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Topical Review

Modulation of Intracellular Calcium-Release Channels by Calmodulin

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Introduction

The release of Ca^{2+} from intracellular stores is a key step in a wide variety of biological functions, most notably excitation-contraction and excitation-secretion coupling as well as transcription and apoptosis. This release is mediated by two related families of calcium release channels, the ryanodine receptors (RyR) and the inositol l,4,5-trisphosphate receptors (IP_3R). Both the RyRs and IP_3Rs are expressed in specialized subcompartments of the smooth endoplasmic reticulum, containing micromolar to millimolar concentrations of both free and bound Ca^{2+}. Ca^{2+} release through these high-conductance channels is triggered by ligand binding, either via a voltage-sensing molecule in the surface membrane or Ca^{2+} in the case of RyRs, or via the concerted action of IP_3 and Ca^{2+} in the case of IP_3Rs, resulting in rapid increase in cytosolic free Ca^{2+} content before the channels close. Given the fundamental importance of the release events, both channel subfamilies are modulated by myriad pathways through small molecules and protein-protein interactions [Fig. 1 and reviews of RyRs (Coronado et al., 1994; Meissner, 1994; Sutko & Airey, 1996; Franzini-Armstrong & Protasi, 1997; Zucchi & Ronca-Testoni, 1997) and IP_3Rs (Mikoshiba, 1997; Yoshida & Imai, 1997; Taylor & Broad, 1998; MacKrill, 1999)]. This review focuses on the regulation of the two intracellular calcium-release channel subfamilies by calmodulin (CaM), a small calcium-binding protein that is a key modulator of many steps in a wide variety of Ca^{2+}-signaling pathways.

The RyR family of calcium-release channels is composed of at least three isoforms: RyR1, which is expressed primarily in skeletal muscle, RyR2, which is the dominant isoform in cardiac muscle, and RyR3, which is found in a wide variety of tissues, but which is mostly associated with diaphragm and brain (Sorrentino & Volpe, 1993). Each of the three isoforms, which are encoded by different genes, are comprised of approximately 5,000 amino acids with a molecular mass of approximately 560 kDa. The first 4,000 amino acids form large cytosolic domains, which are the putative binding sites for many channel regulators, while the C-terminal 1,000 amino acids hold the transmembrane domains that constitute the channel pores. The functional channel complexes are composed of tetramers of 560 kDa monomers and four FK506 binding proteins (FKBPs). The RyRs display a characteristic bell-shaped, biphasic dependence on cytosolic Ca^{2+} concentration with μM Ca^{2+} being required for channel activation and elevated Ca^{2+} concentrations resulting in channel inhibition. Each of the three isoforms is characteristically modified by the plant alkaloid ryanodine, being converted into a subconductance state by nM ryanodine with an open channel probability (P_o) near 1, which would be expected to result in a dramatic increase in Ca^{2+} flux under most conditions. The second phase of ryanodine modification of RyRs is complete channel closure induced by μM ryanodine concentrations. The RyRs are inhibited by ruthenium red and high cytosolic Mg^{2+} and are sensitized to Ca^{2+} activation by adenine nucleotides and caffeine.

The IP_3Rs encode proteins of approximately 2,500 amino acids with the C-terminal 1,000 constituting the transmembrane domain. This region has fairly high homology with the three RyRs with regions of near identity and pockets of high divergence; there is very little homology between the N-terminal 1,500 residues of the IP_3Rs and the RyRs. There are also three known IP_3R isoforms with differences in their regulation as is observed for the RyRs. Typically, multiple IP_3R isoforms are expressed in the same tissue, confounding attempts to resolve the differences between isoforms in pharmacological and channel properties. Each of the three IP_3R isoforms requires that IP_3 and low Ca^{2+} (10–100 nM) be present for

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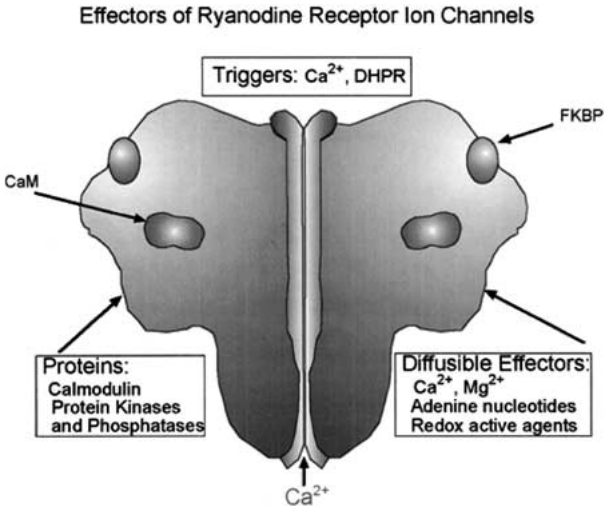

Fig. 1. Regulation of RyR ion channels. Prominent forms of regulation are indicated, including regulation by both small molecules and proteins. Also indicated are the approximate locations of the FKBP12 and CaCaM binding sites in RyR1 identified by cryo-electron microscopy (Wagenknecht et al., 1997).

channel activation. In single-channel measurements, type 2 (Ramos-Franco, Fill & Mignery, 1998) and type 3 (Hagar et al., 1998) IP_3Rs were maximally activated by 0.1 and 10 μM cytoplasmic Ca^{2+} concentrations without showing significant inactivation at 10 and 100 μM, respectively, while the more extensively studied IP_3R1 displayed a biphasic regulation by cytosolic Ca^{2+} with a maximal activity at ~0.1 to 10 μM Ca^{2+}, depending on the IP_3 concentration (Kaftan, Ehrlich & Watras, 1997; Mak, McBride & Foskett, 1998; Ramos-Franco et al., 1998). Michikawa et al. (1999), however, found that the purified IP_3R1 is not directly inactivated by Ca^{2+}. Purified cerebellar IP_3R1 channels reconstituted into planar lipid bilayers were activated by Ca^{2+} without inhibition at concentrations as high as 200 μM. Addition of CaM inhibited channel activity, which indicated that Ca^{2+}-dependent inactivation is mediated by CaM.

In addition to similarities in Ca^{2+}-release activity, the RyRs (Fig. 1) and IP_3Rs share tight regulation by both small molecules and proteins. Both families are modulated by the proteins CaM and FKBP12 as well as by Mg^{2+} and adenine nucleotides, in addition to Ca^{2+}. Major pharmacological differences lie in the sensitivity to specific exogenous compounds such as caffeine, ryanodine and dantrolene (RyRs) and adenophostin A or heparin (IP_3Rs) (*see* reviews cited above).

Calmodulin is a small (16.7 kDa) cytosolic Ca^{2+}-binding protein, which mediates Ca^{2+}-dependent regulation of numerous biological processes. CaM is found in all eukaryotic cells and among animals the amino-acid sequence is 100% conserved. Structurally, CaM is comprised of two globular domains separated by a central alpha-helical domain. Each of the N- and C-terminal domains contains two EF-hand Ca^{2+}-binding domains (Babu et al., 1985). CaM binds to its target proteins through three distinct mechanisms, with Ca^{2+} bound (CaCaM), without Ca^{2+} bound (ApoCaM) or constituently bound (Ca^{2+}-independent binding). Following binding to the target protein (or Ca^{2+} binding to or dissociation from constitutively bound CaM) a conformational change is induced in the target protein, altering its function. There is no clear consensus CaM-binding motif although CaM typically interacts through an amphipathic helix of approximately 20 amino acids or through a so-called IQ motif (Rhoads & Friedberg, 1997; Jurado, Chockalingam & Jarrett, 1999).

Ryanodine Receptors Bind Calmodulin

An early study showed that two high-molecular-weight proteins in cardiac and skeletal SR vesicles bind CaM, as determined with the use of the photoaffinity probe azido-[^{125}I]CaM (Seiler et al., 1984). Subsequent studies have shown that the two protein bands represented the cardiac and skeletal 560 kDa ryanodine receptor polypeptides and one of their proteolysis products (Meissner, 1994). The initial studies quantifying CaM binding to the RyR1 used either fluorescently (Yang et al., 1994) or ^{125}I-labeled (Tripathy et al., 1995; Zhang et al., 1999) CaM. These studies indicated that RyR1 binds a single CaCaM per tetramer and 2 to 6 ApoCaM per submit. More recent studies using metabolically labeled [^{35}S]CaM showed that RyR1 binds a single CaM per subunit with nanomolar affinity, independent of cytosolic Ca^{2+} (Moore et al., 1999a; Balshaw et al., 2001). These results suggest that chemical modification of CaM increases the number of ApoCaM binding sites of the RyR1, resulting in nonphysiological binding.

Studies investigating the binding properties of RyR2 conflict, suggesting that cardiac SR membranes bind either 1 CaCaM and 0.25 ApoCaM per RyR2 subunit (Fruen et al., 2000) or 2 CaCaM and 1 ApoCaM (Balshaw et al., 2001) per subunit. Balshaw et al., (2001) found that following purification, the number of CaCaM sites decreased from approximately 2 to 1 per RyR2 subunit, suggesting that other CaM binding proteins were removed, or alternatively, one of the two CaCaM binding sites in RyR2 was conformationally destroyed or buried during purification. ApoCaM and CaCaM bind to and dissociate from RyR1 and RyR2 on a time scale of seconds to minutes (Tripathy et al., 1995; Balshaw et al., 2001; Yamaguchi et al., 2001). It is therefore likely that CaM remains bound to the receptors during a muscle excitation-contraction cycle or other events that change cellular Ca^{2+}. Direct binding of CaM to RyR3 has not yet been reported since no tissue expresses RyR3 alone.

3-Dimensional reconstructions based on cryo-electron microscopy studies have indicated that, in the presence of 100 μM free Ca^{2+}, RyR1 shows the addition of four major differences in images recorded in the presence or absence of CaM, each with a density correlating to the mass of CaM. These major differences are nearly indistinguishable and are symmetrically located within a cleft on the cytosolic domain of the receptor (Wagenknecht et al., 1997). This CaM-binding domain was approximately 9 nm away (on center) from the FKBP12 binding domain, also located on the cytosolic domain of RyR1 (*see* also cartoon representation of Fig. 1 based on Wagenknecht et al., 1997). Both proteins flank (on opposite ends) a RyR1 structural domain (domain 3) which connects directly with the transmembrane assembly, providing a possible structural explanation for the effects of these proteins on channel activity.

IP_3Rs Bind Calmodulin with a Lower Affinity than RyRs

Studies of CaM binding to the IP_3Rs have been hampered by the significantly lower affinity these channels have for CaM than is observed for the RyRs. IP_3R1 bound 1 CaM per subunit in the absence of Ca^{2+}, and an additional CaM in the presence of Ca^{2+} with comparable affinity ($K_D \sim 1$ μM) (Cardy & Taylor, 1998). These binding studies used [^{125}I]CaM, which in the case of RyRs has overestimated the number of ApoCaM binding sites, as discussed above. CaM binding to the three known IP_3R isoforms has also been monitored by their ability to bind to a CaM-Sepharose column in the presence of Ca^{2+} and to elute in the absence of Ca^{2+}. Using this technique, IP_3R1 and a fragment derived from IP_3R2 were shown to bind CaM in a Ca^{2+}-dependent manner, however, Ca^{2+}-independent binding of CaM was not determined (Yamada et al., 1995). Direct binding of CaM has not been reported for the intact IP_3R2, while full-length IP_3R3 did not bind to CaM-Sepharose (Yamada et al., 1995) or [^{125}I]CaM (Cardy & Taylor, 1998). Lin, Widjaja & Joseph (2000) found that IP_3R3, expressed in COS-7 cells, did not bind to CaM-Sepharose, while $IP_3R1/3$ hetero-oligomers did.

Calmodulin Binds to Ryanodine-Receptor Fusion Proteins and Peptides

Several studies have attempted to map the location of potential CaM binding domains by using bacterially expressed fusion proteins and peptides derived from the full-length protein; these are summarized in Fig. 2*A* for RyR1 and RyR2. One initial study by Chen and MacLennan (1994) localizing CaM binding domains in RyR1 found 6 regions capable of binding [^{125}I]CaM. Each bound only in the presence of Ca^{2+}, three of these (aa 2063–2091, 3611–3642 and 4303–4328) showed a stronger signal than did the other three (921–1173, 2804–2930 and 2961–3084).

In an independent study, overlays performed with digoxigenin-labeled CaM revealed three potential CaM-binding domains (amino-acid sequences have been converted to the numbering system reported by Takeshima et al., (1989) including a 5-amino-acid insertion from amino acids 3481–3485 not found in the sequence of rabbit RyR1 reported by Zorzato et al. (1990) for ease of comparison). A fusion protein corresponding to amino-acid residues 2937–3225 bound CaM both in the presence and absence of Ca^{2+}, a second fusion protein (aa 3552–3661) required μM Ca^{2+}, whereas a third fusion protein (4431–4627) bound only in high Ca^{2+} (100–500 μM) (Menegazzi et al., 1994). In a follow-up to this latter study, these CaM-binding domains were refined into CaM binding peptides (aa 3042–3057, 3617–3634, 4540–4557), all of which bound CaM in the presence of Ca^{2+} (Guerrini et al., 1995), with no or very little change in dansyl-calmodulin fluorescence in the absence of Ca^{2+}. The peptides corresponding to these sequences from RyR2 and RyR3 were also synthesized; each of these also bound CaCaM.

Further evidence localizing the RyR1 CaM binding domain was provided by the finding that CaM protects RyR1 from tryptic cleavage at Arg 3630 and 3637 both in the presence and absence of Ca^{2+} (Moore et al., 1999a), a region which overlaps fusion protein 3611–3642 and peptide 3617–3634. A recently published article has followed up on this finding by examining the ability of peptides derived from this area to bind CaM in the presence and absence of Ca^{2+}. This study indicates that, upon binding Ca^{2+}, CaM shifts its points of interaction N-terminally (Rodney et al., 2001).

We have recently repeated the CaM overlay experiments using metabolically ^{35}S-labeled CaM, which avoids the artifacts that can result from using chemically modified CaM, and found Ca^{2+}-dependent binding to RyR1-derived fusion protein 3225–3662 and Ca^{2+}-independent binding to fusion protein 4302–4430 (Balshaw et al., 2001). The same study showed that fusion proteins corresponding to RyR2 amino acid residues 263–615 (analogous to RyR1 248–604), 3298–3595 (analogous to RyR1 3337–3628), 3543–3961 (analogous to RyR1 3588–4006) and 4548–4748 (analogous to RyR1 4614–4817) all strongly bound CaCaM, with much weaker binding to ApoCaM. Fusion protein 2724–3016 (analogous to RyR1 2758–3051) bound CaM independently of Ca^{2+}. A truncated form of fusion protein 3298–3595, comprised of aa 3298–3577 (analogous to RyR1 3337–3610), failed to bind CaM in either the presence or absence of Ca^{2+}, indicating that the binding to fusion proteins 3298–3595 and presumably 3543–3961 requires the RyR2 sequence 3578–3595

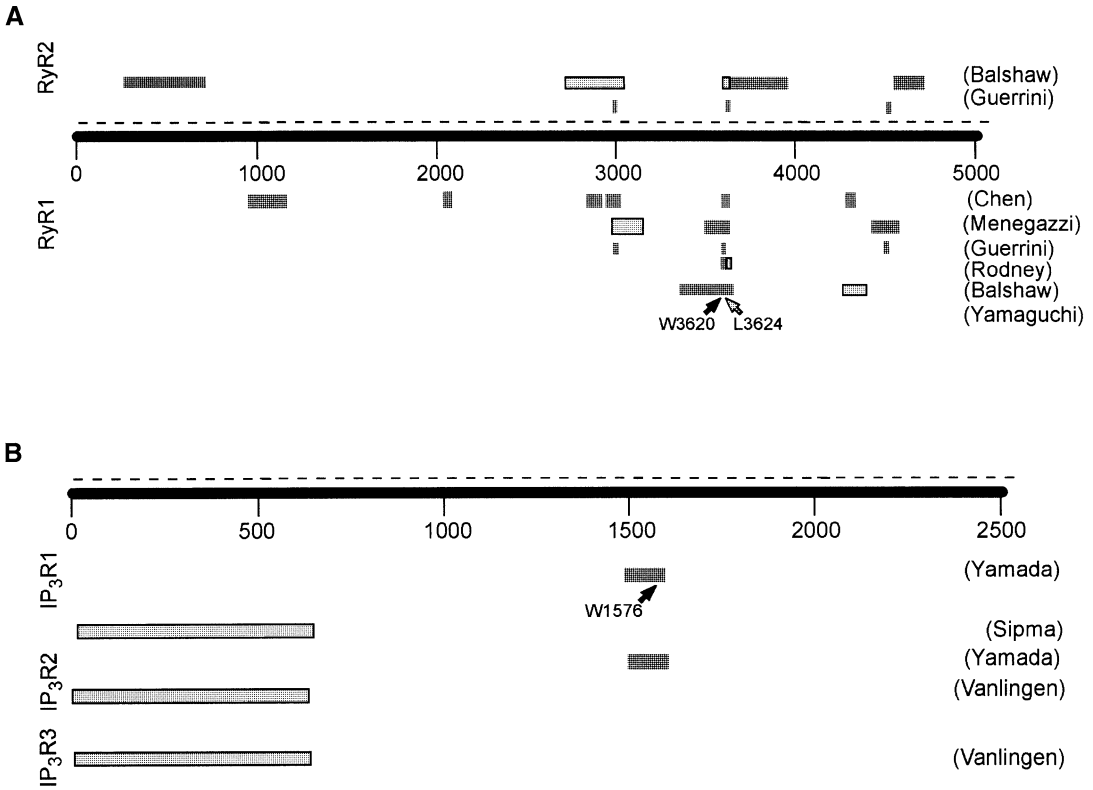

Fig. 2. Putative CaM binding domains in RyR (*A*) and IP_3R (*B*) families of Ca^{2+}-release channels. Domains derived from both RyRl and RyR2 that have been suggested in multiple studies (Chen & MacLennan, 1994; Menegazzi et al., 1994; Guerrini et al., 1995; Balshaw et al., 2001; Rodney et al., 2001, Yamaguchi, et al., 2001) and the three IP_3R isoforms (Yamada et al., 1995; Sipma et al., 1999; Vanlingen et al., 2000) to bind CaM are indicated. Sites specifically binding CaCaM are indicated by dark grey boxes, while those that are Ca^{2+} independent are indicated by light grey boxes with a dark outline. Results derived from mutagenesis studies with full-length receptors are indicated with a black arrow for CaCaM binding and a light grey arrow for Ca^{2+}-independent CaM binding.

(HPQRSKKAVWHKLLSKQR). Mutation of two of these residues in full-length RyR1 resulted in decreases in [^{35}S]CaM binding to the channel. Mutation of Trp 3620 to Ala specifically reduced the affinity of binding to CaCaM without altering the binding of ApoCaM, while mutation of Leu 3624 to Asp greatly decreased the affinity of both ApoCaM and CaCaM (Yamaguchi et al., 2001). Each RyR1 subunit has, therefore, a single Apo- and CaCaM binding site, located between amino acids 3611 and 3644 in RyR1, and presumably in the homologous domain in RyR2, as summarized in the Table. Cryo-electron microscopy and 3-dimensional reconstruction studies of RyR1 have shown that calmodulin is shifted approximately 32 Å, on center, between Apo- and Ca-CaM (Samso, Berkowitz, & Wagenknecht, 2000), suggesting major conformational changes on binding of ApoCaM and/or CaCaM.

Calmodulin Binds to IP_3R Fusion Proteins and Peptides

A Ca^{2+}-dependent CaM-binding site with a $K_D \sim 0.7$ μM at 2 mM Ca^{2+} was localized to amino acids 1564–1585 in IP_3R1 by testing the ability of fusion proteins to bind to a CaM-Sepharose column and by measuring changes in Trp fluorescence of peptide 1564–1585 in the presence of increasing concentrations of CaM (Fig. 2*B*; Yamada et al., 1995). The full-length W1576A IP_3R1 mutant did not bind to CaM-Sepharose, indicating that W1576 is essential for CaCaM binding. The corresponding domain (1558–1596) from IP_3R2 also bound to CaM-Sepharose, while the full length IP_3R3 did not. This putative IP_3R CaM-binding site is conserved between IP_3R1 and IP_3R2 but has very little homology with any of the RyR isoforms and is, therefore, unlikely to be a conserved CaM binding domain among the Ca^{2+}-release channel superfamily.

CaM inhibited [^{3}H]IP_3 binding to a bacterially expressed N-terminal domain comprised of the first 581 amino acids from IP_3R1 in a Ca^{2+}-independent manner (Sipma et al., 1999) (Fig. 2*B*). Similarly, [^{3}H]IP_3 binding to the first 581 amino acids of IP_3R3 was inhibited by CaM in the presence of 1 mM EGTA, while only a small inhibition was observed for this domain from IP_3R2 (Vanlingen et al., 2000). Lin et al. (2000) compared the CaM-binding properties of the regulatory domain of two splice variants of IP_3R1 (long, aa 1314–1905 and short, aa 1693–

Table I. Sequence of a potential CaM binding domain in the RyRs

RyR Isoform	N-term Residue	Sequence	C-term Residue	CaCaM	ApoCaM	Reference
RyR1	3611	HPYKSKKAVWHKLLSKQRRRAVVACFRMTPLY	3642	+	–	Chen
RyRl	3616	KKAVWHKLLSKQRRRAVVA	3634	+	–	Guerrini
RyRl	3625	SKQRRRAVVACFRMTPLYNL	3644	+/–	+	Rodney
RyRl	3614	KS KKAVWHKLLSKQRRRAVVA	3634	+	–	Rodney
RyRl	3614	KSKKAVWHKLLSKQRRRAVVACFRMTPLYN	3643	+	+	Rodney
RyR2	3578	HPQRSKKAVWHKLLSKQR	3695	+	+	Balshaw
RyR2	3581	RSKKAVWHKLLSKQRKRAVVACFR	3604			Nakai
RyRl	3614	KSKKAVWHKLLSKQRRRAVVAC	3635			Takeshima

Sequences of a series of overlapping CaM binding sites from studies using fusion proteins (Chen & MacLennan, 1994; Balshaw et al., 2001), peptides (Guerrini et al., 1995; Rodney et al., 2001) and primary sequence predictions (Takeshima et al., 1989; Nakai et al., 1990). The Ca^{2+} dependence of binding is indicated by a plus or minus in either the CaCaM or ApoCaM column.

1733 deleted). Both the long and short fusion proteins and full–length IP_3R1s bound CaM in a Ca^{2+}-dependent manner as determined by CaM-Sepharose chromatography and, for the fusion proteins, also by dansyl-CaM fluorescence. Interestingly, CaM-Sepharose bound a greater percent of the added short form (70% *vs.* 30% for the long form), which suggests an increase in affinity or possibly the unmasking of a latent CaM binding site in the shorter splice variant.

Calmodulin Affects Ryanodine Receptor Activity

The initial studies of the effect of CaCaM on the ryanodine receptors indicated that 1 μM CaM resulted in approximately a fourfold decrease in the rate of $^{45}Ca^{2+}$ release from both skeletal (Meissner, 1986) and cardiac (Meissner & Henderson, 1987) SR vesicles. In single-channel measurements, CaCaM inhibited RyR1 and RyR2 ion channels without an effect on single-channel conductance (Smith, Rousseau & Meissner, 1989). Inhibition occurred in the absence of ATP, showing that CaM inhibited the cardiac and skeletal muscle RyRs via a direct interaction rather than through phosphorylation. Further Ca^{2+}-release experiments and [^{3}H]ryanodine-binding to SR vesicles (Tripathy et al., 1995; Fruen et al., 2000) as well as single-channel measurements (Buratti et al., 1995; Tripathy et al., 1995) indicated that CaM regulation of RyR1 is Ca^{2+} dependent. At low free Ca^{2+} concentrations (<1 μM), CaM had a stimulatory effect on channel activity, increasing ryanodine binding and single-channel open probability (P_o) by approximately 3-fold (Tripathy et al., 1995). At higher Ca^{2+} concentrations (50 to 100 μM), CaM had an inhibitory effect similar to that observed in Ca^{2+} efflux assays with a 2–4 fold decrease in [^{3}H]ryanodine binding and P_o. Both the low and high Ca^{2+} effects of CaM on RyR1 activity occurred with nanomolar affinity. Use of a Ca^{2+}-insensitive mutant CaM (B1234Q) indicated that the stimulation of channel activity at low Ca^{2+} is indeed due to Ca^{2+}-free CaM binding while the increase in the CaM binding affinity to RyR1 is largely due to Ca^{2+} binding to the RyR1 (Rodney et al., 2000). Ikemoto, Iino & Endo (1995) observed that CaM was able to activate and inhibit the RyR1 in chemically skinned fibers of rabbit psoas muscle, as indicated by a potentiation of SR Ca^{2+} release by exogenously added CaM at low Ca^{2+} concentrations (<3 μM) and inhibition at elevated Ca^{2+} concentrations (3–30 μM).

Recent Ca^{2+}-release, [^{3}H]ryanodine-binding and single-channel measurements have indicated that RyR2 is also inhibited by CaM at high Ca^{2+}. However, rather than being stimulated at low Ca^{2+}, RyR2 was unaffected (Fruen et al., 2000) or inhibited (Balshaw et al., 2001) by ApoCaM. Ikemoto et al. (1998) studied the effects of CaM on Ca^{2+}-induced Ca^{2+} release in permeabilized mutant mouse skeletal muscle cells expressing either RyR1 or RyR3. In the presence of Mg^{2+}, CaM had a similar effect on the two ryanodine receptor isoforms stimulating Ca^{2+} release at low Ca^{2+} concentrations while inhibiting release at high Ca^{2+}. RyR3 heterologously expressed in HEK293 cells also showed a pattern of CaM regulation similar to that of RyR1 with a nearly 30-fold stimulation at 88 nM Ca^{2+} and 30% inhibition at 50 μM Ca^{2+} (Chen et al., 1997).

Calmodulin Affects IP_3R Activity

Calmodulin inhibited IP_3-evoked Ca^{2+} release from cerebellar microsomes where IP_3R1 is the predominantly expressed isoform, suggesting that CaM exerted its effects through IP_3R1 (Patel et al., 1997). Ca^{2+} release mediated by each of the three IP_3-receptor isoforms was inhibited by CaM in a "cytosol-like" medium (free [Ca^{2+}], 200 nM), using cell lines that predominantly express a particular isoform (Adkins et al., 2000). CaM inhibition of Ca^{2+} release by IP_3R1 and IP_3R2 agrees with CaM-binding studies (*see* above) but are difficult to reconcile with binding studies of IP_3R3, as this isoform apparently

does not bind CaM (Cardy & Taylor, 1998). Missiaen et al. (1999) found that CaM inhibited IP_3-evoked Ca^{2+} release in permeabilized A7r5 cells with an IC_{50} of 4.6 μM at cytosolic Ca^{2+} concentrations greater than 0.3 μM.

Other Channel Effectors Affect The Functional Interaction Between Ca^{2+}-Release Channels And Calmodulin

The modulation of the RyRs by CaM is affected by redox state and various other molecules that influence channel activity. Redox-active molecules may have profound effects on channel activity in situations such as ischemia and exercise-induced fatigue. These conditions lead to an accumulation of oxidized metabolic products and the generation of highly reactive oxygen and nitrogen intermediates. It has been shown that superoxide anions activate RyR2 only in the presence of CaM (Kawakami & Okabe, 1998) and that CaM can protect RyR1 from oxidation-induced crosslinking (Zhang et al., 1999) and N-ethylmaleimide alkylation of C3635 (Moore et al., 1999b). However, these interventions may not be of direct physiological relevance.

Physiological redox state is regulated by the tripeptide glutathione (Glu-Cys-Gly) that exists in either a reduced form, GSH, or an oxidized, disulfide-linked form, GSSG. Oxidizing conditions, simulated by high concentrations of GSSG, reduce the affinity of RyR1 and RyR2 for CaM in both very low free Ca^{2+} (<10 nM) (3–9 fold increase in K_D) and elevated Ca^{2+} (100 μM) (2–3 fold increase in K_D), leading to a substantial difference between the affinity for CaCaM and ApoCaM (Balshaw et al., 2001).Functionally, at low free Ca^{2+} concentrations (~100 nM), the stimulatory effect of CaM on RyR1 activity is maintained in the presence of reduced or oxidized glutathione. Activation was observed in the presence of caffeine and adenine nucleotides, interventions that increase the sensitivity of the channel to cytosolic Ca^{2+} (Tripathy et al., 1995; Fruen et al., 2000; Balshaw et al., 2001). In the case of RyR2 conflicting results have been reported. At 100 nM Ca^{2+}, CaM was found to have an inhibitory effect (Balshaw et al., 2001), while in another study CaM had no or effect or had a slightly activating effect on [^{3}H]ryanodine binding in the presence of caffeine or AMPPCP (Fruen et al., 2000).

As stated above, both RyR1 and RyR2 (as well as RyR3) are inhibited by CaM at high Ca^{2+} concentrations (100 μM). For RyR1, inhibition was maintained in reducing (GSH) or oxidizing (GSSG) conditions in the presence of caffeine or MgAMPPCP (Balshaw et al., 2001). CaCaM inhibition of RyR2 depended on Ca^{2+} concentration, the receptor's redox state and the presence of MgAMPPCP. In the absence of MgAMPCP, inhibition was observed in either reducing or oxidizing conditions at submicromolar to millimolar Ca^{2+} concentrations, while inhibition in the presence of MgAMPPCP at [Ca^{2+}] > 10 μM was significant only in the presence of GSH (Balshaw et al., 2001). Modulation of CaM regulation of the IP_3Rs by other effectors has not been systematically studied.

When the functional effects of altered redox state are coupled with the direct effects of GSH and GSSG on CaM binding as discussed earlier, one can hypothesize some possible effects of oxidative stress on Ca^{2+} release. In skeletal muscle, shifting the GSH/GSSG ratio to a more highly oxidizing state will have the effect of a pronounced increase in peak Ca^{2+} release through a lower CaM affinity and direct effects of oxidation on channel activity. In the case of cardiac muscle, in the presence of Mg^{2+} and adenine nucleotide, a shift from reducing to oxidizing conditions will reduce the CaM binding affinity and attenuate inhibition by CaM, sensitizing the channel to activation by cytosolic Ca^{2+}. This could lead to an increase in the sensitivity of RyR2 to Ca^{2+} provided by the sarcolemmal Ca^{2+} channel.

An additional form of redox-sensitive modulation of RyRs occurs through S-nitrosylation, the addition of NO to a reactive thiol (cysteine), which is a rapidly reversible posttranslational modification that has been shown to modulate the activity of both RyR2 and RyR1 (Eu et al., 1999; Stamler & Meissner, 2001). Activation of RyR1 by S-nitrosylation is dependent on the presence of CaM, and in the presence of saturating concentrations of CaM, RyR1 is activated by S-nitrosylation at <10 μM Ca^{2+} and inhibited at >100 μM Ca^{2+} (Eu et al., 2000). This would have the functional effect of stimulating both muscle contraction and relaxation through increased sensitivity of Ca^{2+}-release activation and increased sensitivity to inactivation at high Ca^{2+}.

Conclusion

The release of Ca^{2+} from intracellular stores is a crucial signaling pathway involved in a multitude of biological processes including muscle contraction, hormone secretion, gene expression and egg fertilization. Two related families of ion channels mediate this calcium-release channel activity: the ryanodine receptors and the inositol 1,4,5-trisphosphate receptors. Given the importance of the release event, it is subjected to a very tight regulation via many different pathways, summarized in Fig. 1. Among the most complex modulators of Ca^{2+} release is regulation by the Ca^{2+} binding protein CaM. Direct CaM regulation of Ca^{2+} release, as is reviewed here, is a complex combination of direct effects on channel activity and modulation of these effects by other channel effectors. This allows a wide range of effects of CaM on calci-

um release depending on tissue type and metabolic state from activation to inhibition.

In addition to the direct binding and regulation of the Ca^{2+}-release channels, CaM also influences Ca^{2+} release through interactions with multiple other proteins that in turn are regulators of Ca^{2+} release. Although there are several such targets of CaM regulation, major contributors to regulation are the sarcolemmal voltage-dependent Ca^{2+} channel (DHPR), calmodulin-dependent protein kinase (CaMKII), and calmodulin-stimulated protein phosphatase (calcineurin).

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