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Calcium, troponin, calmodulin, S100 proteins: From myocardial basics to new therapeutic strategies

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Abstract

Ca^{2+} acts as global second messenger involved in the regulation of all aspects of cell function. A multitude of Ca^{2+}-sensor proteins containing the specific Ca^{2+} binding motif (helix-loop-helix, called EF-hand) developed early in evolution. Calmodulin (CAM) as the prototypical Ca^{2+}-sensor with four EF-hands and its family members troponin-C (TNC), myosin light chains, and parvalbumin originated by gene duplications and fusions from a CAM precursor protein in prokaryotes. Rapid and precise regulation of heart and skeletal muscle contraction is assured by integration of TNC in the contractile structure and CAM in the sarcolemmal L-type Ca^{2+} entry channel and in the sarcoplasmic Ca^{2+} release channel RYR. The S100 proteins as evolutionary latecomers occur only in the animal subphylum vertebrates. They are not involved in switching on and off key cell functions but rather operate as modulators. In the heart S100A1 modulates Ca^{2+} homeostasis, contractile inotropy, and energy production by interaction with the elements involved in these functions. The binding properties of different Ca^{2+}-sensor proteins associated with specific regulatory and modulatory functions in muscle are discussed in detail. Some of these sensor proteins are critically involved in certain diseases and are now used in clinical diagnostics.

Keywords: Calcium signalling; Calmodulin; Cardiac contractility; Parvalbumin; S100 proteins; Troponin; Myosin light chains; Evolution of calcium-sensor proteins

Tribute to professor Setsuro Ebashi

With the present overview on calcium binding proteins involved in Ca^{2+} signalling in the heart we want to pay due deference to the late Prof. Setsuro Ebashi from the University of Tokyo, Japan, whose original views and unrelenting scientific pursuit were decisive in unraveling how calcium regulates muscle contraction. Ebashi played a decisive role in the discovery of three major aspects, (i) description of the so-called "relaxing factor", (ii) Ca^{2+} functioning as signal messenger, and (iii) discovery of the troponin complex [1].

After establishment of Ca^{2+} as intracellular second messenger, a breathtaking endeavour successively discovered an ever increasing number of proteins functioning as Ca^{2+} signal targets leading up from basic research to translation into clinical relevance particularly in the field of heart diseases. Here we summarise this saga of "calciomics" as it evolved from troponin-C over calmodulin to dozens of S100 proteins as known today, a story paradigmatically echoing the biologic evolution of one of the largest protein superfamilies with 66 subfamilies and over 200 human genes coding for proteins containing this particular type of Ca^{2+} binding motif [2–5]. This is preceded by a brief account of the general basics of Ca^{2+} distribution and intracellular Ca^{2+} handling in vertebrates which sets the stage for the function of the Ca^{2+} sensing proteins. Their phylogenetic evolution will be briefly outlined for a deeper insight into the functional diversity among the individual Ca^{2+}-sensor proteins.

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Calcium signalling for muscle contraction, how come?

At the end of the 19th century Sidney Ringer (1883) obtained the first hint that Ca^{2+} was involved in regulation of contraction in the isolated rat heart [6]. Cardiac muscle failed to contract when the bathing saline was free of Ca^{2+}, whereas action potential could still be observed [7]. This notion came ahead of its time and it took another 60 years to demonstrate that indeed Ca^{2+} may serve as trigger for contraction when added to the cut ends of frog muscle [8], and as activator of the ATPase of myosin-B as actomyosin was called these days [9].

In 1951 Bozler [10] demonstrated relaxation of glycerinated muscle fibres by addition of EDTA. Since EDTA binds both Ca^{2+} ($pK_1 = 10.5$) and Mg^{2+} ($pK_1 = 8.7$) with high intrinsic affinity [11] there was still some ambiguity left as to the precise chelating effects of EDTA in the muscle system. Only later, the Schwarzenbach group in Switzerland [12] synthesized a new chelator "glycolcomplexon", now known as EGTA (ethyleneglycol-bis(beta-aminoethylether)-*N*, *N*′-tetraacetic acid), which binds Mg^{2+} ($pK_1 = 5.2$) with an affinity 6 orders of magnitude lower than Ca^{2+} ($pK_1 = 11$) [11]. EGTA then unambiguously allowed in the 1950s to selectively chelate Ca^{2+} over Mg^{2+} and thus demonstrate the requirement of trace amounts (around 10^{-6} to 10^{-5} M) of free Ca^{2+} for activation of the myofibrillar MgATPase [13–15]. Induction of muscle contraction by electrical impulses was known since the work of Galvani in Bologna in the late 18th century [16], but it was Sandow who in 1952 first suggested a direct role of Ca^{2+} in the process of excitation–contraction coupling [17].

Muscle relaxation

Muscle relaxation is another matter. Relaxation appears when tension disappears, but the muscle will not lengthen unless it is pulled out by some external forces. Several different mechanisms including soluble enzymes and nucleotides such as cAMP have been implied in relaxation until it was recognised that Ca^{2+} again plays the major role, but this time by its absence. Anything that removes Ca^{2+} efficiently from free solution without damaging the rest of the system allows for relaxation of glycerol-extracted muscle models. The relaxing factor first described by Marsh in 1951 [18] turned out to represent the lipid-rich granular or microsomal fraction containing a potent Mg^{2+}-activated ATPase originally isolated 1948 by Kielley and Meyerhof [19], now called the sarcoplasmic reticulum (SR) with its ATP consuming Ca^{2+}-pump (SERCA). The entangled path leading to the discovery of the SR and its function as Ca^{2+}-store in muscle has been competently portrayed by Davies [20] and Ebashi [1].

Why was calcium chosen to serve as second messenger?

Why has nature chosen a simple inorganic divalent cation such as Ca^{2+} as highly specialised intracellular signal transmitter [21–24]? First, calcium is one of the most abundant elements on earth and in biological systems. The adult human skeleton comprises ~1 kg of Ca^{2+} mainly in the form of hydroxyapatite representing 99% of the total Ca^{2+} in the body. Outside the skeleton, in the extracellular fluids (ECF) and in the cells, Ca^{2+} amounts to 10–15 g while the total amount of Mg^{2+} in the body is about 25 g one-third of it being intracellular. Second, divalent cations such as Ca^{2+} or Mg^{2+} can form more stable and specific complexes with organic substances including proteins than monovalent cations such as Na^+ or K^+, but they do not form covalent bonds in biology. Third, calcium as member of the 4th element period (four electron shells) has a more complex electron configuration than magnesium (3rd period with three electron shells) allowing for greater flexibility of calcium in coordination with protein ligands (coordination numbers usually 6–8, but up to 12 are possible) in an irregular pentagonal bipyramid. In contrast, Mg^{2+} (ionic radius = 0.64 Å) is much smaller than Ca^{2+} (ionic radius = 0.97 Å) displaying an ionic volume of only 44% compared to that of Ca^{2+}, and requires a fixed octahedron geometry with six coordinating oxygen atoms in binding to proteins. Fourth, ligand binding by Ca^{2+} is ~1000 times faster than with Mg^{2+}. Finally, energy production in all living cells relies on phosphoryl bond breakdown and Ca^{2+} more readily than Mg^{2+} forms insoluble salts with the originating inorganic phosphate or pyrophosphate which limits its solubility to around 10^{-3} M. This lower solubility of calcium salts required Ca^{2+} to be ejected from the cytoplasm while Mg^{2+} serves as cofactor in enzyme reactions involving the MgATP complex.

Excitation–contraction coupling

The eukaryotic cells and the excitable myocytes of skeletal and heart muscle, in particular, evolved an intricate and well-balanced pump and channel system which controls the ion fluxes and ion gradients across the cell membrane [23–27] (Fig. 1). This ensemble ensures a 10,000-fold gradient between the extracellular ionised biologically active Ca^{2+} concentration of around 10^{-3} M and 1–2×10^{-7} M in the cytoplasm at rest, and entertains a resting potential of around −80 mV at the inner side of the surface membrane (sarcolemma). On the other hand, the concentration of Mg^{2+} is around 10^{-3} M both inside and outside the muscle cell. In the cytoplasm Mg^{2+} is largely buffered by complexing with ATP and proteins. The lipid bilayers of the cell surface membrane as well as of the intracellular organelles like SR and mitochondria are virtually impenetrable for charged ions. The ionised Ca^{2+} in the ECF and in the blood of around 0.9 mM is one of the most tightly controlled physiological parameters involving the endocrine system comprising parathormone, calcitonin, and vitamin D3 (cholecalciferol).

For induction of muscle contraction, depolarisation of the sarcolemma during the action potential (AP) lets small amounts of Ca^{2+} enter the cell via the voltage-dependent

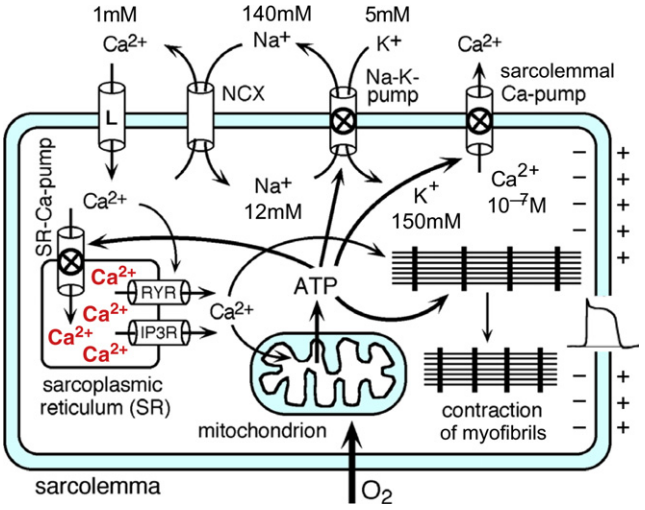

Fig. 1. Simplified schematic of a cardiomyocyte displaying the major ion pumps, channels, and exchanger for intracellular Ca^{2+} handling including approximate intra- and extracellular ion concentrations. The Ca^{2+} concentration in the cytoplasm at rest ($\sim 10^{-7}$ M) is almost 10 times lower than in ultrapure bidistilled water after passage over an ion exchange resin. In humans one-third of the Ca^{2+} required for one heartbeat enters via the sarcolemmal Ca^{2+}-channel during depolarisation and activates Ca^{2+}-release from the SR via RYR. As much Ca^{2+} as enters must be ejected mainly by the Na^{+}–Ca^{2+}-exchanger (the sarcolemmal Ca^{2+}-pump PMCA contributes only negligibly to Ca^{2+} extrusion from the myocyte). Ca^{2+} can also be released from SR by the second messenger inositol trisphosphate (IP3) via the IP3R Ca^{2+} release channel. Charge distribution at rest and shape of action potential are given at the sarcolemma on the right. ATP using ion pumps are marked by encircled crosses. IP3R, SR inositol-trisphosphate Ca^{2+}-release channel (in heart IP3R2); L, L-type Ca^{2+}-channel (DHPR); NXC, Na^{+}–Ca^{2+}-exchanger; RYR, SR ryanodine Ca^{2+}-release channel (in heart RYR2).

L-type Ca^{2+}-channels (DHPR) that activate the SR Ca^{2+}-release channel (RYR) leading to a Ca^{2+} wave that sweeps through the entire myocyte (Ca^{2+}-induced Ca^{2+}-release from the SR) transiently increasing the cytoplasmic Ca^{2+} about 20-fold to over 10^{-6} M. The L-type Ca^{2+}-channels are referred to as dihydropyridine (widely used Ca^{2+}-channel blocking drugs) receptor (DHPR) and the SR Ca^{2+}-release channel as ryanodine (poisonous plant alkaloid) binding receptor (RYR). RYR1 is the skeletal muscle isoform while RYR2 is expressed in the heart [24]. The stored Ca^{2+} in the SR may reach concentrations higher than 10 mM mainly bound with relatively low affinity ($K_D \sim 10^{-3}$ M) to calsequestrin. Cardiac calsequestrin ($\sim$45 kDa) is a highly acidic protein with over 30% negatively charged Asp and Glu residues and can bind $\sim$40 Ca^{2+} per molecule. In this article binding affinities are given as dissociation constants, i.e. the ligand concentration at half saturation at the binding sites.

By going from rest to activity the energy requirement in skeletal muscle increases by several tenfold [28]. This requires a coordinated stimulation of energy production in the mitochondria which is assured by the same Ca^{2+} transients involved in excitation–contraction coupling (Fig. 1). In the heart contractions follow rhythmically neither reaching maximal force development at systole nor complete relaxation during diastole. Thereby 20–30% of the total energy used by the working heart gets consumed by the ion pumps [24,29,30]. Also in the heart the energy production in the mitochondria is controlled by Ca^{2+} that spills over from the cytoplasmic Ca^{2+} transients for control of some Ca^{2+}-dependent key enzymes of the tricarboxylic acid cycle and the ATP synthase (complex V of the respiratory chain).

Ca^{2+} is thus vital for the regulation of muscle contraction and energy production, it also regulates many other cellular functions including secretion- and transcription-coupling, synaptic transmission, hormonal regulation, control of cell cycle, fertilization, and vision [27]. It can, however, only function as intracellular messenger against its low cytoplasmic background level. Since in the extracellular compartment Ca^{2+} occurs in the millimolar concentration range it cannot function as signal transmitter, though it does bind to many proteins with low to moderate affinities ($K_D = 10^{-4}$ to 10^{-3} M) providing structural–functional integrity (e.g. proteins in the blood clotting cascade). In its millimolar extracellular concentration Ca^{2+} represents, however, the most toxic substance. Any uncontrolled influx of Ca^{2+} induces a cell's irrevocable death. Most of this scenario was not known in the 1960s of last century when the race was on, what and where might be the target sites for the Ca^{2+} signal in muscle. Conceptually the intracellular Ca^{2+}-sensors must respond to the Ca^{2+} transients triggered by the action potential and this requires Ca^{2+} binding sites with affinities in the range between 10^{-6} and 10^{-5} M.

Discovery of troponin-C and calmodulin

Since Ca^{2+} triggered the activity of crude actomyosin but not that of reconstituted actomyosin prepared from separately purified actin and myosin, the concept of Ca^{2+} acting as specific messenger was not readily accepted. In order to tackle this problem, in the early 1960s, Ebashi rather "reluctantly" (as laid down in his own words in the Croonian Lecture [1]) turned his interest to protein chemistry. In Perry's laboratory it was observed that low ionic strength extraction at pH 8 from rabbit myofibrils yielded a viscous solution containing actin, tropomyosin, and a third fraction provisionally called component-C [31], now known as troponin. It was not immediately realised that this protein fraction was able to confer Ca^{2+}-sensitivity to pure actomyosin until Ebashi in 1963 reported that a muscle mince extract containing mainly tropomyosin ("native" tropomyosin as it was first called) restored Ca^{2+}-sensitivity to ATP-induced superprecipitation of reconstituted actomyosin [32]. Subsequently, mainly the three laboratories of Ebashi (Tokyo), Gergely (Boston), and Perry (Birmingham, UK) were involved in purifying and characterising troponin. By 1973 a general consensus was reached that troponin consists of three subunits whose names indicate their roles: the Ca^{2+} binding troponin-C (TNC $\sim$18.4 kDa), the inhibitory troponin-I (TNI $\sim$24 kDa), and the tropomyosin binding troponin-T

(TNT ~35.9 kDa) [33–36]. Detailed descriptions of regulation of cardiac muscle contraction by the Ca^{2+}-sensor TNC and the mechanistic interaction with its two partners TNI and TNT on the actin filament have recently been published [37–39].

Calmodulin (CAM ~16.8 kDa) was the second intracellular Ca^{2+}-sensor to be discovered in 1970 by Cheung [40] and Kakiuchi's group [41] in the brain. It was found to activate the brain cyclic nucleotide phosphodiesterase. The Ca^{2+} affinity (K_D 1–10 μM) to the purified activator protein from bovine heart muscle was determined three years later [42]. Based on its physico-chemical and functional properties this protein has successively been called cyclic phosphodiesterase activator, modulator protein, calcium-dependent regulator, and troponin-C-like protein until its close relation to TNC was corroborated by amino acid sequence homology in 1980 ([43], and references therein). Calmodulin was then commonly adopted as acronym for "Ca^{2+}-dependent modulator". At that time CAM was known to activate a number of enzymes in a Ca^{2+}-dependent manner such as cyclic nucleotide phosphodiesterases, plasma membrane ATPases, myosin light chain kinase, NAD kinase in plants, and muscle phosphorylase kinase and protein kinase activities in synaptosomal preparations. Today we know that CAM can interact with more than 100 different proteins, enzymes as well as structural proteins. It regulates numerous fundamental cellular activities including glycogen metabolism, intracellular motility, calcium transport, cyclic nucleotide metabolism, protein phosphorylation and dephosphorylation, cell cycle progression, regulation of gene expression, and many others.

CAM is a cytoplasmic protein interacting with soluble as well as structural components in different cell compartments, while TNC is specifically integrated in the sarcomeric actin filament structure of striated muscle (heart and skeletal muscles) for the specialised function of regulation of contraction.

Parvalbumin and the EF-hand motif

Parvalbumin (PARV) was the first Ca^{2+} binding protein to have its amino acid sequence [44] and atomic 3D-structure [45] resolved in 1973. PARV-like proteins were found in large quantities in the muscles of fishes and amphibians [46]. These proteins, earlier called "myogens", are highly water-soluble and have an isoelectric point between 4.0 and 4.5. Due to its size (~12.1 kDa) that was much smaller than for example bovine serum albumin (~66.4 kDa), the main component was then renamed parvalbumin (parvus = latin for small) [46]. Two PARV isoforms from two different genes exist in vertebrates including humans, alpha and beta, mostly with 109 amino acid residues (AA) and 108 AA, respectively. The PARVbeta is also called oncomodulin as it was first found in tumour tissue. In the teleost zebrafish there are nine paralogue PARV genes yielding isoforms with overall protein identity of 31% [47]. The considerable variations occurring at the nucleotide level in the 5′- and 3′-untranslated regions (UTR) of the nine genes are thought to allow for developmental- and tissue-specific regulation.

On the basis of the PARV structure Kretsinger developed the concept of the Ca^{2+} binding EF-hand (helix-loop-helix) motif [45]. The PARV structure contains six consecutive alpha-helices interspersed by short loops and linker regions. The helices are labelled A through F with the C-terminal pair (E and F) forming a Ca^{2+} binding site with a 12 AA loop between the two helices. This configuration can be depicted by extending the index finger (representing helix-E) and extending the thumb (representing helix-F) from the fist, while the bent middle finger embraces the bound metal, thus the EF-hand. From sequence comparison this domain structure has become a highly conserved Ca^{2+} binding motif found to date in more than 600 Ca^{2+} receptor proteins grouped into at least 66 subfamilies; the human genome alone may contain up to 100 EF-hand containing proteins [21,48]. In fact, PARV contains three homologous EF-hand motifs, but only the two C-terminal ones (with helices CD and EF) are able to bind Ca^{2+} with high affinity.

Calcium ligation to the canonical EF-hand

CAM represents the prototypical intracellular Ca^{2+}-sensor containing four canonical Ca^{2+} binding sites. The canonical EF-hand motif consists of 29 AA of which residues 1–10 form the entering helix-E, residues 10–21 (12 AA) the loop around the Ca^{2+}, and 19–29 the exiting helix-F [21,48,49]. Ca^{2+} is bound in the loop with seven oxygen ligands in a pentagonal bipyramidal arrangement, with six of the ligands from the protein and one oxygen atom supplied by a water molecule. In all four binding loops of CAM with 12 AA each, metal coordination is provided at positions 1, 3, 5, 7, 9, and 12. Residues 1, 3, and 5 provide monodentate ligands, and residue 12 a bidentate ligand, via side-chain carboxylates. In all four sites Asp (D) is at positions 1 and 3, and Glu (E) is at position 12, while position 5 contains either Asp (D) or Asn (N). At position 7 either Thr (T), Tyr (Y) or Gln (Q) provides a backbone carbonyl oxygen ligation. The water-mediated coordination at position 9 involves a different AA in all four sites. The consensus sequence of the 12 residues in the four binding loops is presented in the AA one-letter code with X for any AA: DXDGDGTIXXXE.

The EF-hands are organised in pairs that together form a stable four-helix bundle, one such domain in the N- and a second in the C-terminal part of the molecule (Fig. 2). The pairing of EF-hands enables cooperativity in the binding of Ca^{2+} ions [50]. In certain proteins, however, EF-hand motifs may also occur in odd numbers or even as single motif. Proteins with up to 12 EF-hands have been described, whereby some EF-hands may display variations in the AA sequence incompatible with Ca^{2+} binding [48,49].

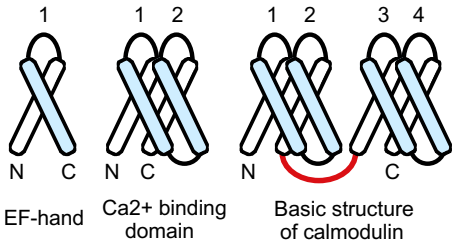

Fig. 2. Hierarchical structure of EF-hands in the prototypical calmodulin. The fundamental Ca^{2+} binding motif is the helix-loop-helix EF-hand. Usually the EF-hands appear in adjacent pairs, one pair being referred to as N-(terminal), the other as C-(terminal) domains. A linker (symbolised in red) connects the two domains by exiting from the downstream helix of EF-hand 2 and entering into the upstream helix of EF-hand 3. Helices are symbolised by straight bars; EF-hands are numbered; and N- and C-termini are indicated.

In CAM two Ca^{2+} bind with a tenfold lower affinity (K_D $\sim 10^{-5}$ M) to the N-domain than the two Ca^{2+} binding to the C-domian (K_D $\sim 10^{-6}$ M). This allows CAM to sense Ca^{2+} transients in the cytoplasm over a relatively wide concentration range [21,49–51]. At low cytoplasmic Ca^{2+} in the resting state, most of the CAM molecules will be in the apo-form devoid of Ca^{2+}. In the apo-form CAM assumes a dumbbell structure with an extended alpha-helical linker between the N-domain and the C-domain. When Ca^{2+} binds to CAM the linker between the two domains bends round and CAM assumes a more globular shape ready to wrap around a substrate recognition site. At the same time the two domains undergo conformational changes exposing hydrophobic patches that favour target protein interaction [52]. As Ca^{2+} binds to the four sites with cooperativity almost all CAM molecules will be fully activated during a Ca^{2+} transient ensuring a tightly controlled "all-or-none" response thus a clean separation between the "on" and "off" states.

CAM must be able to selectively bind Ca^{2+} in the background of at least 1000 times greater concentrations of Mg^{2+}. As the binding affinity of CAM for Mg^{2+} is about 10,000-fold lower than for Ca^{2+}, it will not seriously interfere with the Ca^{2+} signal. However, during the resting state some sites of CAM may become occupied by Mg^{2+}. Since the binding kinetics of Mg^{2+} are much slower than for Ca^{2+}, the slow exchange rate at those sites occupied by Mg^{2+} could delay the spread of the Ca^{2+}-CAM signal. Some AA variations in the metal binding loop as well as in the flanking two helices may in fact not only modulate the affinity for Ca^{2+} but also that for Mg^{2+}.

Adaptation of calcium and magnesium binding to specialised function

Modulation of the binding characteristics for both Ca^{2+} and Mg^{2+} by deviations in the AA sequence from that of the canonical EF-hand in CAM was found in several Ca^{2+}-sensor proteins including PARV and TNC. The specific function of TNC in regulation of muscle contraction required some modification of the prototypic CAM model.

There are two types of TNC in striated muscle encoded by two separate genes, skeletal (sTNC) and cardiac (cTNC) (for reviews, see [37,39]). The cTNC is the common Ca^{2+}-sensor also for slow contracting skeletal muscle. Both TNC isoforms (~18 kDa) comprise four EF-hands, sites 1 and 2 in the N-domain and sites 3 and 4 in the C-domain like in CAM (Fig. 2). In contrast to CAM, the TNC isoforms exhibit considerable structural modifications not only in comparison to CAM but also among their tissue-specific isoforms (over 30% differences in AA sequences among all three proteins) presumably due to functional selection pressure [43,55]. Isolated sTNC is still able to bind four Ca^{2+}, while cTNC binds only three Ca^{2+} with the first EF-hand motif being defunct (Fig. 3). The C-terminal (carboxy-terminal) two EF-hands bind Ca^{2+} in both sTNC and cTNC with very high intrinsic affinity ($K_D \sim 10^{-8}$ M). However, Mg^{2+} also binds to the two C-terminal sites with moderately high affinity ($K_D \sim 10^{-4}$ M), thus in presence of physiological concentrations of Mg^{2+} (1–2 mM), the apparent affinity for Ca^{2+} binding is lowered by two orders of magnitude [54,55]. On the other hand, Ca^{2+} binding to the N-terminal (amino-terminal) sites in sTNC or to the single N-terminal site in cTNC ($K_D \sim 10^{-6}$ M) is not affected by the presence of Mg^{2+} because its affinity to these sites is as low as $\sim 10^{-2}$ M. Full contractile response occurs with Ca^{2+} bound to the N-terminal EF-hands irrespective of the occupancy of the C-terminal sites with either Ca^{2+} or Mg^{2+}. Taken together, the N-terminal EF-hands

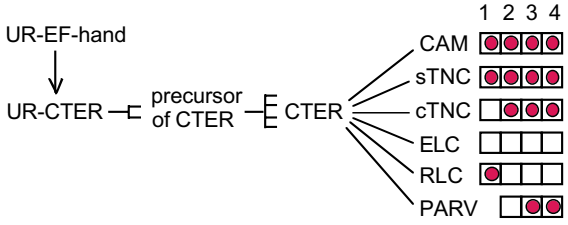

Fig. 3. Evolution of the EF-hand protein family CTER comprising calmodulin (CAM), troponin-C (TNC), essential (ELC), and regulatory (RLC) myosin light chains. For TNC are given the two isoforms skeletal (sTNC) and cardiac (cTNC). Parvalbumin (PARV) is also included. All these proteins originally contained four EF-hand motifs and form a congruent protein family. The single UR-EF-hand duplicated without fusion to form the single motif UR-CTER. The UR-CTER subsequently duplicated with fusion to form the two-motif precursor CTER. Two EF-hand units facing one another comprise a globular Ca^{2+} binding domain. The precursor CTER duplicated with fusion once more to give rise to the common four EF-hand CTER family. Subsequent gene duplications without fusion in organisms ancestral to animals, plants, fungi, and protists produced the genes for the subfamilies of CAM, TNC, ELC, RLC, and PARV. PARV seems to have undergone a subsequent deletion of the EF-hand 1. The number of EF-hands (squares) and those of them that have preserved the ability to bind Ca^{2+} (red dots) are indicated for contemporary proteins of the CTER family. Adapted from references [21,48,53]. For further explanations, see text.

are the Ca^{2+}-specific regulatory sites with a sufficiently high affinity for sensing Ca^{2+} signals, while the C-terminal EF-hands are always occupied by a divalent metal ion be it Mg^{2+} or Ca^{2+}. The C-domain of both sTNC and cTNC thus needs the divalent metal ion for structural integrity within the troponin complex, which is attached via TNT to tropomyosin in the actin filament.

PARV is mostly found in nervous tissue and in fast contracting muscle of small animals (e.g. rodents). It may serve as buffer in tissues where the trigger Ca^{2+} needs to be removed quickly. PARV consists of three EF-hands (numbered 2, 3, and 4), the first of which (number 2) does not bind Ca^{2+}. The number-1 motif is inferred to have been deleted altogether [48,49]. PARVs from different sources bind either two Ca^{2+} or two Mg^{2+} both with high intrinsic affinity (K_D for Ca^{2+} $\sim 10^{-7}$ M and for Mg^{2+} $\sim 10^{-5}$ M) in the two C-terminal sites 3 and 4 (Fig. 3) [56,57]. In mixtures both metals compete for the same two sites as is also the case for the two C-terminal sites in TNC. It is thus concluded that in the myocytes at rest with basal ion concentrations of ~100 nM for Ca^{2+} and 1 mM for Mg^{2+}, two Mg^{2+} are bound to PARV. An increase of cytoplasmic free Ca^{2+} levels to 1 μM or higher results in dissociation of Mg^{2+} from PARV and subsequent binding of two Ca^{2+} ions. Because of the slow exchange rate of bound Mg^{2+}, the Ca^{2+} trigger for contraction first binds to the low affinity regulatory Ca^{2+} sites of TNC in the myofibrils. Fast relaxation is initiated and enhanced by shifting the Ca^{2+} from the low affinity regulatory sites of TNC to the high affinity sites of PARV in the cytoplasm. The delay in Ca^{2+} binding to PARV explains why Ca^{2+} can still act as trigger for contraction in fast muscles. Subsequently, the Ca^{2+} from PARV is taken up into the SR by the SERCA Ca^{2+}-pump, which has a Ca^{2+} affinity of around 10^{-7} M [58]. In general, low affinity Ca^{2+}-specific sites that bind Ca^{2+} with very fast on-rates of $\sim 10^{8}$ M^{-1} s^{-1} (Ca^{2+} binding is only limited by diffusion) are ''triggering sites'' [59], while high affinity Ca^{2+}–Mg^{2+} sites may be seen as either ''relaxing sites'' (in PARV) or ''structural sites'' (in the C-domain of TNC).

Evolution of the CTER protein family

The EF-hand is one of the most frequently used motifs in multiple proteins found in bacteria, archaea, and eukaryotic systems [5,60]. As mentioned earlier, every living cell must protect its cytoplasm from the poisonous Ca^{2+}. It is therefore not surprising that also prokaryotes tightly control their cytoplasmic Ca^{2+} concentrations by use of EF-hand Ca^{2+} binding proteins. In bacteria the cytoplasmic Ca^{2+} seems to play a major role in chemotaxis, differentiation, and cell division, although the function of most of their Ca^{2+} binding proteins remains elusive.

The origin of the CAM gene still lies in the dark. Almost 500 canonical EF-hand Ca^{2+} binding motifs with diverse cellular functions, but not a single pseudo EF-hand, have been identified to date in various bacterial genomes [5,60]. From this it may be concluded that pseudo EF-hand motifs as found in the large family of S100 proteins are phylogenetically younger than the canonical EF-hands. Besides several hundred prokaryotic proteins with one predicted EF-hand, around 40 contain multiple EF-hand motifs ranging from two up to six and can phylogenetically be divided into several groups. The appearance of penta- (calpain) and hexa-EF-hand (calretinin and calbindin-D28k) proteins could tentatively be explained by phylogenetically gene replications. The number of AA in the Ca^{2+} binding loop can vary from 11 up to 14 in these EF-hand proteins [52]. In the majority of the known EF-hand motifs the Ca^{2+} binding loop is coupled to two flanking alpha-helices which confer a strong propensity to dimerisation. Nevertheless, the isolated loop from the third CAM motif was transplanted into a host scaffold protein and shown to bind Ca^{2+} while remaining monomeric in solution [61].

Since the eukaryotic protists, which are thought to derive from a monophyletic origin, contain CAM, it is assumed that the compact structure of CAM with four EF-hand motifs welded together (Fig. 2) may have surfaced already in prokaryotes. The abundant single EF-hand-like motifs in the genomes of bacteria could provide clues for the origin of the prototypical EF-hand [62–64]. Furthermore, this prokaryotic CAM must have been functionally so successful that it was carried over into the eukaryotic lineage and, consequently, is present in all five kingdoms of life including fungi, plants, and animals [5,65,66].

On the basis of sequence alignment of both nucleotides and AA the four-motif CAM structure must have arisen by two gene duplications and fusions (Fig. 3). The EF-hands 1 and 3 and the hands 2 and 4 have the highest internal homology [48,67]. About a dozen extant EF-hand proteins including TNC, essential myosin light chain (ELC), regulatory myosin light chain (RLC), and PARV have arisen by duplications of the CAM gene without subsequent fusion that also must have occurred in organisms ancestral to animals, plants, fungi, and protists. However, this gene diversification was coupled with subfunctionalisation leading to considerable modifications of genes and proteins. The first EH-hand in cTNC is defunct, in RLC only the first EF-hand has preserved Ca^{2+} binding, the ELC has totally lost Ca^{2+} binding capacity, and only the last two EF-hands still bind Ca^{2+} in PARV (Fig. 3). This family of Ca^{2+} binding proteins is called CTER, an acronym composed from the first character of CAM, TNC, ELC, and RLC [5,48].

How does calmodulin overcome its conservatism?

CAM is one of the most conserved proteins with 100% AA sequence identity among all vertebrates [67–71]. It possesses 149 AA including the N-terminal Met which may be removed in the mature protein (~16.8 kDa). Around 500 million years ago, a common ancestor bequeathed three CAM genes for all vertebrates. Mammals and fish separated 450 million years ago, and birds separated from mammals about 150 million years later. Mammals includ-

ing humans still display three separate genes all coding for a 100% identical CAM molecule. Frogs and birds might have lost two of the genes. Comparison of the three mammalian bona fide CAM genes with the single gene from chicken and frog indicated that the latter are most closely related to the mammalian CAM-II gene, suggesting that CAM-I and CAM-III were derived from CAM-II by gene duplication [67]. On the other hand, the teleost zebrafish genome contains six separate genes which were derived from the three precursor orthologues, all producing a single CAM protein that is 100% identical to those of the other vertebrates [70]. Some more AA substitutions were permitted in CAM of fungi, plants, and invertebrates. But the AA sequence of CAM from the worm *Caenorhabditis elegans* still shares 96% identity with the common vertebrate form [68]. The genome of the plant *Arabidopsis thaliana* contains seven CAM genes coding for only four almost identical CAM isoforms that are still 89% identical to vertebrate CAM [66].

How is CAM able to orchestrate the intracellular calciome of diverse multicellular organisms in view of its conserved protein structure, and why do many of these organisms conserve multiple genes for producing one single protein? Two plausible explanations may be available. First, comparison of the 5′- and 3′-UTRs from vertebrate and zebrafish genes revealed gene-specific differences. In species with several genes these distinct UTRs may permit regulation of CAM levels at discrete cellular sites during differentiation and in highly specialised cell types such as neurons or striated muscle cells [67,69,70].

Second, CAM not only changes conformation on Ca^{2+} binding in solution but its structure is also massively affected by the binding to various substrates. The N- and C-domains of CAM wrap around the target peptides. The target peptides themselves often present as random coil in solution, adopting alpha-helical structures in the complex with CAM [72,73]. More recently a specific CAM binding motif (IQ) was defined that in many structural proteins including different types of myosins and cytoskeletal proteins can bind CAM in the presence or absence of bound Ca^{2+} [74,75]. The consensus sequence of this binding motif starts with IQ that is used for the acronym: IQXXXRGXXXR (I = Ile, Q = Gln, R = Arg, G = Gly, and X for any AA). In myosins either one or multiple IQ motifs are located on the heavy chains between the motor domain and the tail domain. In conventional myosin-II the ELC and RLC bind to the IQ motifs, while in most other myosin classes CAM binds to the IQ motifs [76]. Thus CAM does not only bind to targets when it is saturated with Ca^{2+} but it firmly associates with many proteins in its apo-form as Ca^{2+}-sensor subunit in a multimer. Relevant for regulation of Ca^{2+} homeostasis in the heart is its association with cardiac RYR2, Cav1.2 (pore forming subunit of the DHPR Ca^{2+}-channel), cardiac IP3R2 (inositol trisphosphate-operated Ca^{2+} release channel of the SR), and phosphorylase kinase [24]. Apart from this, single EF-hand motifs are also found integrated in the primary structure of RYR2, calcineurin-B, dystrophin, calpain proteases and vertebrate phosphodiesterases. The function and putative Ca^{2+} binding capacity are poorly understood.

As Ca^{2+}-sensor CAM has fast binding kinetics with an on-rate for Ca^{2+} of $\sim 10^8\ M^{-1}\ s^{-1}$ ensuring that pre-bound CAM can activate or modify the associated proteins on a millisecond time scale as has been shown in the case of the DHPR Ca^{2+}-channel [77]. The Ca^{2+} off-rate is variable between different EF-hands over a 1000-fold range depending on the nature of the AA in the ninth position (water-mediated coordination) in the EF-hand [78]. Similar variability in off-rate for Ca^{2+} can be seen in CAM depending on its interaction with different target proteins. Dissociation rates for Ca^{2+} may become so slow that the activation of CAM and its target persists even after the cytoplasmic Ca^{2+} concentration has returned to low resting levels [79]. CAM is thus able to individually regulate a myriad of intracellular Ca^{2+}-sensitive processes despite its conserved protein structure. This is not en example of neo- or sub-functionalisation but rather a case of the much rarer co-functionalisation (a multigene—one protein system).

The S100 protein family, an evolutionary latecomer

The first S100 protein was isolated from bovine brain by Moore in 1965 [80] and is now known as S100B. It was a small (~10.7 kDa) acidic protein soluble in 100% saturated ammonium sulphate solution and thence the name "S100" survived. The S100 proteins are expressed exclusively in vertebrates and represent the largest subgroup within the superfamily of EH-hand Ca^{2+} binding proteins displaying unique properties distinct from the EF-hand proteins in the CTER family [4]. To date over 20 human S100 genes are known, of which 17 are tightly clustered in a region of the human chromosome 1q21 that is frequently rearranged in cancers [81–83]. The rapid development of the field with a steady flow of new family members appearing required the nomenclature to be continuously adjusted [4,82,84]. The adopted nomenclature designates the S100 genes in the chromosomal cluster 1q21 as S100A followed by Arabic numbers (S100A1, S100A2, S100A3, etc.). In contrast, S100 genes from other chromosomal regions outside this cluster carry the stem symbol S100 followed by a single letter (S100B, S100C, etc.).

A related gene family is present in the same gene cluster (1q21) and encodes for proteins containing an S100-like domain fused to a larger peptide. These proteins include trichohyalin, filaggrin, and repetin, which are multidomain proteins involved in epidermal differentiation and are classified as a separate family [85].

The S100 proteins (molecular weights varying between 9 and 13 kDa) contain two Ca^{2+} binding motifs, a classical C-terminal EF-hand with a canonical Ca^{2+} binding loop (with 12 AA as in CAM) and an S100-specific N-terminal EF-hand with a modified Ca^{2+} binding loop (with 14 AA) called "pseudo EF-hand". These pseudo EF-hands

are exclusively found in the N-domain of S100 and S100-like proteins [5,85]. The canonical EF-hands are highly conserved among all S100 proteins, while significant sequence variation may occur in the Ca^{2+} binding loop of the pseudo EF-hands. Some pseudo EF-hands have even lost the Ca^{2+} binding ability as in S100A10.

Since no pseudo EF-hands are found in the bacterial genomes the tissue- and cell-specific expression profiles of the S100 proteins are not predicted by the Ca^{2+} binding proteins found in bacteria. In addition, no S100 genes were found in *Arabidopsis thaliana*, *Drosophila melanogaster*, *Caenorhabditis elegans*, or *Saccharomyces cerevisiae*. In evolutionary terms, the lowest organism reported thus far containing a pseudo EF-hand protein closely related to S100A1 is a chondrichthye (dogfish Squalus acanthias) [2,5]. As the vertebrates originated ~500 million years ago in the Ordovician period it must be assumed that this also marks the evolutionary start of the S100 protein family that now can be found in fishes, amphibians, reptiles, birds, and mammals. Pseudo EF-hands always pair with canonical EF-hands, but canonical EF-hands already occur in pairs in bacterial genomes. Thus in contrast to the ancient canonical EF-hand the pseudo EF-hand must come from a more recent origin. Evolution of EF-hands creating the sequence diversity of pseudo EF-hands might have been achieved by gene duplication or exon recombination from a CAM-type precursor protein with subsequent loss of two of the four EF-hands. Thus the S100 proteins end up with only two Ca^{2+} binding sites.

The fact that several S100 proteins are present in human, but absent in rat and mouse (S100A2, S100A12, S100P, S100Z), lineages that separated ~75 million years ago, may illustrate progressing evolution of this protein family. Duplication of the human S100A7 gene into four additional genes (including two pseudo genes) supports the hypothesis of its rapid evolution and expansion [2,4,86]. These additional S1007-like gene copies, some of which seem to be nonfunctional in humans, are absent in rodents. Most S100 genes cluster on different chromosomes in human (1q21), rat (2q34), and mouse (3F1-F2). In each case these chromosomal regions are close to the skin development gene cluster implicating the origin of S100 proteins in the evolution of the skin (ectodermal germ layer). Human and rodents exhibit similar expression patterns with high levels of S100B in the brain, S100A1 in the heart, and skin-specific expression of S1007.

Structure and function of S100 proteins

The basic structural and functional unit of the S100 proteins is a symmetric antiparallel dimer of two molecules each containing one canonical and one pseudo EF-hand connected by a central hinge region [4,50,87]. Starting from the N-domain the helices flanking the Ca^{2+} binding loops are numbered 1 through 4. In the dimer helix-1 of the N-terminal pseudo EF-hand in one subunit and helix-4 of the C-terminal canonical EF-hand of the second subunit form together a stable four-helix bundle. A second interaction occurs vice versa at the other end of the two subunits thus forming together a stable eight-helix bundle. The large dimer interfaces are highly hydrophobic inducing dimerisation even at picomolar concentrations. Upon Ca^{2+} binding, the C-terminal canonical EF-hands expose a concave hydrophobic surface that is required for target recognition, while the N-terminal pseudo EF-hands do not drastically change shape. In fact, one dimer unit saturated with Ca^{2+} presents two symmetrically disposed target binding sites.

The pseudo EF-hands evolved largely varied Ca^{2+} affinities from μM to mM in order to meet the versatile requirements of cells from different tissues and subcellular compartments. Dimeric S100 proteins bind four Ca^{2+} per dimer [50,86,87]. In both canonical and pseudo EF-hands the Ca^{2+} is coordinated in a pentagonal bipyramidal configuration. In the canonical EF-hands of the C-domain Ca^{2+} is coordinated like in CAM. However, in the N-terminal pseudo EF-hand Ca^{2+} binding occurs in the loop via backbone oxygen atoms and only by one carboxylate side group of Glu in position-14 (the last AA of the loop entering helix-2) providing an oxygen for strong bidentate coordination. The predominant involvement of backbone carbonyl oxygens in Ca^{2+} binding may allow for much greater AA sequence variability in the loop of the pseudo EF-hands concomitant with modulation of metal binding affinities. The affinity for Mg^{2+} is usually rather low (K_D > mM), so that at resting low Ca^{2+} levels no metal may bind to the N-domain.

All S100 proteins with the exception of S100G form functional homo- as well as heterodimers. Interestingly, in contrast to all other known S100 proteins, S100G does not act as signalling protein but rather as a Ca^{2+} buffer [4,86]. Even higher oligomers like tetramers, hexamers and octamers are observed displaying particular structures which might be required for specific functions [88–90]. In addition to their intracellular functions, several S100 proteins are secreted upon Ca^{2+} signalling via vesicle fusion with the cell membrane into the extracellular space, where they might acquire oligomeric structures specialised for extracellular functions [4]. For instance, the crystal structures of the tetrameric S100A8/S100A9, the hexameric S100A12, and the octameric S100B complexes are all different. However, all of these multimeric structures bind Ca^{2+} ions in addition to those in the EF-hands. These additional Ca^{2+} ions are bound at the interfaces of the subunits presumably stabilising the larger structures. It is hypothesized that the oligomerisation requires high Ca^{2+} levels as present in the extracellular space, where these complexes act as pro-inflammatory signalling components through activation of RAGE (receptor for advanced glycation end-products) and Toll-like receptor-4 [86,88,91].

Zinc affects calcium signalling by S100 proteins

Beside Ca^{2+}, several S100 proteins also bind Zn^{2+} with a wide range of affinity ($K_D = 4$ nM to 2 mM) [87,92].

S100A3 displays by far the highest affinity for Zn^{2+} ($K_D = 4$ nM) and at the same time, the lowest affinity for Ca^{2+} ($K_D \sim 20$ mM), implying that S100A3 functions rather as Zn^{2+} signalling than as Ca^{2+} signalling protein. S100A2 mainly localises to the nucleus and functions as tumour suppressor by interacting with p53 [93]. Two Zn^{2+} ions can bind to S100A2 with high affinity (apparent $K_D = 25$ nM) being coordinated by Cys and His residues between the two subunits outside the Ca^{2+} binding loops ([92], and references therein). No common structural Zn^{2+} binding motif was detected. Binding of Zn^{2+} to S100A2 reduces the Ca^{2+} affinity by 300 times at one EF-hand and induces tetramer formation.

The total intracellular Zn^{2+} concentration is of the order of several hundred μM, but the majority of Zn^{2+} is bound to zinc finger proteins, metallothioneins, and to reduced glutathione. The free intracellular Zn^{2+} concentration is low ranging from pM to nM levels. However, the concentrations of free Zn^{2+} change rapidly upon cellular stimulation and may reach μM levels sufficiently high to be sensed by the nuclear S100A2 [92,94]. The large number of Zn^{2+}-dependent DNA binding and regulatory proteins including S100A2 suggests that changes in free Zn^{2+} may directly be translated into altered gene expression. Interestingly, Zn^{2+} binding to other S100 proteins like S100B or S100A12 (coordinated by His and Asp or Glu) increases their affinity for Ca^{2+} and thus directly links Zn^{2+} and Ca^{2+} signalling. In the extracellular space the concentrations of Zn^{2+} are much higher in the μM to mM range. Here Zn^{2+} is able to precipitate oligomer formation of secreted S100 proteins (S100B or S100A12) for extracellular functions [88,91]. An example for Ca^{2+}-independent but Zn^{2+}-dependent target protein recognition is the interaction of S100B with the Tau protein from neurons (Tau protein is involved in neurofibrillary tangle formation in Alzheimer's disease) [95]. Some S100 proteins are also able to bind Cu^{2+}. S100B highly expressed in brain was shown to efficiently sequester Cu^{2+} and suppress Cu^{2+}-induced cell damage [96].

Function of S100A1 in the heart

Generally, S100 proteins are associated with multiple targets that promote cell growth and differentiation, motility, regulation of cell cycle, and transcription as well as cell surface receptor activities [50,97,98]. Abnormal S100 gene expression has been associated with various diseases including chronic inflammation, tumour progression, Alzheimer, psoriasis, and cardiomyopathy [97,99].

S100A1 (~10.5 kDa, [94]) is preferentially abundant in the human heart (cardiomyocytes), although it is also found in lower amounts in skeletal muscle, brain, and kidney [100,101]. This distribution pattern also holds for mouse, rat, and pig (for further references, see [102]). Much lower amounts of other S100 isoforms are also present in cardiomyocytes including S100A4, S100A6, and S100B. S100A1 was also shown to form heterodimers with S100A4 and S100B [103]. S100A1 mRNA and protein levels steadily increase during embryonic development and reach a plateau in the postnatal state with the highest expression in left ventricles and descending concentrations in the right ventricle and the atria. The predominant location of S100A1 in the human heart was recently confirmed by genome-wide transcriptome analysis [104,105].

On the subcellular level, S100A1 was reported to associate, besides its cytoplasmic occurrence, with the sarcolemma, junctional and longitudinal SR, sarcomere, intercalated discs, and mitochondria of ventricular cardiomyocytes ([106], and references therein). At the molecular level, S100A1 was shown to interact in a Ca^{2+}-dependent manner with the cardiac isoforms of RYR2, SERCA2A, phospholamban (PLN), titin, and the mitochondrial F1-ATP synthase in complex V of the respiratory chain [99,102]. The major locations of S100A1 in the cardiomyocyte are schematically given in Fig. 4 (red symbols). These target proteins represent the important regulators of cardiac excitation–contraction coupling and energy homeostasis. Subsequent intensive research over the last decade employing gain- and loss-of-function studies on intact hearts and on isolated cardiomyocytes in vivo and ex vivo yielded a deeper insight into the mechanisms involved in control of cardiac contractility by S100A1 competently reviewed in [102].

RYR2

S100A1 was shown to exert a dual effect by interacting with RYR2. Stimulation of RYR2 by S100A1 increases systolic performance through enhanced Ca^{2+}-induced Ca^{2+} release (CICR) from the SR [102]. On the other hand, by enforcing closure of the RYR2 channel during diastole S100A1 reduces the Ca^{2+} spark frequency thus reducing Ca^{2+} leak from the SR [107]. Consequently, more Ca^{2+} will

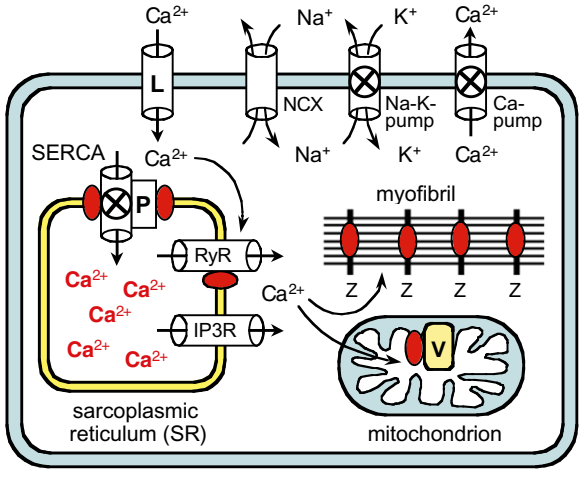

Fig. 4. Schematic showing the major interaction sites of S100A1 protein in the cardiomyocyte (red symbols). In immunostaining S100A1 localises to the Z-disc, functionally it interacts with the extensible region of titin (see text). In mitochondria S100A1 interacts with F1-ATP synthase in complex V of the respiratory chain. P, phospholamban. Other symbols and abbreviations as in legend to Fig. 1. For further explanations, see text.

be retained in the SR that is available for enhancement of subsequent contractions.

SERCA2A

S100A1 stimulates Ca^{2+} uptake into the SR not only by direct interaction and stimulation of the Ca^{2+}-pump SERCA2A, but also by combining with PLN and thus derepressing its inhibitory effect on SERCA2A [102]. Both effects together significantly fasten relaxation by rapid removal of cytoplasmic Ca^{2+} after contraction.

Titin

Ca^{2+}-dependent interaction with titin presents an additional mechanism furthering contractility not involving Ca^{2+} cycling. The giant protein titin (~3000 kDa) comprises a third sarcomeric filament system (in addition to actin and myosin filaments) with elastic properties. The titin molecules start with the N-terminus at the Z-disc and run near the actin filaments in the I-band (non-overlap region) and along the myosin filaments in the A-band (actin and myosin filaments overlapping) over a distance of more than 1 μm up to the middle of the sarcomere in the M-band. In the extensible region in the I-band (between Z-disc and the tips of the myosin filaments), cardiac titin contains a cardiac-specific splice element, either N2B or N2BA, followed by the elastic PEVK (with over 70% Pro, Glu, Val, and Lys) domain [108]. The PEVK domain joining the N2B isoform contains 163 AA. N2BA titin contains a much longer PEVK domain with 600–800 AA, plus an additional immunoglobulin domain in the N2BA element. As a result, N2BA titin has a longer extensible region and higher molecular mass than N2B titin (~3300 versus ~2970 kDa). Both N2B and PEVK elements interact with the actin filaments in the I-band near the Z-disc [109]. This interaction with actin contributes to passive tension and resists filament sliding during contraction. S100A1 can specifically bind to the N2B (or N2BA) and PEVK regions of titin in a Ca^{2+}-dependent manner. Binding of S100A1 to the extensible region releases titin from the actin filaments thereby reducing passive tension. This action of S100A1 contributes to facilitation of contraction. Interestingly, no tension producing interaction with actin is observed with skeletal muscle titin lacking the N2B element [109].

ATP synthase in mitochondria

The significant fraction of S100A1 in mitochondria suggests their involvement in the energy metabolism [102,106,110]. Indeed, S100A1 directly interacts in dependence of Ca^{2+} in the mitochondrial matrix with the alpha- and beta-chains of the F1-ATPase at the inner mitochondrial membrane stimulating the ATP production. Consequently, increased and decreased S100A1 protein concentrations result in a reciprocal energetic phenotype with augmented and reduced ATP levels, respectively [110].

Taken together, S100A1 supports positive inotropy (contractile force) and lusitropy (relaxation) by a concerted interaction at different target sites. Moreover, the contractile reserve that can be mobilised by S100A1 occurs in the absence of beta-adrenergic signalling involving cAMP and the protein kinase-A (PKA) and CAM-dependent kinase-II (CaMKII) phosphorylation systems [102]. The intracellular actions of S100A1 are independent of trans-sarcolemmal Ca^{2+} fluxes. S100A1 does not affect Ca^{2+} entry via DHPR, or efflux via the Na^{+}–Ca^{2+}-exchanger (NCX) in forward or reverse mode [111]. Finally, S100A1 couples cardiac Ca^{2+} cycling with Ca^{2+}-dependent mitochondrial energy production [102].

Pathophysiological relevance of S100A1 in heart failure

The lesson learnt from transgenic animal models indicates that heterozygous and homozygous S100A1 knockout (SKO) mice have unaltered baseline heart functions, but they display impaired contractile function in response to beta-adrenergic stimulation in vivo [112]. Thus heterozygous SKO hearts with only 50% of S100A1 protein levels compared to wild-type hearts exhibit an identical defective phenotype to homozygous SKO hearts. This indicates that over 50% of normal left ventricular S100A1 protein levels are required for cardiac adaptation to acute haemodynamic stress, a finding that challenges the current understanding, according to which beta-adrenergic stimulation suffices to cope with increased mechanical demand by signalling via cAMP and downstream phosphorylation cascades [24,102]. On the other hand, transgenic overexpression of S100A1 (STG) provides chronically improved contractile function without concomitant development of cardiac hypertrophy as it occurs under long-term beta-adrenergic overstimulation. Thus the S100A1-induced cytoplasmic Ca^{2+} fluxes seem to bypass the effects of chronically elevated Ca^{2+} levels leading to hypertrophy as occurring not only on beta-adrenergic stimulation, but also under increased activity of angiotensin-II, endothelin-1 or prostaglandin PGE2 [24,113].

In conclusion, defective mobilisation of Ca^{2+} from intracellular stores may be responsible for the impaired inotropic reserve in S100A1-deficient hearts [102]. Indeed, failing human myocardium displays a marked loss of S100A1 expression at both the transcriptional and translational levels [114]. In the mouse heart infarct model signalling via Gq-coupled receptor agonists (e.g. endothelin-1) and protein kinase-C (PKC) was recently shown to lower S100A1 mRNA and protein abundance leading to greatly accelerated progression towards cardiac failure accompanied by excessive mortality [115]. Intracoronary S100A1 gene delivery in clinically relevant experiments with animal models of chronic and acute heart failure restored the diminished S100A1 protein levels, resulting in short- and long-term rescue of cardiac function [116–118]. These findings might be taken as proof-of-hypothesis for providing new therapeutic approaches to heart failure [4,102].

Clinical implications of CTER members (CAM, TNC, ELC, RLC, PARV)

Calmodulin-dependent protein kinase-II in adverse heart remodelling

CAM consists of 149 AA (16.8 kDa). Due to its central position as Ca^{2+}-sensor in all types of cells it is assumed to be involved in many diseases including Parkinson, Alzheimer, and rheumatoid arthritis [119–121]. However, no disease-related mutations in either of the three CAM genes have been reported so far. Instead, rather defects in some of the many reaction partners of CAM might be responsible for disease symptoms. Several classes of hydrophobic drugs including phenothiazines, naphthalene sulphonamides, imidazoles, and dihydropyridines bind to CAM with affinities in the micromolar range and all of them inhibit its ability to activate target enzymes [51]. However, unwanted side effects rather than specific therapeutic use may derive from these relatively nonspecific drug interactions with CAM.

A particularly prominent regulator of cardiac function is the CAM-dependent protein kinase-II (CaMKII) relaying the Ca^{2+} signal to cardiac targets by phosphorylation [24,122,123]. On activation by Ca^{2+}-loaded CAM (CaCAM), CaMKII progressively phosphorylates its subunits (autophosphorylation of 6 up to 12 subunits, ~55 kDa each, in the oligomeric CaMKII) in dependence on frequency and intensity of the Ca^{2+} transients. Autophosphorylation increases the affinity of CaCAM to CaMKII about 1000-fold up to K_D $\sim 10^{-10}$ M (called "CAM trapping") keeping the enzyme on sustained activity far beyond the duration of a single Ca^{2+} transient [24]. Unlike S100A1, whose actions remain restricted to intracellular Ca^{2+} handling, the multifunctional CaMKII activates besides RYR2, SERCA2A and PLN also the DHPR producing a sustained increase in intracellular Ca^{2+}. CaMKII phosphorylates in several instances the same target sites as PKA, which latter via cAMP is directly regulated by beta-adrenergic signalling [24,123]. However, activity and expression of CaMKII are also increased by beta-adrenergic stimulation [124]. Collectively, PKA and CaMKII seem to function in a temporally coordinated way with PKA responding directly and rapidly to beta-adrenergic stimulation, while CaMKII depending on CaCAM subsequently prolongs the adrenergic effects in a positive feed-forward loop [24].

Chronic beta-adrenergic overstimulation as occurring in various heart diseases, including hypertrophy, dilation, and contractile dysfunction, induces adverse remodelling, a powerful clinical predictor of increased morbidity and mortality [125]. Transgenic overexpression of CaMKII in mice causes myocardial dilation, contractile dysfunction, and abnormal intracellular Ca^{2+} homeostasis that mimics changes in remodelled myocardium in heart failure patients and in animal models of structural heart disease [123]. Based on these observation, it was hypothesized that CaMKII is a critical downstream element in the beta-adrenergic signalling pathway determining clinically relevant heart disease phenotypes. Indeed, genetically inhibited CaMKII (delta and gamma isoforms predominant in heart) prevented maladaptive remodelling from excessive beta-adrenergic stimulation and myocardial infarction by preserving baseline as well as physiological increases in cardiac function [123]. In conclusion, CaMKII inhibitory therapy could present a new approach for treating structural heart disease and myocardial dysfunction. This case also illustrates the necessity to focus therapeutic strategies on individual CAM targets rather than modulating CAM activity itself since it serves as ubiquitous carrier of the intracellular Ca^{2+} signal.

Troponin in hypertrophic cardiomyopathy

Mutations associated with familial cardiomyopathies are reported to occur in the genes of all three troponin components expressed in the heart, cTNC (~18.4 kDa, 161 AA), cTNI (~24 kDa, 210 AA), and cTNT [37,126,127]. cTNC is present in the heart as well as in slow-contracting skeletal muscle, while cTNI is specific for myocardium. cTNT exists in 10 isoform variants generated from the gene TNNT2 with cTNT3 (~35.9 kDa, 297 AA) being the main species in the adult heart. In the last decade many cardiomyopathy-related mutations were found in at least 10 genes encoding sarcomeric proteins including myosin heavy (MHC) and light (ELC, RLC) chains, and myosin binding protein-C (MyBPC). Fewer mutations occur in genes encoding the cardiac troponin components, actin and tropomyosin [126,128]. Correlation between gene defect and phenotype is difficult to establish, not least because the mutations vary widely in prevalence and penetrance. Collectively, as all these altered proteins are constituents of the sarcomere, their genetic modifications affect force generation leading to hypertrophic cardiomyopathies (HCM) with some phenotypic variation such as specific morphological traits, sudden cardiac death or transition to dilated cardiomyopathy (DCM) with progression of the disease.

The only disease-related mutation in the cTNC gene (TNNC1) so far leads to a replacement of a nonpolar Leu at position 29 to a polar Gln residue (L29Q). Position 29 is the second AA in the defunct Ca^{2+} binding loop of the first EF-hand of cTNC ([129], and references therein) where the cardiac-specific N-terminus of cTNI interacts with its two phosphorylatable serines (positions 23 and 24 in the unprocessed protein). The functional consequences of the L29Q mutation render cTNC refractory to the modification of contractility by the interaction with cTNI that has been phosphorylated by PKA. In vivo this might abolish one of the beta-adrenergic regulatory mechanisms involved in positive inotropy [24].

Troponin as diagnostic marker for myocardial infarction

During ischaemic periods resulting in cell death (myocardial infarction), a variety of intracellular enzymes and

proteins including the troponin components are released into the blood. The temporal release pattern of these cellular components depends on the extent of hypoxia, their subcellular localisation, and their physico-chemical characteristics [130,131]. For several reasons cTNI and cTNT have become the "gold-standard" as markers for acute myocardial infarction (MI). cTNI and cTNT are the only markers specific for heart muscle (cTNC is expressed in heart and slow skeletal muscle). Most of the other markers (creatine kinase, creatine kinase isoform CKMB, myoglobin and others) are not heart-specific but also increase after damage of skeletal muscles. The levels of cTNI and cTNT release predict the extent of heart muscle damage. Blood levels in healthy persons are negligible, so pathological increases are easily detectable. Troponin levels start rising within four to six hours after beginning of chest pain or heart damage and stay elevated for at least one week. The early rise of the markers is essential for early therapeutic treatment and survival of patients, and the following sustained elevation allows detection of myocardial infarction that occurred days earlier. Recently the cardiac-specific S100A1 (monomer ~10.5 kDa, 94 AA) was proposed to complement the troponin markers in heart damage diagnostics [130]. S100A1 shows high sensitivity (significantly higher than troponin) to cardiac damage in the early time window (0–6 h) while cTNI and cTNT are currently the best late markers. The earlier release of S100A1 compared to troponin may be due to its cytoplasmic fraction in cardiomyocytes in contrast to troponin, which is stoichiometrically incorporated in the actin filaments.

Myosin light chains in hypertrophic cardiomyopathy

Both ELC (upstream) and RLC (downstream) bind in myosin to two successive IQ motifs located at the MHC, where the motor domain joins the tail domain. In the two-headed myosin two light chains are associated with each MHC. The light chains are thought to stabilise this so-called neck region. The ventricular ELC (MLC1V, ~21.9 kDa, 195 AA) and RLC (MLC2V, 18.8 kDa, 166 AA) are also expressed in slow skeletal muscle, while the atria contain their own isoforms (MLC1A and MLC2A) (for review, see [132]). As mentioned earlier, the RLC has preserved metal binding capacity only in the first EF-hand motif (Fig. 3). In cardiac myosin Ca^{2+} binds to the MLC2V with an affinity of $K_D \sim 10^{-7}$ M and Mg^{2+} with $K_D \sim 10^{-5}$ M, thus the two ions compete with one another for binding to myosin. Mg^{2+} may be bound during relaxation at low Ca^{2+} levels, while on sustained increase of cytoplasmic Ca^{2+} the Mg^{2+} might become replaced by Ca^{2+} [133,134]. On the basis of the affinities for Ca^{2+} and Mg^{2+} the single functional EF-hand of the MLC2V may classify as structural site that will always be occupied by one of the two metals. It could, however, also function as relaxation site by taking up the activating Ca^{2+} from cTNC with some delay because of the slow exchange rate of Mg^{2+}.

FHC-related gene mutations are reported for both MLC1V and MLC2V. While only few variants seem to occur in the MLC1V, at least 10 mutations have been identified for the MLC2V [135–137]. However, Ca^{2+}-sensitivity and contractility change with the location of the mutation in the protein. The largest disturbances are seen with two mutations located directly in (Asn47Lys at position 11 in the canonical Ca^{2+} binding loop with 12 AA) or near (Arg58Gln at the end of the exiting helix) the Ca^{2+}–Mg^{2+} binding site resulting in loss of metal ion binding. In reconstituted cardiac muscle preparations the two mutants increased Ca^{2+}-sensitivity of the contractile apparatus and force development. With mutations located farther downstream from the first EF-hand the MLC2V preserves its ion binding capacity. Another mutation (Phe18Leu) near the phosphorylation site at Ser15 decreases the cooperativity during activation of force generation [138]. These findings point to the importance of the N-terminal domain including an intact Ca^{2+} binding site of MLC2V in the regulation of cardiac muscle contraction and imply its possible role in the MLC2V-linked pathogenesis of FHC.

Parvalbumin in gene therapy of heart failure

Although PARV (~12 kDa, 110 AA) is absent from heart and slow skeletal muscle of larger animals including human, it is abundantly present in the myocardium and in fast-contracting skeletal muscle of small animals like mouse and rat as well as in muscles of fishes and amphibians. That the two high affinity mixed Ca^{2+}–Mg^{2+} sites in the C-domain of PARV function as Ca^{2+} buffer, enhancing relaxation, was suggested by the concomitant increase in cellular PARV content with the speed of contraction and relaxation of various muscles [139]. To prove this concept, mRNA and PARV protein were induced by direct gene transfer in slow-contracting rat soleus muscle in vivo that does not normally synthesize PARV. The de novo formed PARV dose-dependently increased the speed of relaxation without affecting the time to reach the peak of contraction [140].

Drawing from these findings, it was hypothesized that PARV, normally not present in the human heart, could be therapeutically introduced in order to speed up relaxation after each heartbeat in diastolic heart failure (HF) [141]. Although causes and symptoms are quite diverse, diastolic dysfunction is estimated to occur in about 40% of HF patients [142]. Diastolic dysfunction is characterised by a slowing of the relaxation phase in the contractile cycle. The consequence of slow relaxation is an increase in wall stiffness, resulting in impaired filling and a depression of stroke volume [143]. On the other hand, systolic dysfunction is predominantly characterised by reduced force development and attenuation of the force–frequency relationship concomitant with a marked decrease of SERCA2A activity and expression, and an increase in expression and function of the Na^{+}–Ca^{2+}-exchanger (Fig. 1).

These two types of HF intermix and present a continuum of disease phenotypes changing from the one to the other extreme. An impaired intracellular Ca^{2+} handling represents the common molecular basis for both types of HF that culminates in adverse structural remodelling of the myocardium. Collectively, resting Ca^{2+} levels are increased in the cytoplasm, the amplitude of the Ca^{2+} transients is reduced and its duration is prolonged [144–146].

Intensive work is presently directed to engineered gene delivery in order to redress the disturbed Ca^{2+} balance in the cardiomyocytes addressing different key elements in Ca^{2+} regulation including SERCA2A, PLN, and PARV [147–149]. Contractility including relaxation has recently been improved in isolated cardiomyocytes from human failing hearts by overexpression of SERCA2A [150]. On the other hand, in a heart failure mouse model ablation of PLN (inhibits the SERCA2A Ca^{2+}-pump when unphosphorylated while its phosphorylation by PKA at Ser16 derepresses the inhibition) or in vivo expression of a PLN mutation (Val49Ala) incapable of interacting with SERCA2A, both rescued contractile dysfunction by release of pump inhibition [151]. One possible drawback of these approaches is that by activation of the SERCA2A an increased amount of ATP will be required. The therapeutical usefulness may thus be limited due to the compromised energetic state of the failing heart [148,152]. Transgenic de novo expression of PARV at a tissue concentration of 30–80 μM efficiently enhanced relaxation in rat hearts in vivo without affecting the time course of force development or contraction amplitude [148]. Similar results were obtained with a hyothyroid rat model resembling the characteristics of impaired Ca^{2+} handling associated with heart failure. Ectopic expression of PARV in the myocardium of patients with diastolic dysfunction is considered as a novel therapeutic strategy.

Parvalbumin is a major food allergen

Food allergy is the most common cause of life-threatening anaphylaxis involving IgE-mediated hypersensitivity [153]. Anaphylactic reactions are particularly frequent when the primary sensitisation process occurs via the gastrointestinal tract. PARV is the major cross-reactive allergen in fish. PARV with bound Ca^{2+} is remarkably resistant to heat, denaturing chemicals, and proteolytic enzymes, which might contribute to its allergenic properties. It shares the allergenic potential with many other Ca^{2+} binding proteins isolated from pollen (grasses, trees, and weeds), parasites, and fish [154].

The allergenicity of carp PARV, which like the human species has 109 AA, was drastically modified by preventing Ca^{2+} binding to the two C-terminal EF-hands (Fig. 3) [153]. The first EF-hand (AB helices) is naturally defunct, and in the two functional sites (helices CD and EF) Ca^{2+} binding was prevented by replacing the Asp (providing oxygen coordination) residues at positions 1 and 3 of the 12 AA Ca^{2+} loop by nonpolar Ala residues (at positions 51 and 53 in CD, and 91 and 93 in EF). The histamin release activity of PARV with mutations in either CD or EF (binding only one Ca^{2+} each) was shifted to higher concentrations by 1–2 orders of magnitude in comparison to wild-type PARV, but eventually reached the same maximal level. On the other hand, PARV with mutations in both CD and EF sites (PARV-CD/EF) that bound no Ca^{2+} at all exhibited a 95% reduced IgE reactivity and represented the derivative with the least allergenic activity. PARV-CD/EF was nevertheless sufficiently allergenic for immunisation to rise IgE antibodies in the mouse which cross-reacted with wild-type PARV from various fish species. Thus using the hypoallergenic carp PARV-CD/EF, it may become possible to treat fish allergy by immunotherapy [153]. PARV-CD/EF displays a significant decrease of alpha-helical content compared to wild-type PARV or to those mutants that still bound one Ca^{2+}. The loss of fold was associated with the 95% reduction of allergenic activity. These findings underscore the notion that the bound metal ions in mixed Ca^{2+}–Mg^{2+} high affinity sites contribute to the structural integrity of the protein [52].

Conclusions

Ca^{2+} acts as global second messenger involved in the regulation of all aspects of cell function. A large number of Ca^{2+}-sensor proteins containing the specific Ca^{2+} binding motif (helix-loop-helix, called EF-hand) developed early in prokaryotes. CAM as the prototypical Ca^{2+}-sensor with four EF-hand motifs originated in prokaryotes by successive domain duplications and fusions. It is one of the most conservative proteins and was carried over to the eukaryotic world, now being present in plants, fungi, and animals. Before entering the eukaryotes, the prokaryotic CAM gene further duplicated several times without fusion giving rise to a Ca^{2+} binding protein family (CTER) whose members individually acquired new functions. The S100 proteins represent a new creation by the subphylum vertebrates and are found nowhere else. They seem to have originated from a CAM-type precursor protein by gene duplication or exon recombination with subsequent loss of two EF-hands [2]. Thus the S100 proteins comprise only two Ca^{2+} binding sites and require dimerisation (or higher oligomerisation) to gain functionality. The S100 proteins constitute the largest subfamily of Ca^{2+} binding proteins and seem to evolve rapidly in vertebrates by gene duplications accompanied by neo- or sub-functionalisation. In contrast, the evolution of multiple genes producing identical CAM molecules able to combine with and regulate over 100 reaction partners rather indicates co-functionalisation. The presence of different genes for a single CAM might allow for its differential expression in a developmental- and tissue-specific manner.

Muscle contraction (heart and skeletal muscle) requires fast and precise regulation in the millisecond time range. In this case the extracellular information acts by opening the voltage-dependent Ca^{2+} entry channels DHPR via

membrane depolarisation. Incorporation of the Ca^{2+}-sensing proteins within the structures responsible for generating the signal (association of CAM with the sarcolemmal DHPR and with the SR Ca^{2+}-release channel RYR) and for switching on and off contraction (TNC in the actin filament of the contractile sarcomere) assures a coordinated fast signalling path from Ca^{2+} entry into the cell (bypassing ligand-operated surface receptor mechanisms involving primary messengers like transmitters and hormones), Ca^{2+}-induced Ca^{2+}-release from the SR and binding of Ca^{2+} to the contractile machine for activation. The additional presence of PARV supporting relaxation by taking up the activatory Ca^{2+} from TNC with high affinity is optional and only found in fast contracting muscle and heart of small rodents and fishes. The integrated sensor protein responds to binding of Ca^{2+} with a conformational change that is immediately transmitted to the target structure, thus meeting the required preciseness and speed of signalling. Ca^{2+} binding to the cytoplasmic fraction of both CAM and S100 proteins induces them to expose the hydrophobic surfaces required for translocation and specific interaction with their target proteins [52].

Signalling involving second messengers, such as cAMP, cGMP, G-proteins, diacylglycerol, and others, is usually processed by cell surface receptor systems connecting to phosphorylation cascades. This type of signalling involves covalent protein modification depending on two enzyme systems, protein kinases and phosphatases, and consequently, operates at a slower time scale than Ca^{2+} signalling [24].

In contrast, the S100 proteins as evolutionary latecomers are not directly involved in switching on and off key cell functions like CAM and TNC, but rather operate as modulators. Such modulatory functions may be reflected by the relatively mild phenotypes in mice in response to overexpression or knockout, or alternatively, redundancy may exist among the different S100 isoforms [4]. Nevertheless, in humans specific S100 proteins are associated with serious diseases including cancer, psoriasis, inflammatory disorders, neurodegeneration, and cardiomyopathy. These disease-associated S100 proteins made their way as markers into clinical diagnostics.

S100A1 as main cardiac isoform, unlike the beta-adrenergic signalling cascade, is involved in chronic rather than acute inotropic modulation of cardiac contractile performance without causing pathological remodelling [102]. However, the cardiac S100A1 levels in the non-diseased myocardium are indispensable for the cardiac stress response both in ischaemic damage and chronic haemodynamic overload. Consequently, restoration of the low S100A1 levels in the diseased myocardium appears as a promising new therapeutic strategy.

Outlook

Although transgenic interventions to remedy defective Ca^{2+} handling components have demonstrated the proof-of-concept in isolated cardiomyocytes or in small animal models in vivo, there is still a long way to go before such therapeutic approaches will become possible in human medicine. Intracoronary catheter based application of adenoviral gene transfer to achieve global myocardial transfection has been developed to be ready for human applications. However, it is not known at present how many gene copies will be incorporated in the host genome of different cell types in the heart (the cardiomyocytes comprise 70–80% of the mass but only 20–30% of nuclei besides a majority of other cells including fibroblasts, neurons, smooth muscle, and endothelial cells), nor where these copies will be inserted with regard to genomic regulation. A steady expression at the desired level in the target cells without affecting the endogenous gene activity profile would be required. Even the adenoviral transfection vehicles still pose problems. Genome-wide analysis revealed that in failing rat hearts on top of ~250 differently regulated genes in a transgene-specific manner with SERCA2A transfection, over 3000 additional genes were affected by the viral infection alone [155].

Generally, great efforts are under way to pharmacologically target signalling components, in particular, specific protein kinases. As CAM and S100 proteins each interact with so many different target sites in enzymes and structural protein assemblies in different cell types, it may be impossible to pharmacologically affect these major Ca^{2+}-sensors in order to remedy specific disease processes. As a corollary, such approaches must be laden with unwanted side effects as shown already with those drugs that interact with and inhibit CAM. For future therapeutic improvement in the field of Ca^{2+} signalling it may be more promising to concentrate on the target sites of CAM and S100 proteins. As many targets as possible including their function should be characterised and catalogued for CAM and the individual S100 proteins. This would allow to search for pharmacological interference with the target proteins in order to either inhibit or facilitate their interaction with the Ca^{2+}-sensors. It could be particularly useful to address the individual S100 proteins, for fewer serious unwanted side effects may be encountered by tinkering with their modulatory functions. To date, a beginning of unraveling the interactions and functions of the S100A1 in the heart has just started.

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