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Mini Review

Characterization of calumenin in mouse heart

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Calumenin is a multiple EF-hand Ca^{2+}-binding protein located in the endo/sarcoplasmic reticulum of mammalian hearts. Calumenin belongs to the CREC family of Ca^{2+}-binding proteins having multiple EF-hands. Ca^{2+} homeostasis in the sarcoplasmic reticulum (SR) of mammalian hearts is maintained by RyR2, SERCA2 and other associated SR resident proteins. Evidence suggests that calumenin interacts with RyR2 and SERCA2, and therefore changes in the expression of calumenin could alter Ca^{2+} cycling in mouse heart. In this review, current knowledge of the biochemical and functional roles of calumenin in mouse heart is described. [BMB reports 2010; 43(3): 158-163]

INTRODUCTION

Calcium is an universal second messenger that plays important roles in various cellular processes such as gene expression, signal transduction, exocytosis and muscle contraction (1). In striated muscle cells, muscle contraction and relaxation cycles are governed by Ca^{2+} release and uptake occurring in the SR (2). RyR and SERCA are the two main proteins in the SR responsible for Ca^{2+} release and uptake, respectively. The SR contains also contains various resident proteins in the lumen that are involved in Ca^{2+} buffering. Fluctuation in the luminal Ca^{2+} concentration could modulate the quality and efficiency of protein folding and stability, as well as muscle contraction (3). Calsequestrin (CSQ) (4), calreticulin (CRT) (5), sarcalumenin (6), histidine-rich Ca^{2+}-binding protein (HRC) (7) and calumenin (8, 9) have all been identified as important SR luminal proteins that could regulate Ca^{2+} homeostasis in muscle cells.

Among SR resident proteins, calumenin was recently characterized to be 315 aa in length and has a calculated molecular weight of 37 kDa (10, 11). The N-terminal region of the calumenin protein contains a 19 aa signal sequence, whereas the C-terminal region has a unique 4 aa ER/SR retention signal (10). Calumenin is ubiquitously expressed in different tissues with a higher expression level found in the heart (10). Recent evidence suggests that calumenin is functionally associated with the release and uptake of Ca^{2+} in the SR during the Ca^{2+} cycling process (8, 9, 12).

Additionally, calumenin belongs to the CREC family of Ca^{2+}-binding proteins. The CREC family in mammalian cells is composed of a number of EF-hand proteins. The acronym CREC stands for Ca^{2+}-binding proteins of 45 kDa (Cab45), reticulocalbin, ER Ca^{2+}-binding protein of 55 kDa (ERC-55) and calumenin (13). Recently a number of other proteins also have been included in this protein family, and their physiological and biochemical functions have been under investigation. Regarding gene expression, the CREC family of proteins are encoded by five genes: *RCN1, RCN2, RCN3, SDF4* and *CALU* (13).

This review attempts to summarize the recent advances contributing to the overall knowledge of the biochemical and physiological properties of calumenin in striated muscle, especially focusing on biochemical and functional properties in mouse heart.

Genomic organization of calumenin

Mouse calumenin gene was first cloned and characterized from mouse heart using the signal sequence trap method (10). Subsequent investigations have revealed that the calumenin gene has two alternatively spliced variants of equal length, named calumenin-1 and calumenin-2. Calumenin-2 was previously known as crotoxin-binding protein of 50 kDa (CBP50) or crocalbin (14, 15). In comparing mouse calumenin-1 and -2, the 19 aa signal sequence and 4 aa ER/SR retention sequences are identical (16). Calumenin protein contains one in vivo glycosylated N-glycosylation site at the 131st aa position. The aa sequences of mouse calumenin-1 and -2 have 92% identity and 95% homology (16), with the difference lying in EF-hands-1 and -2. Despite this, the conserved amino acids closely resemble the consensus EF-hand sequence (17).

Although the calumenin gene was initially mapped to the proximal portion of mouse chromosome 7, a recent investigation suggests its presence in chromosome 6 of the mouse genome (16). Localization of calumenin to mouse chromosome 6 as well as that to human chromosome 7 are in conserved synteny with each other. Furthermore, the mouse cal-

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umenin gene contains six exons and five introns (16). Exon 2 is different between calumenin-1 and -2; exon 2 of calumenin-2 is 72 bp before that of calumenin-1 in the 5′ to 3′ direction (16).

Other CREC family members

Among other CREC family member proteins, the *RCN1* gene encodes reticulocalbin protein (331 aa) containing a signal sequence, six EF-hands and the ER retention signal HDEL (18). Although previously reported to be localized only to the ER, reticulocalbin has recently been found to be localized at the surface of bone endothelial cells and prostate cancer cells (19). The translocation protein Sec63p located in the ER apparently interacts with reticulocalbin (20). Similarly, the RCN2 gene encodes the ER Ca^{2+}-binding protein 55 kDa (ERC-55), which is 317 aa long and contains a signal sequence, six EF-hands and the ER retention signal HDEL (21). ERC-55 interacts with the human pappilomavirus (HPV)-encoded oncogenic protein E6, the vitamin D receptor (VDR), the neuronal pentraxins 1 (NP1) and 2 (NP2) and the snake venom taipoxin (22). Another gene, *RCN3*, encodes a 328 aa precursor of reticulocalbin-3 containing a signal sequence, six EF-hands and the HDEL ER retention signal in its C-terminus. This protein interacts with proPACE4. The *SDF4* gene encodes Cab45, a 362 aa protein containing a signal sequence, six EF-hands and the C-terminal ER retention signal HDEF (23, 24). Cab45-C interacts with Munc18a and Munc 18b and is involved in the secretion process.

Calumenin polymorphisms

For the calumenin gene, at least 23 single nucleotide polymorphisms (one repeat or one insertion/deletion polymorphism) have been identified in the general population (25). The effect of calumenin polymorphism on the anticoagulant response was also reported (26). The polymorphism is localized to the 3′-untranslated region of the *CALU* gene.

Ca^{2+}-binding properties of calumenin

Calumenin protein contains six EF-hands, each of which consists of typical helix-loop-helix motifs. The EF-hands undergo possible conformational changes upon Ca^{2+} binding. Specifically, the binding of Ca^{2+} to the individual EF-hands of calumenin was estimated to occur with a Kd of ~600 μM (11). To date, there is no clear structural explanation for the relatively low affinities of the EF-hands of calumenin when compared to the EF-hands found in other Ca^{2+}-binding proteins (13).

Expression and localization of calumenin in striated muscle

Mouse and human calumenins are ubiquitously expressed in multiple tissues (10, 27). Both calumenin isoforms in mouse are abundant in muscle, and transcription levels are higher in cardiac muscle than in skeletal muscle. The mRNA expression level of calumenin is decreased in the adult mouse heart compared to embryonic stages (10). Likewise, calumenin protein expression during developmental stages was significantly decreased in adult mouse heart compared to embryonic and neonatal hearts (9). The level of calumenin protein steadily decreased in mouse heart until reaching a steady state level that was maintained throughout adulthood. This indicates that the pattern of calumenin expression is similar to that of other ER chaperone proteins such as CRT, glucose regulated protein 78, glucose regulated protein 94, protein disulfide isomerase and ER protein 57 (ERp57) (28)

Analysis of rabbit skeletal muscle showed that calumenin is abundant in the junctional fraction of rabbit skeletal SR where RyR1 is enriched, suggesting a possible interaction between calumenin and RyR1 (12). In ventricular myocytes and HL-1 cells, calumenin staining displayed clear localization along the Z-line and longitudinal axis of cardiomyocytes. This results suggest that calumenin is co-localized to areas of cardiomyocytes where SERCA2 and RyR2 are enriched (9).

Calumenin in EC coupling

The muscle sarcotubular system, consisting of the SR and transverse tubules (TT), regulates Ca^{2+} homeostasis within muscle cells and thereby muscle contraction and relaxation (2). Muscle contraction and relaxation along with other physiological parameters are dependent on the composition of the components of the Ca^{2+} handling apparatus. In skeletal muscle, the voltage sensor signal is transmitted to RyR via protein/protein interaction with the II-III loop of the dihydropyridine receptor (DHPR). In contrast, the coupling process in cardiac muscle depends on Ca^{2+} entering the fiber through the DHPR channel and initiating Ca^{2+}-activated Ca^{2+} release through RyR. SERCA proteins are responsible for causing relaxation by pumping Ca^{2+} from the cytoplasm into the lumen of the SR. They are activated by an increase in cytoplasmic Ca^{2+} and inhibited as the luminal Ca^{2+} concentration increases towards maximum levels. The pump is most active during EC coupling when cytoplasmic Ca^{2+} is highest and stored Ca^{2+} is at its lowest. A number of proteins interact with SERCA proteins, thereby regulating Ca^{2+} homeostasis in the SR. Proteins such as CSQ, CRT, calumenin, sarcalumenin, and HRC are known to promote Ca^{2+} buffering in striated muscle (4-9).

Evidence shows that calumenin regulates Ca^{2+} release from the SR. For example, over-expression of calumenin in C2C12 cells significantly increased the storage capacity of Ca^{2+} in the SR, but at the same time decreased depolarization-induced Ca^{2+} release (12). The increased storage of SR Ca^{2+} can be attributed to increased Ca^{2+} buffering power inside the SR lumen due possibly to an increased level of calumenin protein. Although the physiological role of calumenin was not ex-

plicitly revealed by its over-expression in C2C12 cells, it was found to be related to Ca^{2+} homeostasis in the SR. Moreover, any change in calumenin expression could affect Ca^{2+} release from the SR by altering the interaction between RyR and calumenin. Calumenin over-expression in neonatal rat ventricular cardiomyocytes increased SR Ca^{2+} storage capacity while decreasing fractional Ca^{2+} release (8). In both skeletal and cardiac muscle, calumenin over-expression increased the SR Ca^{2+} load while also significantly decreasing Ca^{2+} release, suggesting that calumenin over-expression can inhibit Ca^{2+} release by direct interaction with RyR (Fig. 1). In rat cardiomyocytes, calumenin over-expression was found to inhibit SERCA Ca^{2+} uptake by direct interaction with SERCA2 (Fig. 1).

On the other hand, calumenin knockdown by siRNA in myocytes does not affect SR Ca^{2+} storage capacity (9) or total Ca2+ buffering capacity due to the fact that calumenin is a minor protein in the SR compared to CSQ (11, 29). Calumenin knockdown in cardiomyocytes enhanced Ca^{2+} cycling, as seen by an increased Ca^{2+} transient amplitude and faster time to peak Ca^{2+} transients. Similarly, knockdown of calumenin enhanced SERCA2 activity, as seen by a decreased time to 50% baseline of Ca^{2+} transients in HL-1 cells (9). Knockdown of calumenin changed the Ca^{2+} affinity of SERCA protein without affecting the expression and phosphorylation of phospholamban. These findings elucidate the importance of calumenin in Ca^{2+} cycling in the SR.

The functional role of calumenin in Ca^{2+} cycling in the SR is similar to that of HRC (7, 30). HRC is distributed throughout the SR in a similar expression pattern as that of calumenin. Evidence suggests that HRC also interacts with and modulates the activities of SERCA and RyR, suggesting that regulation of SR function by SR resident Ca^{2+}-binding proteins is physiologically important, as evidenced by the existence of multiple regulatory Ca^{2+}-binding proteins in the SR. Functional studies suggest that calumenin acts in concert with other modulatory proteins as regulatory proteins that control Ca^{2+} release and uptake in the SR.

Characterization of calumenin interaction with SERCA and RyR in muscle cells

Calumenin can interact with various proteins in the SR. GST-ull down and immunoprecipitation assays showed that the interaction between calumenin and SERCA is Ca^{2+} dependent, and that the E1 state of SERCA shows higher association with calumenin than the E2 state (9). A GST-pull down assay of different deleted regions of calumenin and SERCA2 showed that the SERCA-L4 region interacts with the calumenin middle region containing EF-hands 3 & 4. The SERCA-L4 region is the longest luminal loop in the SR and also interacts with ERp 57, which has been shown to regulate SERCA activity (31). Molecular modeling has indicated that the association between calumenin and SERCA is dependent on 4 hydrophobic aa in the SERCA2-L4 region. This finding suggests that calumenin and SERCA interact by hydrophobic interaction. Similarly, calumenin interacts with RyR2 in mouse heart (9).

Functional role of calumenin in non-muscle cells

Calumenin interacts with a group of proteins in non-muscle cells. Serum amyloid P component (SAP) was the first protein reported to interact with calumenin in Ca^{2+}-dependent fashion (32). This interaction indicates that calumenin may participate in the immunological defense response and could be involved

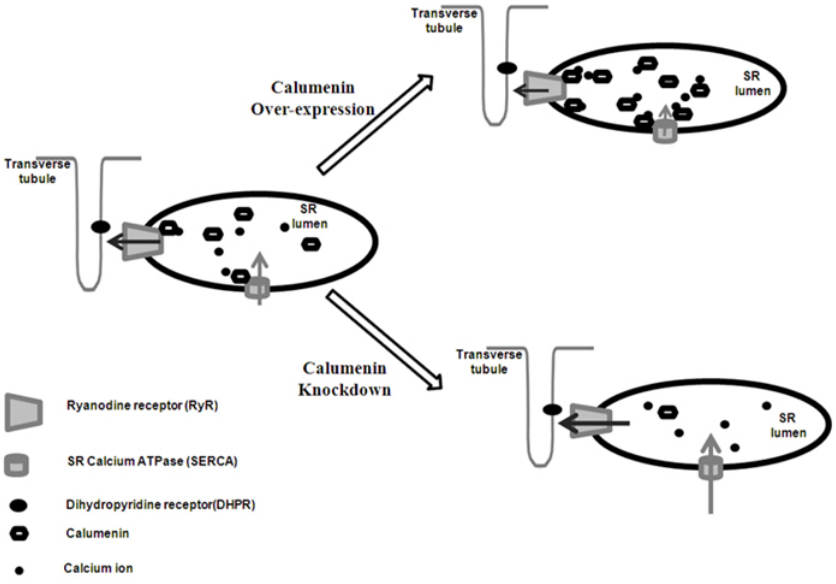

Fig. 1. A hypothetical model showing how calumenin in the sarcoplasmic reticulum (SR) lumen could regulate excitation-contraction coupling of the heart. The SR is a key organelle involved in Ca^{2+}-cycling of muscle cells. In muscle cells, Ca^{2+} release from the SR by RyR initiates muscle contraction, and Ca^{2+} uptake into the SR by SERCA results in muscle relaxation. Recent evidence suggests that calumenin located in the SR lumen directly binds both RyR and SERCA (left panel). In calumenin over-expressing cells, the rates of Ca^{2+} release and Ca^{2+} uptake in the SR are decreased due to the inhibitory role of calumenin on both RyR and SERCA (right panel, top), whereas knockdown of calumenin leads to increased rates of Ca^{2+} release and Ca^{2+} uptake in the SR due to activation of RyR and SERCA (right panel, bottom).

in amyloidosis that causes amyloid deposition in different types of tissues.

Wallin et al. (33) have shown that calumenin is able to regulate γ-carboxylation in rat. The vitamin K-dependent γ-carboxylation system consisting of vitamin K_1 2, 3 epoxide reductase (VKOR) and γ-glutamyl carboxylase is present in the ER (34-36), and γ-carboxylation of proteins promotes complex formation with Ca^{2+}. Over-expression of calumenin in COS-1 cells inhibits γ-carboxylase activity while the siRNA-mediated knockdown of calumenin increases γ-carboxylase activity (37), suggesting an inhibitory role for calumenin. Using immunoprecipitation experiments it was found that calumenin is directly associated with γ-carboxylase. Genetically engineered BHK21 cells over-expressing VKORC1 showed enhanced factor IX production upon calumenin gene silencing (38). The above studies show that calumenin is an important regulator of the γ-carboxylation system, reinforcing the importance of the biosynthesis of functional vitamin K-dependent proteins.

Even though mouse calumenin is localized to the lumen of ER and is not secreted, Vorum et al. showed that calumenin is secreted from the ER in human cultured cells and localized to the secretory pathway (27). This discrepancy is attributed to the inefficient ER retention signal HDEF. A recent finding shows that calumenin forms a Ca^{2+}-dependent complex with thrombospondin-1 (TSP-1). Both calumenin and TSP-1 are released from thrombocytes upon stimulation with thrombin (39). This suggests that calumenin may serve as a major factor in thrombosis.

Proteomic profiling of fibroblasts treated with calumenin shows that calumenin may modulate the cell cycle. This indicates a possible autocrine or paracrine effect for calumenin on nearby cells, which indicates possible involvement in the pathophysiology of thrombosis or wound healing (40). Differential expression of calumenin is observed in cardiomyopathy, fracture healing and experimental nerve injury (41, 42). Calumenin transcription was down-regulated in metastatic cells compared to primary tumor cells (43). Calumenin was also down-regulated in hepatocellular carcinoma cells with high metastatic potentials compared to similar cells with low metastatic potentials (44). Mass spectrometry analysis revealed that calumenin could be phosphorylated at Ser-44 and Thr-65 (45, 46). A chemical genetic screening of v-src substrates identified calumenin as a tyrosine phosphorylation target, suggesting that calumenin is involved in signaling pathways (47). Evidence also shows that calumenin is associated with the ER protein translocase, which leads to the translocation of calumenin from the cytosol to lumen (20).

Summary

Calumenin is a CREC family Ca^{2+}-binding protein that has diverse functions in different tissues. Evidence shows that abnormal expression of calumenin is associated with various pathological conditions such as cardiomyopathy. In striated muscle, calumenin interacts with RyR and SERCA in the SR and regulates the major pathways of Ca^{2+}cycling involved in contraction-relaxation cycles. Based on the finding that expression of calumenin is gradually reduced from the embryonic stages to developmental stages, the functional role of calumenin could change dynamically during the development of striated muscle. Characterization of calumenin function in vivo is an interesting area for further investigation.

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