Molecular architecture of Ca$^{2+}$ signaling control in muscle and heart cells

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Ca$^{2+}$ signaling in skeletal and cardiac muscles is a bi-directional process that involves cross-talk between signaling molecules in the sarcolemmal membrane and Ca$^{2+}$ release machinery in the intracellular organelles. Maintenance of a junctional membrane structure between the sarcolemmal membrane and the sarcoplasmic reticulum (SR) provides a framework for the conversion of action potential arrived at the sarcolemma into release of Ca$^{2+}$ from the SR, leading to activation of a variety of physiological processes. Activity-dependent changes in Ca$^{2+}$ storage inside the SR provides a retrograde signal for the activation of store-operated Ca$^{2+}$ channel (SOC) on the sarcolemmal membrane, which plays important roles in the maintenance of Ca$^{2+}$ homeostasis in physiology and pathophysiology. Research progress during the last 30 years had advanced our understanding of the cellular and molecular mechanisms for the control of Ca$^{2+}$ signaling in muscle and cardiovascular physiology. Here we summarize the functions of three key molecules that are located in the junctional membrane complex of skeletal and cardiac muscle cells: junctophilin as a “glue” that physiologically links the SR membrane to the sarcolemmal membrane for formation of the junctional membrane framework, mitsugumin29 as a muscle-specific synaptophysin family protein that contributes to maintain the coordinated Ca$^{2+}$ signaling in skeletal muscle, and trimeric intracellular cation channel as a novel cation-selective channel located on the SR membrane that provides counter-ion current during the rapid process of Ca$^{2+}$ release from the SR.

Introduction

Control of locomotion and muscle contraction is an elemental process in human physiology. Defined regulation of Ca$^{2+}$ ion in muscle and heart cells is crucial for the maintenance of voluntary contractile activity of skeletal muscle and for the rhythmic contraction of the heart. In muscle cells, Ca$^{2+}$ ions are stored inside the sarcoplasmic reticulum (SR), which can be utilized for fast contraction and other physiological activities. Excitation-contraction (E-C) coupling is a bridging process between depolarization of sarcolemma and transient elevation of intracellular Ca$^{2+}$ via opening of the ryanodine receptor (RyR)/Ca$^{2+}$ release channel on the SR. After each contraction, majority of the Ca$^{2+}$ ions is sequestered back to SR by the sarco/endoplasmic reticulum Ca$^{2+}$ ATPase (SERCA). Various Ca$^{2+}$ exchange systems across the sarcolemmal membrane, such as the Na$^+/$/Ca$^{2+}$-exchanger and store-operated Ca$^{2+}$ entry (SOCE), are required to maintain a low resting cytosolic Ca$^{2+}$ level and sufficient Ca$^{2+}$ storage inside the SR.

Triad/diad junctions are specialized membrane complex that originates from close association between the transverse-tubular (TT) invagination of the sarcolemma membrane and the terminal cisternae of the SR. Maintenance of a proper junctional membrane structure allows for efficient execution of E-C coupling, as well as the relay of retrograde signaling from SR to activation of SOCE. While considerable progress has been made over the past few decades on understanding the cellular and molecular mechanisms of E-C coupling in cardiac and skeletal muscles, it is conceivable that for effective signal transduction, the physical structure of triad/diad junction has to be maintained.

In the course of searching for membrane-bond proteins participating in E-C coupling of striated muscles, we took a reductionist approach by producing a monoclonal antibody library against the triad junction proteins, in order to understand their function in muscle physiology and cardiovascular diseases. Here we summarize the role of three novel proteins that were identified from such an immunoproteomic approach: JP as a glue that physiologically links the TT and SR membrane together, MG29 as a master regulator of the overall Ca$^{2+}$ signaling in muscle physiology and aging, and trimeric intracellular cation channel (TRIC) as a counter-ion channel that provides a pathway for efficient Ca$^{2+}$ release across the SR membrane (Fig. 1). While these proteins were primarily identified in muscle cells, their functions in non-muscle cells may also be applied.

Junctophilins

Junctional membrane complexes (JMCs) composed of close associations of plasma membrane and ER/SR are shared by excitable cell types, i.e., “triad” in skeletal muscle, “diad” in cardiac muscle, “peripheral coupling” in smooth and immature striated muscle, and “subsurface cistern” in neurons. In skeletal muscle, Ca$^{2+}$ signals during E-C coupling is generated by
direct stimulation of RyRs upon activation of cell-surface L-type Ca\(^{2+}\) channels, while Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels activates RyRs in cardiac muscle. Thus, close proximity of the L-type Ca\(^{2+}\) channels and RyRs in JMCs is particularly important since otherwise the proper physical interaction or efficient Ca\(^{2+}\)-diffusion path may be disrupted in skeletal and cardiac muscle cells.

Previously, RyR was identified as the “foot” protein that is embedded in the junctional membrane gap of the triad.\(^5\) However, its role in the formation of JMCs may be limited since that mutant skeletal muscle-lacking RyRs still retain the triad structure,\(^9\)\(^10\) and cultured cells stably transfected with RyR cDNAs do not generate JMC.\(^11\) One important molecule isolated from our immuno-proteomic library is junctophilin (JP), which physically associates with rapid exit of Ca\(^{2+}\) across the SR membrane. TRIC provides counter-current links the TT and SR membrane together, allowing the formation of triad junctions.\(^5\)\(^\text{12-14}\) Our previous studies demonstrated that preservation of an intact triad junction is essential for the maintenance of quiescent Ca\(^{2+}\) release machinery during the resting state of skeletal muscle contraction, as well as for the efficient control of E-C coupling during the activation process of muscle contraction.\(^6\)\(^\text{15}\) JP-mediated formation of junctional membrane structure is essential for operation of SOCE, and therefore contributes to the long-term maintenance of Ca\(^{2+}\) homeostasis in muscle cells.\(^6\)

The mammalian genome contains four tissue-specific JP subtype genes, namely JP1–4.\(^\text{14,16}\) Mammalian JP subtypes are composed of 628–744 amino acid residues, containing conserved eight repeats of membrane occupation and recognition nexus (MORN) sequence of 14 residues at the amino-terminal region, and a transmembrane segment spanning the ER/SR membrane at the carboxyl-terminal end. The amino-terminal region containing MORN motifs interacts specifically with the plasma membrane likely due to its binding affinity to a broad spectrum of phospholipids.\(^\text{17}\) Our previous data on knockout mice lacking JP subtypes indicate that JPs are essential for the physiological communication between plasma-membrane ion channels and RyRs in skeletal and cardiac muscles,\(^5\)\(^\text{6,12,13}\) hippocampal CA1 neurons and cerebellar Purkinje cells.\(^\text{19-21}\) Moreover, JP can efficiently generate JMCs by interacting with the plasma membrane and spanning the ER in an expression system using amphibian embryos.\(^5\) The triad is generated in skeletal muscle expressing both JP1 and JP2, while the diad is formed in cardiac muscle containing JP2 alone. This observation, together with the role of JPs in JMC formation, may suggest that JP1 is a major determinant for the structural maturation from diads to triads in skeletal muscle. However, typical triads were not observed in cardiomyocytes overexpressing ectopic JP1,\(^\text{13,22}\) suggesting that complex cellular mechanisms including membrane biogenesis and transport are also essential for the formation of JMC formation. Based on available data, channel communications in JP-mediated JMCs in excitable cell types are illustrated in Figure 2.

Recent studies had identified the association between JP dysfunction and several pathological conditions. Specific knock-down of JP1 in skeletal muscle cells significantly reduces SOCE\(^6\) and leads to altered ultrastructure and increased muscle fatigability.\(^\text{23}\) Reduction of JP2 expression correlates well with progressive TT remodeling in failing rat heart,\(^\text{24}\) and Ca\(^{2+}\) influx becomes a less efficient trigger for RyR activation when JP2 expression is reduced in cardiomyocytes.\(^\text{25}\) In mice model and human patients with cardiomyopathy, JP2 expressions are remarkably downregulated in the heart.\(^\text{26,27}\) Moreover, JP2 mutations leading to amino-acid substitutions was likely associated with weakened E-C coupling efficiency of the heart and pathogenesis of hypertrophic cardiomyopathy.\(^\text{15,28,29}\) Furthermore, triplet-repeat expansion mutations of the neural JP3 gene are direct causes of Huntington’s disease-like 2, a disease characterized by abnormal movements, dementia and psychiatric syndromes\(^\text{30,31}\) and germ-line ablation of JP3 activates membrane potential in neurons,\(^\text{17}\) which might lead to the late-onset neurodegeneration observed in Huntington’s disease-like 2. Thus, proper function of JP subtypes is essential for the architectural integrity of the junctional membrane in excitable cells, which may be the underlying mechanism of altered excitability and disrupted Ca\(^{2+}\) signaling in human diseases.

**Mitsugumin 29**

MG29 is enriched in the triad junctional region of skeletal muscle. MG29 shows 45% sequence homologies and shares structural features with synaptophysin, a major membrane protein involving in synaptic vesicles dynamics. Immunochemical and RNA blot data revealed that MG29 is expressed almost exclusively in the skeletal muscle.\(^\text{32}\) Immunofluorescence microscopy and immunogold electron microscopy further showed that MG29 is located specifically in the triad junction. While it is located mainly at SR membranes during the early stages of
skeletal muscle development, the protein appeared preferentially at the triads in mature muscle. The skeletal muscle from MG29-deficient mice demonstrated abnormalities in ultra-structure of triad junction, including swollen TTS, partial defects in the triads at the A-I junction, incomplete formation of the SR networks with fragmented or vacuolated structure. These ultra-structural defects occurred during early myogenesis and remained until the adult stage. The appearance of the protein at different muscle developing stages and analysis of skeletal muscle cell in MG29-deficient mice suggest that MG29 is essential for the formation and maintenance of SR networks and triad structure. In addition to involving in formation and maintenance of triad junction, MG29 probably also interact with triad junctional proteins directly. We found that the purified MG29 protein enhanced activity of the RyR channel incorporated into the lipid bilayer membrane. Co-expression of MG29 and RyR in CHO cells caused cell apoptosis due to depletion of ER Ca²⁺ stores. These data suggested a functional interaction between MG29 and RyR, which could have important implications in the Ca²⁺ signaling processes of muscle cells. Others also reported that MG29 may interact with TRPC3.

The impacts of MG29 on physiological functions of the muscle were investigated in our previous studies. Skeletal muscles from MG29-deficient mice performed poorly in muscle contractility measurements, i.e., with reduced twitch tension and increased susceptibility to fatigue. The mutant muscles not only fatigued to a greater extent but also recovered significantly less than the wild-type muscles. In further studies, we discovered that the increased fatigability of mutant muscles were resulted from a defective movement of Ca²⁺ across the plasma membrane, which is likely through SOCE. Using fluorescent based intracellular Ca²⁺ measurements and manganese quenching methods, we found that SOCE was dramatically reduced in mg29⁻⁻ myotubes. Interestingly, removal of extracellular Ca²⁺ or inhibition of SOCE by pharmacological blockers 2-APB or skf-96365 significantly reduced the difference in fatigue sensitivity between wild-type and MG29-deficient muscles. Therefore, we demonstrated a dysfunction of SOCE in MG29-deficient skeletal muscles and established a physiological role of SOCE in long-term skeletal muscle Ca²⁺ homeostasis.

MG29 also involves in other aspect of Ca²⁺ signaling, i.e., Ca²⁺ sparks, the elemental units of Ca²⁺ release from SR. In young and healthy skeletal muscles, Ca²⁺ sparks are active and plastic upon osmotic challenge while they remain silent under resting condition. The dynamic nature of Ca²⁺ sparks appears to be lost in MG29-deficient muscle. In addition, we identified a segregated Ca²⁺ pool that uncoupled from the normal E-C coupling process in skeletal muscles from MG29 null mice. It is noteworthy that all these structural and functional abnormalities are found in aged skeletal muscle as well. Recently we discovered that decreased SOCE may contribute to Ca²⁺ dysregulation associated with muscle aging, and ablation of MG29 led to dysfunction of SOCE in neonatal and adult muscles derived from the mg29⁻⁻ mice. These studies, together with another finding that MG29 protein is significantly decreased in aged skeletal muscles make us to believe that MG29 may act as a sentinel during muscle aging. Reduced homeostatic capacity for effective intracellular Ca²⁺ signaling following reduced MG29 expression may underlie the progression of sarcopenia and contractile dysfunction during muscle aging. The critical role of MG29 was evidenced by the remarkably resemble phenotype of aged mice vs. young mg29⁻⁻ mice as mentioned above. Identification of compromised Ca²⁺ sparks signaling, segregated intracellular Ca²⁺ release, and defective SOCE may provide unique targets for therapeutic interventions against the effects of aging on muscle performance. The primary amino acid sequence for MG29 is highly conserved in human and rodents. The skeletal muscle-specific nature of MG29 expression minimizes concerns that targeting MG29 to
Both demonstrate preferred conductivity for K+. Single-channel rent associated with SR/ER Ca\(^{2+}\) release.

The link between TRIC channel and Ca\(^{2+}\) shuttling activity of the SR was established in several studies. Specifically, TRIC-A and B form homotrimers that conduct monovalent ions, with a high conductance for K\(^{+}\). Thus TRIC may be a candidate for the SR K\(^{+}\) channel that mediates the countercurrent associated with SR/ER Ca\(^{2+}\) release.

The link between TRIC channel and Ca\(^{2+}\) shuttling activity of the SR was established in several studies. Specifically, in tric\(^{-a/-}\)b\(^{-/-}\) cardiomyocytes, compromised Ca\(^{2+}\) release was observed accompanying Ca\(^{2+}\) accumulation in the SR store, and similar pathology was also identified in tric\(^{-a/-}\) skeletal muscle and tric\(^{-b/-}\) alveolar epithelial cells. The two isoforms of TRIC protein share more than 40% of homology and both demonstrate prefered conductivity for K\(^{+}\). Single-channel properties of TRIC-A and TRIC-B reveal distinct permeation and modulatory functions. TRIC-A channel is more sensitive to changes in membrane potential whereas TRIC-B channel is more sensitive to control by luminal and cytoplasmic Ca\(^{2+}\). Thus, it is likely that different gating mechanisms allow coordinated responses to the development of negative charge within the SR lumen as well as changes in luminal Ca\(^{2+}\) concentration.

The physiological relevance of TRIC-A and TRIC-B was also investigated in our previous studies. First, we found that TRIC-A and TRIC-B had distinct tissue distribution patterns, i.e., expression of TRIC-A protein was detected primarily in striated muscles, while TRIC-B protein was detected in the lung, brain and other tissues, and TRIC-B mRNA was also seen in testis, stomach and liver tissues. The tric\(^{-a/-}\)tric\(^{-b/-}\) double knockout mice died at E9.5 day due to cardiac arrest and substantial Ca\(^{2+}\) deposit was observed in SR of the heart, accompanied by significantly compromised spontaneous Ca\(^{2+}\) oscillation in isolated cardiomyocytes from the tric\(^{-a/-}\)tric\(^{-b/-}\) mice. Tric\(^{-b/-}\) neonates died shortly after birth due to respiratory failure, as revealed by the deflated lungs and alveolar type II epithelial cells of the tric\(^{-b/-}\) neonates with severe histiological and functional defects. More recently, a unique phenotype was identified in skeletal muscle from the tric\(^{-a/-}\) mice, which are viable probably due to the more specialized expression of TRIC-A protein. Specifically, intensive fatiguing stimulation causes a drastic alternan behavior in isolated tric\(^{-a/-}\) skeletal muscle, i.e., transient and drastic increase in contractile force appeared within the decreasing force profile during repetitive fatigue stimulation. Inhibition of SERCA function could lead to aggravation of the stress-induced alternans in tric\(^{-a/-}\) muscle, indicating that altered SR Ca\(^{2+}\) transport independent of the SERCA pump is associated with the stress-induced alternans in the tric\(^{-a/-}\) skeletal muscles. In addition, absence of the K\(^{+}\) current through TRIC-A possibly leads to reduced sensitivity to voltage gating, contributing to the development of alternans (Fig. 3). In terms of the effect of TRIC knockout on Ca\(^{2+}\) release events, our functional assays revealed a synergistic effect of TRIC-A and TRIC-B deletion in various tissues, i.e., double knockout cells seem to have more dramatic reduction of the Ca\(^{2+}\) release, while single knockout only leads to partially decreased Ca\(^{2+}\) release from the intracellular store. It is likely that an orchestra of countercurrent channels works in coordination to facilitate the Ca\(^{2+}\) release function responding to different stimulations.

Thus, current data allow us to establish the physiological functions of TRIC-A and TRIC-B K\(^{+}\) channels and their contribution as a countercurrent for intracellular Ca\(^{2+}\) release in different tissues. However, the exact regulatory mechanisms of these two channels and how they respond to different gating mechanisms remain unknown. Furthermore, whether TRIC-A and TRIC-B involve in other functions aside from providing counter-current activity for SR Ca\(^{2+}\) release requires further investigation.

**Conclusions and Future Directions**

The junctional membrane structure in skeletal and cardiac muscle harbors many Ca\(^{2+}\) signaling-related proteins that form the unique “channelsome” for control of Ca\(^{2+}\) homeostasis and signaling in...
muscle physiology and cardiovascular diseases. Beneficiated by the powerful marriage of molecular genetic approaches with functional studies, three key molecules have been identified in this complex: JPs as a "glue" that physiologically links the SR membrane to the sarcolemmal membrane for formation of the junctional membrane framework; MG29 as a muscle-specific synaptophysin family protein that contributes to maintain the coordinated Ca\(^{2+}\) signaling in skeletal muscle; and TRICs as cation-selective channels located on the ER/SR membrane that provide counter-ion current during the rapid release of Ca\(^{2+}\) from the SR. As abnormal expression and/or mutations of JPs and MG29 have been linked to a growing number of diseases, including hypertrophic cardiomyopathy,\(^{15,28,29}\) Huntington’s disease-like 2\(^{30,31}\) and sarcopenia,\(^{32,33}\) further understanding the mechanisms that control the expression, subcellular distribution, and their functional interaction with other Ca\(^{2+}\) regulatory components should increase the translational value of these findings. It should be noted that many of the findings can be extended beyond the content of striated muscle physiology and diseases. For example, recent studies have linked the function of TRIC-A channel to vascular biology for control of blood pressure,\(^{34,35}\) and TRIC-B channel to secretory function in the lung tissue.\(^{48}\) One can expect that mutations or polymorphisms in these genes may cause dysregulation of Ca\(^{2+}\) signaling for the underlying human diseases. These studies will be an important task for future investigation. Moreover, development of TRIC-A and TRIC-B specific channel blockers or agonists could be of considerable clinical benefit for treatment of human diseases.

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