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CALMODULIN MODULATION OF PROTEINS INVOLVED IN EXCITATION-CONTRACTION COUPLING

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1. ABSTRACT

Muscle excitation-contraction coupling is, in large part, regulated by the activity of two proteins. These are the ryanodine receptor (RyR), which is an intracellular Ca^{2+} release channel and the dihydropyridine receptor (DHPR), which is a voltage gated L-type calcium channel. In skeletal muscle, the physical association between RyR1 and L-type Ca^{2+} channels is required for muscle excitation-contraction coupling. RyRs also regulate intracellular Ca^{2+} homeostasis, thereby contributing to a variety of cellular functions in different tissues. A wide variety of modulators directly regulate RyR1 activity and, consequentially, alter both excitation-contraction coupling and calcium homeostasis. Calmodulin, one of these cellular modulators, is a ubiquitously expressed 17 kDa Ca^{2+} binding protein containing four E-F hands, which binds to RyR1 at both nanomolar and micromolar Ca^{2+} concentrations. Apocalmodulin (Ca^{2+} free calmodulin) is a partial agonist, while Ca^{2+}calmodulin is an inhibitor of RyR1. This conversion of calmodulin from an activator to an inhibitor is due to Ca^{2+} binding to the two C-terminal sites on calmodulin. Calmodulin can also modulate the L-type Ca^{2+} channel in the transverse tubule membrane, producing either inactivation or facilitation of the channel upon elevation of the local Ca^{2+} concentrations. Calmodulin binds to a region on RyR1 corresponding to amino acids 3614-3643 and to a region in the carboxy-terminal tail of the L-type Ca^{2+} channel α_1 subunit. However, these calmodulin binding motifs on both proteins bind to undetermined motifs on the other protein, suggesting that they represent more general protein-protein interaction motifs. These findings raise questions about the role of calmodulin in excitation-contraction coupling in skeletal muscle.

2. RYANODINE RECEPTORS

Ryanodine receptors (RyRs) are Ca^{2+} release channels that reside in endoplasmic/sarcoplasmic

reticulum. These proteins have high affinity for ryanodine, a plant alkaloid, and are homotetramers of a subunit with a molecular mass greater than 500 kDa. To date there are three isoforms of ryanodine receptors identified in mammalian tissues: RyR1, RyR2 and RyR3. The three RyRs are encoded by separate genes and have different tissue distributions. The overall sequence homology among these RyRs is about 60%. Although RyR1, RyR2 and RyR3 are often referred to as skeletal, cardiac and brain isoforms (1-5), respectively, this designation is misleading since all of these isoforms are found in other tissues. RyR1 is found in some parts of brain, such as Purkinje cells of the cerebellum (6, 7), and in smooth muscle (8), while RyR2 is the most widely distributed form in brain (6, 7). RyR3 is a minor isoform in both brain and skeletal muscle (6, 7, 9). In skeletal muscle RyR3 appears to exist in the highest concentration in the diaphragm (9). RyR1 and RyR2 are essential proteins in skeletal and cardiac excitation-contraction coupling, respectively. In RyR1 deficient mice, E-C coupling is completely abolished resulting in perinatal death from respiratory failure. Muscular degeneration is also found in these mutant mice, suggesting a role for RyR1 in skeletal muscle morphogenesis (10). RyR2 is not required for E-C coupling in embryonic heart, but appears to serve as a regulator of internal Ca^{2+} stores for other aspects of calcium homeostasis (11). RyR3 is expressed in relative abundance in neonate skeletal muscle and may play an amplifying or auxillary role in E-C coupling (12). RyR3 has also been implicated in spatial learning and hippocampal synaptic plasticity (13). In nonexcitable cells, RyRs are involved in the Ca^{2+} wave propagation needed for other cellular functions, such as secretion activity in pancreatic acinar cells (14).

3. DIHYDROPYRIDINE RECEPTORS

Dihydropyridine receptors (DHPRs) are oligomeric proteins that are found primarily in the transverse tubules in skeletal muscle. They are Ca^{2+} channels of the L-type, which means that they are activated by high voltage, show slow voltage-dependent inactivation and are modulated by phenyalkylamines (eg, verapamil), benzothiazepines (eg, diltiazem), and dihydropyridines (eg, nitrendipine) (15). The DHPR is composed of a 190 kDa α_1 subunit, a 55 kDa β subunit, a 170 kDa disulfide-linked $\alpha_2\delta$ dimer and a 33 kDa transmembrane γ subunit (16). In skeletal muscle DHPRs in the t-tubules are arranged in regular arrays above RyR1 in the SR membranes. In the arrays the DHPRs are found in groups of four, called tetrads, and are positioned above every other RyR1 (17, 18). In the absence of the DHPR α_1 subunit (dysgenic muscle), other subunits of DHPR are no longer anchored to the junctional region and tetrads are absent (19).

4. EXCITATION-CONTRACTION COUPLING

The process whereby depolarization of the muscle membrane leads to contraction of the muscle is known as excitation–contraction (E-C) coupling. There are two kinds of E-C coupling: mechanical coupling, where a change in the conformation of the DHPR directly signals the Ca^{2+} release channel to open, and Ca^{2+}-induced calcium release (CICR), where Ca^{2+} entering through the L-type channel activates the Ca^{2+} release channel (20, 21). Mechanical coupling is required for skeletal but not cardiac muscle E-C coupling (21). The functional interactions between RyR1 and DHPR are believed to be reciprocal. DHPR opens RyR1, defined as orthograde signaling, and RyR1 can prevent DHPR inactivation, defined as retrograde signaling (22, 23). Chimeras of RyR1 and RyR2 or DHPR α_{1sk} and α_{1c} have been used extensively to map the regions on RyR1 and DHPR responsible for these interactions. This approach is based on the findings that only RyR1 and α_{1sk} can restore mechanical E-C coupling in skeletal muscle (23-26). The II-III loop of the α_{1sk} subunit has been shown to be required for the mechanical coupling. Several regions on RyR1 are thought to be involved in either orthograde or retrograde signaling, or both. A region between amino acids 1635-2636 on RyR1 has been shown to be required for both orthograde and retrograde signaling. In addition, a region between residues 2659-3720 on RyR1 has been implicated in retrograde signaling (23). Other regions of both proteins may be involved in their coupling (27, 28). A synthetic peptide representing a sequence between amino acids 3614 and 3643 on RyR1 has been shown to directly interact with the DHPR (29) and conversely, the carboxy-terminal tail of the DHPR α_1 subunit has been shown to interact with RyR1 (28, 29).

As mentioned previously, only every other RyR1 appears to be physically associated with a tetrad of DHPRs (17, 18), producing two different populations of RyR1 (coupled and uncoupled). These two populations must be regulated in different ways. Uncoupled RyR1s are likely to be activated by CICR, with the triggering Ca^{2+} released from neighboring coupled RyR1s. An additional level of complexity, however, comes from the finding that both of the primary players in E-C coupling are modulated by other proteins. One example of a protein that modulates the activity of both the DHPR and RyR1 is the Ca^{2+} binding protein, calmodulin (CaM). CaM in both its Ca^{2+} bound and Ca^{2+} free states can bind and regulate both RyR1 and DHPR (30-34). Studies of the role of calmodulin in E-C coupling have concentrated primarily on its interaction with uncoupled proteins, raising the question of how calmodulin regulates coupled channels. Determining the role of modulatory proteins in E-C coupling remains a major challenge to the understanding of the molecular mechanisms involved in E-C coupling in skeletal muscle.

5. CALMODULIN

Calmodulin (CaM) is a 17 kDa ubiquitously expressed Ca^{2+} binding protein with a single 148-amino-acid polypeptide chain. It contains four calcium binding EF hands between residues 20-31, 56-67, 93-104 and 129-140 (35). An EF hand is defined as two α-helical sequences oriented in a perpendicular way and connected by a Ca^{2+} binding loop. Calmodulin consists of two globular (N and C) lobes connected by an eight-turn α-helix. Each lobe has two calcium binding sites (35). Calmodulin goes through Ca^{2+} dependent conformational changes upon binding to Ca^{2+}, resulting in the exposure of several hydrophobic

residues in the helices of both lobes (36). It has numerous cellular targets and plays an important role of regulating cellular functions (37). CaM binds most target proteins in a Ca^{2+} dependent manner. Upon binding Ca^{2+}, CaM exposes the binding site for its target sequence and can modulate the function of the target. Ca^{2+}CaM binding proteins include calcineurin and CaM dependent kinase II (37, 38). Other proteins, such as neuromodulin, primarily bind the Ca^{2+} free form of CaM (39). Still other proteins bind both the Ca^{2+} free and Ca^{2+} bound forms of CaM. Both RyR1 and the DHPR fit into this latter category. One type of calmodulin binding site is an amphipathic helix with two clusters of positive charges separated by a hydrophobic region (40). Another motif that can bind either apoCaM, Ca^{2+}CaM or both is the IQ motif, which has a consensus sequence of IQXXXRGXXXR (40).

5.1. Functional effects of Calmodulin on RyR1

CaM directly interacts with RyR1 and modulates its function. CaM increases RyR1 activity at low Ca^{2+} concentrations (nM) and inhibits channel activity at high Ca^{2+} concentrations (μM) (30, 41, 42). Since both CaM and RyR1 are Ca^{2+} binding proteins (36, 43), these Ca^{2+} dependent functional effects could arise from Ca^{2+} binding to CaM, RyR1 or both. Using a CaM mutant, which does not bind Ca^{2+} at any of the four Ca^{2+} binding sites, we demonstrated that Ca^{2+} binding to CaM converts it from an activator to inhibitor of the RyR1 (30). We also found that Ca^{2+} binding to sites 3 and 4 on CaM is responsible for its conversion from an activator to an inhibitor. Ca^{2+} binding to RyR1 does, however, alter its interaction with CaM. Ca^{2+} binding to RyR1 increases its affinity for both apoCaM and Ca^{2+}CaM and, conversely, the binding of CaM to RyR1 increases the affinity of the Ca^{2+} binding site on RyR1 (30, 31).

Closely related to the modulation of RyR1 by CaM is its regulation by oxidants and nitric oxide (NO). Skeletal muscle produces reactive oxygen intermediates (ROI) and nitric oxide (NO) even at rest. Reactive oxidant production increases upon strenuous contraction, leading to muscle fatigue (44). RyR1 is believed to be one of the target proteins of both oxidants and NO. Oxidants, NO and calmodulin appear to work together to finely tune the RyR1 activity during the dynamic changes of skeletal muscle. Oxidants, such as H_2O_2, increase RyR1 activity and produce intersubunit disulfide bonds within the RyR1 tetramer (45-47). Calmodulin can protect the channel from oxidation-induced intersubunit cross-linking and, conversely, oxidation can prevent calmodulin binding to RyR1 (48).

The effect of NO, however, on RyR1 function is controversial. Both activating and inhibiting action on the channel have been reported (49, 50). Eu *et al.* (51) demonstrated that under physiological O_2 tension (~10mmHg), NO activated the RyR1 and this modulation appeared to be calmodulin dependent. NO has been shown to oppose the ROI effect of enhancing muscle contractile function (44). Consistent with this, NO blocks oxidation activation of RyR1 (46).

5.2. Calmodulin binding sites on RyR1

Ryanodine receptors were first suggested to be calmodulin-modulated proteins by photo affinity labeling studies (52). Using azido-[^{125}I]calmodulin, Seiler *et al*. (52) demonstrated that high molecular proteins in both cardiac and skeletal muscle, later known as RyRs, could bind calmodulin and were the principal bands labeled in junctional SR. Although several earlier papers suggested that there were multiple apocalmodulin binding sites per subunit of RyR1 (41, 45, 53), more recent studies (30, 54) have shown that both apoCaM and Ca^{2+}CaM bind to a single site per subunit of RyR1. A number of laboratories have attempted to identify CaM binding sites in the primary sequence of RyR1. Analysis of primary structure of RyR1 identified several putative calmodulin binding sites between residues 2807-2840, 2909-2930, 3031-3049, 3614-3637 and 4295-4325 on RyR1 (2, 55). Based on calpain digestion pattern of RyR1 and CaM's ability to inhibit calpain digestion, three more candidate CaM binding sites were proposed between residues 1383-1400, 1974-1996 and 3358-3374 (56). Using [^{125}I]calmodulin overlays, Chen *et al.* identified six potential calcium-dependent CaM binding sites, three strong CaM binding domains in regions between residues 2063-2091, 3611-3642, and 4303-4328, and three weaker CaM binding domains in regions between residues 921-1173, 2804-2930, and 2961-3084 (57). Zorzato and his group identified three calmodulin binding sites, residues 2937-3225 binding to both apoCaM and Ca^{2+}CaM, residues 3546-3655 binding only to Ca^{2+}CaM, and peptides with amino acids 3610-3629 and 4534-4552 interacting directly with dansylcalmodulin under micromolar Ca^{2+} based on fluorescence spectra (58). Our laboratory found that calmodulin bound to RyR1 could protect a site on RyR1 from tryptic cleavage. Both Ca^{2+}CaM and apoCaM prevented tryptic cleavage after amino acids 3630 and 3637, suggesting that apoCaM and Ca^{2+}CaM bind to the same or overlapping regions on RyR1 and this site contains residues 3614-43 (48, 54, 59, 60). Non-denaturing gel shift assays using a synthetic peptide corresponding to amino acids 3614-3643 on RyR1 confirmed that this sequence could bind both forms of calmodulin (59). Point mutations in this region abolish CaM binding (54). This sequence is highly conserved in the different RyR isoforms, suggesting that all three are modulated by CaM.

Our previous studies with oxidizing agents showed that calmodulin could protect RyR1 from oxidation-induced intersubunit crosslinking. C3635, one of the cysteine residues that form intersubunit disulfide bonds, is protected from oxidation by CaM binding (60). This suggests that CaM binds to a RyR1 intersubunit contact site. This cysteine residue is also the primary site for NO nitrosylation of RyR1 (61).

A crucial aspect needed to evaluate the molecular mechanism by which CaM regulates RyR1 activity is the location of CaM in the three dimensional structure of RyR1. Wagenknecht and coworkers showed that the CaM binding sites on RyR1 were located in the cytoplasmic domain of RyR1 and that the apoCaM and Ca^{2+}CaM binding sites are closely spaced to one another

(62, 63). The regulation of RyR1 activity by CaM is, therefore, likely to be allosteric.

5.3. Calmodulin and DHPR

CaM can bind to both cardiac and skeletal muscle DHPR, although most of the functional effects of CaM on DHPR have been studied in cardiac muscle. CaM serves as a Ca^{2+} sensor for both Ca^{2+} dependent inactivation and facilitation of the cardiac L-type Ca^{2+} channel (32, 64). A mutated CaM that can not bind Ca^{2+} at any of the four Ca^{2+} binding sites blocks the effects of Ca^{2+}CaM on the cardiac L-type Ca^{2+} channel (64), suggesting that both Ca^{2+}CaM and apoCaM bind to L-type Ca^{2+} channels. It has been proposed that CaM is tethered to the channel under resting Ca^{2+} and the elevation of intracellular Ca^{2+} leads to Ca^{2+} binding to CaM, producing cardiac L-type channel inactivation and facilitation (34). The carboxy tail of DHPR α_1 subunit is required for both apoCaM and Ca^{2+}CaM interactions. Two Ca^{2+}-dependent CaM binding sites have been identified in the carboxy-terminal tail of the α_1-subunit of DHPR, the CB region (between the amino acids 1484-1509 or 1627-1652 of the human skeletal muscle or cardiac α_1 subunit, respectively.) and IQ-like motif (between the amino acids 1522-1542 or 1665-1685 of the human skeletal muscle or cardiac α_1 subunit, respectively.) (33, 65, 66). Another region, called the A motif (between the amino acids 1558-1579 of the rabbit cardiac α_1 subunit) may also contribute to the interaction of CaM with the DHPR α_1 carboxy-terminal tail (34). Synthetic peptides, corresponding to the CB region and the IQ motif bind both partially and fully Ca^{2+}-saturated CaM (33). Mutation of the isoleucine 1672 of the cardiac IQ motif to alanine abolishes Ca^{2+}/CaM dependent inactivation and unmasks a strong facilitation by CaM. Mutation of this isoleucine to a glutamate abolishes both facilitation and inactivation (65). Peptides with either mutation still bind to Ca^{2+}CaM as well as wild type IQ peptide. Neither the CB nor IQ peptide has a high affinity for apoCaM (33, 67). Peptide A (1558-1579) and peptide C (1585-1606) from rabbit cardiac α_1 subunit may bind CaM at low Ca^{2+} concentrations, making them candidates for the CaM tethering site on cardiac DHPR under resting conditions (34). A recombinant protein which encompasses the Ca^{2+} binding EF hand, the A and the CB motif of the skeletal muscle DHPR α_1 subunit was found to bind to CaM at less than 10 nM Ca^{2+}(29). A functional effect of calmodulin on the skeletal muscle DHPR has not yet been demonstrated.

5.4. CaM and E-C coupling

A number of studies have shown conclusively that CaM is able to bind to both the DHPR and RyR1. These studies have, however, been performed with uncoupled proteins. Recent studies in our laboratory have revealed another possible role for the CaM binding motifs on both of these proteins (29). We have shown that these motifs can be used for interaction between RyR1 and DHPR and that CaM is competitive for this interaction. Hence the carboxy-terminal tail of the DHPR α_1 subunit, a well established CaM interaction domain, binds to RyR1 and conversely, the CaM binding motif on RyR1 interacts with the DHPR α_1-subunit carboxy-terminal tail. These two CaM binding motifs do not bind directly to each other, and therefore, each must have another binding partner on the other protein. These findings raise the possibility that CaM regulation of coupled channels is very different from that of uncoupled channels. In uncoupled channels CaM is a Ca^{2+} sensor for inactivation and facilitation of the L-type channel and an activator or inhibitor of RyR1 (depending on the Ca^{2+}concentration). However, when these two ion channels are coupled to one another, CaM at sufficiently high concentrations would tend to disrupt one site of DHPR-RyR1 interaction. Since both the CB peptide and an expressed carboxy-terminal tail fragment of the DHPR α_1 inhibit RyR1 channel activity and [^{3}H]ryanodine binding to RyR1 (28, 29), the interaction of the carboxy-terminal tail of the DHPR α_1 subunit may serve to stabilize a closed state of RyR1. If this is true, CaM might be expected to facilitate RyR1 channel opening at low Ca^{2+}, both by disrupting this interaction and by direct effects on channel activity.

6. SUMMARY

The role that CaM plays in E-C coupling is likely to be extremely complex since CaM can interact with both the DHPR and RyR1 and it can do so at both high and low Ca^{2+}. Not only can both Ca^{2+}CaM and apoCaM interact with the two channels, but in both cases the functional consequences of the interaction are also altered by Ca^{2+} binding to CaM. In addition, the CaM binding motifs may be used by coupled channels to interact with each other rather than with CaM. In this situation CaM would be capable of disrupting one of the sites where the DHPR couples to RyR1. These findings demonstrate the remarkable flexibility of CaM as a Ca^{2+} sensor, but emphasize the difficulty in clearly defining its role in E-C coupling. Disruption of CaM binding sites would be expected to alter CaM binding to both uncoupled and coupled channels and to affect the interactions between the DHPR and RyR1. Interpretation of the molecular mechanisms of altered E-C coupling by CaM could be, therefore, misleading. Mutation of CaM or decreasing its expression would be expected to alter a number of Ca^{2+} sensitive processes that use CaM as a Ca^{2+} sensor, possibly producing secondary effects as well as primary effects on E-C coupling. These changes would alter both Ca^{2+} independent and Ca^{2+} dependent effects of CaM on both RyR1 and the DHPR. Interpretation of the molecular mechanisms involved in CaM regulation of the DHPR and RyR1 requires additional structural information. We do not know the molecular details of the interaction of CaM with either channel. Particularly important will be the elucidation of both the molecular determinants for apoCaM versus Ca^{2+}CaM binding on both RyR1 and the DHPR and the molecular mechanisms for the movement of CaM from an apoCaM binding site to a Ca^{2+}CaM binding site. The intriguing structural data obtained with the small conductance Ca^{2+} activated K^+ channel (68) clearly show the importance of high-resolution structure for the interpretation of the complex role of CaM as a Ca^{2+} sensor.

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