in actin avidity	137
α-Actin-1	NP_001091	39	1 and/or 2	Pro-apoptotic effect	138
Protein kinase C α, β, γ	XP_001081588, etc.	309, 316, 324 , etc.	1 and 2	Activation	139
PDLIM1	NP_066272	271	3	Reduced avidities to interacting molecules?	140
Connectin/titin	NP_596869	8563, 8651, 8652; 8506	1 and 3	Myofibril turnover?	44,80

[a]'1', '2', and '3' stand for CAPN1/S1[μ-calpain], CAPN2/S1[m-calpain], and CAPN3[p94], respectively.
[b]The referenced reports describe calpain cleavage sites, and do not necessarily describe their relevance to muscles.

S1[m-calpain],[99] and is also expressed to coronary arteries. Therefore, it is possible that inhibition of calpain ameliorates ACS, or at least suppresses acceleration of ACS.

Another example of the involvement of calpain activity in cardiovascular disorders is myocardial stunning (also called broken-heart syndrome, stress/Takotsubo cardiomyopathy, or transient left-ventricular apical ballooning),[100] which is a form of dysfunction caused by post-ischaemic reperfusion. Several reports point to proteolytic degradation of cardiac troponin I (cTnI) by calpain as a possible cellular mechanism underlying the depressed contractile function.[101,102] There are, however, some controversial reports, demonstrating that no cTnI degradation was detected during porcine or canine myocardial stunning,[103,104] and that myocardial-specific overexpression of CAPN1[μCL] in Tg mice showed no clear cTnI degradation in the heart.[105] Although cTnI proteolysis probably affects myocardial contractile functions,[106] these counter-evidences suggest that cTnI degradation is not a cause but a result of ischaemia/reperfusion. Ischaemia/reperfusion causes calpain activation resulting in proteolysis of several proteins; however, it is still open to question which protein degradation is responsible for myocardial stunning. As discussed earlier, calpains have certain consensus for preferential sequences for substrates.[64,66] Thus, if knock-in mice that have mutated sequence in the calpain cleavage sites of cTnI are generated, they will show whether or not cTnI degradation is essential for myocardial stunning as they only express cTnI mutant unproteolysed by calpains.

In addition to all of these findings, it has been proposed that calpains negatively impact these cardiovascular diseases by overproducing constitutively active PKCα.[107] Conventional PKCs, which have an intramolecular regulatory domain, are usually latent. Conventional calpains cut off this regulatory domain to produce activated PKC, which is called PKM, the catalytic fragment of PKC.[108] Overactive

calpains produce excess PKM in an ischaemic heart; once produced, the kinase remains active regardless of upstream receptor signals, and causes various substrates, such as myosin-binding protein-C and histone deacetylase 5 (HDAC5), to be overphosphorylated. This triggers cycles of morbid cellular responses such as constitutive nuclear HDAC5 export, and subsequently causes cardiomyopathy.[107,109]

5.2.2 Calpains are essential for the heart!

A few reports have shown that conventional calpain activity is required for cardiovascular health. Galvez *et al.*[105] constructed Tg mice that overexpressed CAPN1[μCL], CAPN2[mCL] or calpastatin. The analysis of these mice demonstrated that calpastatin overexpression using the cardiac α-myosin heavy chain gene (*Myh6*) conventional promoter, which inhibited 58% of the endogenous CAPN1/S1[μ-calpain] activity in these mice, caused slow-progressing dilated cardiomyopathy. This result indicates that the activity of conventional calpains, especially CAPN1/S1[μ-calpain], is essential to heart function. The phenotypes of other Tg mice overexpressing CAPN1[μCL] or CAPN2[mCL], as summarized in *Table 1*, show that too much calpain activity appears damaging, although conditional overexpression of CAPN2[mCL] was tolerated under the examined context.

More direct evidence of the requirement for conventional calpains in cardiac functions against haemodynamic stress was published recently. Using a *Capns1* flox (flanked by loxP sequences) deletion construct and *Myh6* promoter-driven Cre Tg mice, Taneike *et al.*[110] designed a conditional *Capns1* knockout and cardiac-specifically disrupted CAPNS1[30K], thus down-regulating both CAPN1[μCL] and CPAN2[mCL]. These cardiac-specific *Capns1*$^{-/-}$ mice had normal global cardiac structure and function under normal conditions. However, when 10-week-old mice were subjected to pressure overload through transverse aortic constriction (TAC), the resulting fibrosis was significantly larger in the knockout than in the wild-type mice. Intriguingly, both knockout and wild-type mice developed cardiac hypertrophy, indicating that conventional calpain activity was not the primary cause of the TAC-induced hypertrophy. These knockout mice exposed to β-adrenergic stress through isoproterenol infusion developed cardiac dysfunction, again indicating the protective role of conventional calpains under stress. As also seen in cultured fibroblast cells,[73,111] cardiomyocytes from the knockout mice showed defective membrane repair,[110] indicating that one of the conventional calpains' ubiquitous physiological functions relates to membrane repair.

Another clue to protective/pathogenic roles of calpains may involve matrix metalloproteinase-2 (MMP-2). Historically, MMP-2 was considered to function on extracellular matrix substrates; however, several reports recently showed that the detrimental effect of MMP-2 may occur primarily within the myocytes.[112] Furthermore, MMP-2 targets a similar subset of proteins (including cTnI) as calpains, and, surprisingly, calpastatin may inhibit MMP-2 also.[112] Therefore, it is possible that at least part of bad reputation of calpains in cardiovascular disorders is responsible for MMP-2.

6. Conclusions and perspectives

Calpain's involvement in skeletal muscle and cardiovascular systems has been the subject of much interest and research. Many studies have shown that the activation of calpains, especially the conventional calpains, exacerbates pathophysiological conditions. However, recent technical advances have allowed us to delve deeper into the true nature of the calpain system. Genetic manipulations to disrupt *Capns1* constitutively or conditionally in mice cause embryonic lethality[30,38] and cardiac hypertrophy,[110] respectively, indicating that calpains are indispensable both to heart-specific functions and to life itself. In short, calpains are a double-edged sword: they are essential for various aspects of life, especially under stress conditions, yet their activation tends to be destructive in cells undergoing pathophysiological chaos.

In the process of revising this article, another review focusing on the role of calpains in multiple aspects of cardiovascular illness had come out.[113] As was discussed in the article as well as in this review, regulation of calpain activity stands as an important issue in developing therapeutic reagents for heart failure. In this respect, it should be noted that some pathological states may result from insufficient calpain activity. Genetic defects in tissue-specific calpains cause various diseases, which may be improved by activators or stabilizers for these calpains, and cardiovascular system and calpain functions therein would be no exception. One of the most urgent needs in this research field is the ability to detect real-time calpain activity *in vivo* at a high resolution for both physiological and pathophysiological conditions. As calpain research enters this new era, both basic science and translational biomedical studies are well positioned to launch comprehensive calpain studies.

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