Modulation of the Ryanodine Receptor and Intracellular Calcium

Ran Zalk, Stephan E. Lehnart, and Andrew R. Marks

Department of Physiology and Cellular Biophysics, Clyde and Helen Wu Center for Molecular Cardiology, Department of Medicine, Columbia University College of Physicians and Surgeons, New York, New York 10032; email: rz2127@columbia.edu, sel2004@columbia.edu, arm42@columbia.edu

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Abstract

Ryanodine receptors (RyRs)/Ca^{2+} release channels, on the endoplasmic and sarcoplasmic reticulum of most cell types, are required for intracellular Ca^{2+} release involved in diverse cellular functions, including muscle contraction and neurotransmitter release. The large cytoplasmic domain of the RyR serves as a scaffold for proteins that bind to and modulate the channel's function and that comprise a macromolecular signaling complex. These proteins include calstabins [FK506-binding proteins (FKBPs)], calmodulin (CaM), phosphodiesterase, kinases, phosphatases, and their cognate targeting proteins. This review focuses on recent progress in the understanding of RyR regulation and disease mechanisms that are associated with channel dysfunction.

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INTRODUCTION

Ca^{2+} release from intracellular stores controls numerous cellular processes by acting on Ca^{2+}-binding proteins. A steep $[Ca^{2+}]$ gradient of more than four orders of magnitude across the sarcoplasmic/endoplasmic reticulum (SR/ER) membrane, maintained by the SR/ER Ca^{2+} ATPase (SERCA), allows for rapid, localized intracellular Ca^{2+} release via Ca^{2+} release channels. Ca^{2+} release channels on the SR/ER belong to two families: the ryanodine receptors (RyRs) and the inositol 1,4,5-trisphosphate receptors. These channels differ from plasma membrane ion channels by their size and nonselective high-conductance cation transport properties, which allow for rapid release of SR/ER Ca^{2+} into the cytoplasm. Three isoforms of the RyR have been identified and cloned. The predominant isoform in skeletal muscle is RyR1, and in cardiac muscle, it is RyR2. In neurons, RyR1, RyR2, and RyR3 are all present. They are encoded by three distinct genes and share ~70% sequence homology (1).

RyRs are approximately 10 times larger than voltage-gated Ca^{2+} and Na^{+} channels. In asymmetric solutions with Ca^{2+} as the charge carrier, large Ca^{2+} conductances of ~100 pS were measured (compared to ~20 pS for the voltage-gated L-type Ca^{2+} channels). A hinge mechanism at the putative pore-lining helices involving G4866 was suggested to control channel opening and closing (2). Increased concentrations of Ca^{2+} (low μM range) activate isolated RyR channels in vitro in planar lipid bilayers potentially via a gating ring mechanism (3). However, the exact structural determinants of RyR gating are as yet unknown.

RyR channels directly control intracellular Ca^{2+} release in skeletal and cardiac muscles, activating muscle contraction during excitation-contraction (EC) coupling. Different mechanisms linking plasma membrane depolarization with myofiber contraction rely on various forms of L-type Ca^{2+} channel ($Ca_v1.1/1.2$)-RyR interaction in skeletal and cardiac muscles. In both muscles, EC coupling is initiated by depolarization of the plasma membrane by an action potential, which is transmitted deep into the cell body along transverse-tubule (T-tubule) membranes. In skeletal muscle, $Ca_v1.1$ on the T tubules colocalizes with RyR in the terminal cisternae in a three-dimensional organization such that four $Ca_v1.1$ oppose every other RyR1, whereas in cardiac muscle this organization is less regular (4, 5). RyR1 gating in skeletal myofibers is under the control of allosteric protein-protein interaction by the specific $Ca_v1.1$ isoform. In cardiac myocytes, transmembrane Ca^{2+} entry via $Ca_v1.2$ activates Ca^{2+}-induced Ca^{2+} release (CICR) by a ligand-dependent mechanism. RyR type 2 and 3 in nonmuscle cells are activated by elevated Ca^{2+} concentrations and/or by cyclic ADP

Ca^{2+} release: the fast and short flow of Ca^{2+} from intracellular stores, such as the ER and the SR, to the cytosol

SERCA: SR/ER Ca^{2+} ATPase

RyRs: ryanodine receptors

EC coupling: the process that connects myocyte membrane depolarization and muscle contraction

ribose (cADPR) by an as yet undefined mechanism. Ligand-gated CICR makes the kinetics of intracellular Ca^{2+} release slower when compared with protein-mediated activation of RyR1 in skeletal muscle (6). Neuronal RyR Ca^{2+} release modulates action potential, neurotransmitter release, and other Ca^{2+}-dependent cellular activities. Many other cell types (i.e., pancreatic β-cells and T cells) express RyRs, but little is known about the role of intracellular Ca^{2+} release in their function.

RyRs form a macromolecular signaling complex, including kinases and phosphatases that are associated with the cytoplasmic channel region, modulate the channel activity upon extracellular signals via second messengers, and thereby regulate SR Ca^{2+} release. Here, we review RyR structure-function relationships with an emphasis on striated muscle physiology and disease mechanisms.

RYR STRUCTURE AND FUNCTION

The ryanodine receptors are ~2200-kDa homotetrameric complexes of four ~565-kDa subunits, forming a square around a central pore. RyRs have large N-terminal cytoplasmic domains that modulate the gating of the channel pore located in the C terminus. The N terminus forms a large cytosolic scaffold, which interacts with regulatory proteins creating a macromolecular signaling complex. Although definitive structural evidence is currently lacking, 4–12 transmembrane domains have been predicted (7).

RyR Structure and Isoforms

In addition to serving as the major Ca^{2+} release channel required for skeletal muscle contraction, RyR1 is also expressed at lower levels in smooth muscle, cerebellum, testis, adrenal gland, spleen, and ovary. RyR2 is most abundant in the heart, lung, and brain. RyR3 is found in the brain, spleen, heart, and testis (8). Tissue-specific splice variants were identified for RyR1 in the modulatory cytoplasmic domain, generating developmentally regulated sRyRs (spliced RyRs) (9); a dominant-negative isoform of RyR3 was found to regulate Ca^{2+} release in smooth muscle (10).

Recent studies to elucidate the RyR structure, using electron microscopy (EM) image reconstructions both of single particles and of two-dimensional (2D) crystal structures, have revealed the location of several domains and the structural effects of several modulators on the receptor.

The RyR transmembrane and pore region topology is still unresolved. A truncated construct expressing the 1030 C-terminal amino acids formed Ca^{2+}-sensitive channels when incorporated into lipid bilayers (11). Prediction analysis of the RyR membrane topology from the primary amino acid sequence showed that the transmembrane domains are clustered at the C-terminal 10% of the channel, and current models predict 6 to 8 transmembrane domains (12) analogous to tetrameric potassium channels (2). Using single-particle cryo-EM, a high-resolution (9.6-Å) structure of RyR1 in the closed conformation detected five membrane-spanning α-helices (13). Moreover, Ludtke and colleagues (13) predict similarities with the $MthK^+$ channel open-pore X-ray structure, including α-helices at residues M4879-A4893 and residues I4918-E4948 (**Figure 1**). A high-resolution (10-Å) cryo-EM reconstruction structure by Samso et al. (14) also showed structural homology with the KcsA K^+ channel crystal structure at the inner pore region.

RyR isoforms contain three divergent regions, which represent sequences with the highest degree of variability between the isoforms. This sequence divergence potentially explains some of the observed isoform-specific functional differences. In RyR2, divergent regions (DRs) 1–3 have been localized to the cytoplasmic region using a combination of EM and green fluorescent protein (GFP) insertion (15). Accordingly, a similar technique was used to localize DR1

$Ca_v1.1/1.2$: L-type calcium channel dihydropyridine receptor (LTCC/DHPR)

CICR: Ca^{2+}-induced Ca^{2+} release

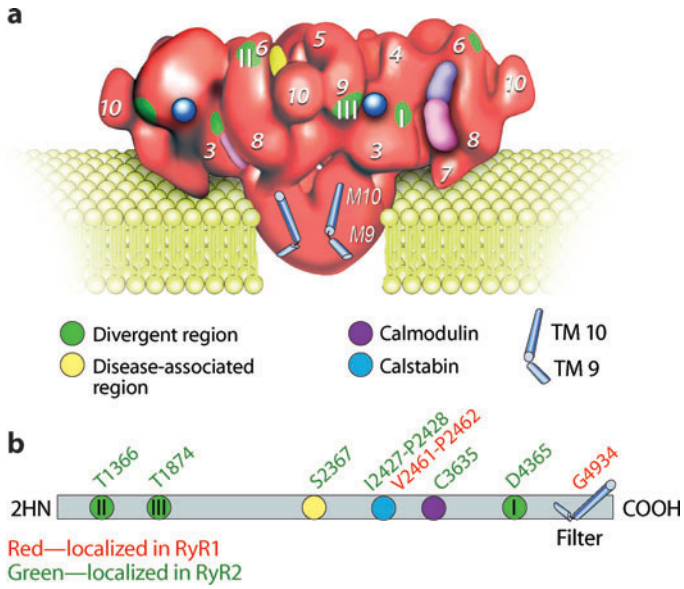

Figure 1

Domains identified in the ryanodine receptors (RyR) structure. (*a*) Schematic representation of the overall structure of the RyR, showing the relative localization of calmodulin (*purple*), Apo-calmodulin, calstabin (*blue*), the three divergent regions (*green*), the central disease-associated mutation region (*yellow*), and the pore region helices. The domain numbers, assigned by Radermacher et al. (150), are indicated. (*b*) Line sequence of the RyR, which indicates the amino acids, localized in RyR1 (*red*) and RyR2 (*green*), that are associated with the domains indicated in panel *a*. Adapted from Sharma et al. (36) with permission from the *Biophysical Journal*.

to domain 3, also known as the "handle" domain (15); DR2, to domain 6 (15a); DR3, to the "clamp region" (domain 9) (15b); and the central disease-associated region, which clusters RyRs missense mutations, to the "bridge" connecting regions 5 and 6 (15c) (**Figure 1**).

EM analysis of the in situ arrangement in the SR membrane revealed that RyRs possess an innate ability to assemble into packed 2D checkerboard-like arrays with the four corners of each receptor contacting the corners of each of its four neighbors (4). Accordingly, at the functional level, coupled channels, opening in concert, were recorded for both RyR1 and RyR2 (16, 17). Reconstruction of 2D crystal studies not only provides a high-resolution electron crystallography tool to resolve the overall structure of the channel but also an in vitro model for 2D array formation. Resembling the native RyR clusters, the purified RyR spontaneously assembles into 2D arrays (18). RyRs in the array may couple via specific domain-domain interactions between the "clamp-like" regions at the corners of the tetramers.

RyR Macromolecular Complex, and Modulation by Protein-Protein Interactions

The N-terminal cytoplasmic domain serves as a scaffold for protein-protein interactions that modulate RyR channel function. These interactions control the channel's activity in response to extracellular stimuli, allowing for adaptation during hormonal cell regulation.

$Ca_v1.1$ II-III Linker Region

Muscle EC coupling is, in large part, regulated by the interaction between RyRs and Cav1s (the voltage-gated L-type calcium channel, isoform $Ca_v1.x$) located on the plasma membrane. At EC coupling sites in the skeletal muscle, every other RyR1 channel in the junctional membrane is associated with a tetrad of four $Ca_v1.1$ channel α_1S-subunits in the T-tubule membrane (19). In contrast to cardiac muscle, skeletal muscle EC coupling occurs through voltage-gated Ca^{2+} release, controlled by direct protein-protein interactions between $Ca_v1.1$ and RyR1 in the absence of significant Ca^{2+} entry through the sarcolemma (20). Voltage-dependent coupling of $Ca_v1.1$ activation occurs through physical interaction of the $Ca_v1.1$ intracellular II–III loop with RyR1 (21). The location of the $Ca_v1.1$ tetrad in relation to the $Ca_v1.1$-binding site on RyR was revealed by comparing the relative organization of the RyR1 and $Ca_v1.1$ arrays (22).

Calmodulin

Calmodulin (CaM) is a ubiquitously expressed 17-kDa Ca^{2+}-binding protein containing four

EF hands, which binds to both RyR1 and RyR2 monomers at a 1:1 stoichiometry. In RyR1, apo-CaM (Ca^{2+} free) is a partial agonist at nanomolar Ca^{2+} concentrations, whereas at higher Ca^{2+} concentrations, Ca^{2+}-bound CaM functions as an inhibitor (23). In contrast to RyR1, CaM inhibits the cardiac RyR2 channel at all Ca^{2+} concentrations (24). CaM also modulates $Ca_v1.1$ gating in the T-tubule membrane, and Ca^{2+}-CaM may inhibit binding of the C-terminal tail of the $Ca_v1.1$ α_1S-subunit to RyR1 (25).

Ca^{2+} binding to RyR results in changes in the localization of CaM while bound to RyR (26). The change in localization could be explained by conformational changes applied to the receptor because it appears that both forms of calmodulin (Ca^{2+} bound and Ca^{2+} free) bind to the same RyR sequence (aa 3630–3637) (27). Additionally, the N-terminal lobe may bind to a neighboring RyR1 subunit (aa 1975–1999) (28). Peptide-binding studies using the 3614–3643 CaM-binding region on RyR suggested distinct activating and inhibitory binding sites (29). In addition, CaM-binding sites may be involved in the RyR1-$Ca_v1.1$ protein interaction sites during EC coupling (30). Elucidation of the exact role of CaM in RyR Ca^{2+} modulation is complex and will require further investigation in the context of tissue-specific EC coupling mechanisms.

Calstabin

The Ca^{2+} channel-stabilizing proteins calstabin1 [FK506-binding protein 12 (FKBP12)] and calstabin2 (FKBP12.6) are enzymes with peptidyl-prolyl-*cis-trans* isomerase activity. They share 85% sequence identity and form amphiphilic β-sheet structures that facilitate protein-protein interactions with RyR1 and RyR2, respectively. Quantitatively, each RyR channel tetramer binds four calstabin proteins (one molecule per monomer) (31). RyR1 and RyR3 bind calstabin1 at a higher affinity than calstabin2 (31, 32), and because calstabin1 is expressed at much higher levels in striated muscles, RyR1 and RyR3 predominantly bind calstabin1 (31). In contrast, RyR2 channels exhibit a higher affinity for and predominantly bind calstabin2 (31, 33).

Calstabin: calcium channel-stabilizing protein

Depletion of calstabin from RyR increases channel open probability and induces subconductance states, indicating that calstabin is required for stabilizing the closed state of the channel (34). Calstabin1 and calstabin2 were localized on the cytoplasmic portion of RyR1 and RyR2, respectively, at the junction between domains 3, 5, and 9 (35, 36), using high-resolution single-particle EM reconstruction. The significant conformational changes observed in the transmembrane domain upon calstabin2 binding and its close localization to the clamp regions of the RyR2 reflect the modulatory effect of this protein on channel function as seen by changes in channel open probability, subconductance states, and coupled gating (17). Fitting the calstabin X-ray structure with the EM-reconstruction of the RyR-calstabin complex as supported by biochemical data (37) suggested calstabin amino acids Q3, Q31, N32, and D37 help mediate the protein-protein interaction. Indeed, single-channel and in vivo data showed the direct involvement of calstabin2 D37 in the binding to RyR2 (37, 38). By contrast, the V2461 and P2462 residues on RyR1 and the homologous I2427 and P2428 on RyR2 have been identified as critical determinants of calstabin binding (39). The locations of these residues correspond to the twisted amide transition-state intermediate of a peptidyl-prolyl bond that allows for calstabin1 and 2 binding with high affinity (40).

We have reasoned that in the wild-type channel the peptidyl-prolyl bond is constrained in the high-energy transition-state intermediate between *cis* and *trans*. Indeed, a V2461G RyR1 mutant channel showed increased mobility around the peptidyl-prolyl bond, allowing for *cis/trans* isomerization, which destabilized the binding of calstabin1 (39).

Ca^{2+} leak: a silent efflux of Ca^{2+} from the SR through RyRs under resting conditions

Molecular-modeling studies have predicted that the proline in the calstabin-binding region on RyR induces a break in a helix, which imposes a twisted amide transition state on the peptidyl-prolyl bond and thus allows for stable calstabin binding (41). Moreover, RyR2 fragment studies have suggested the existence of multiple calstabin2 contact sites within the N terminus (42). Additionally, it was hypothesized that RyR subunit interdomain interactions may contribute to calstabin binding (43).

RyR Phosphorylation by Protein Kinase A or CaMKII

RyR2 functions as a macromolecular signaling complex that controls SR Ca^{2+} release (44). The purified channel complex revealed that RyR is associated with protein kinase A (PKA) and the phosphatases PP1 and PP2A. The interaction is mediated by anchoring proteins, specifically muscle A-kinase-anchoring protein (mAKAP) [which targets PKA and phosphodiesterase 4D3 (PDE4D3) (45)] as well as spinophilin and PR130 (which target PP1 and PP2A to RyR2, respectively). The association of adaptor proteins with RyR is mediated by conserved leucine-isoleucine zipper motifs, which function as specific anchoring sites, thereby allowing for specific, compartmentalized cAMP-dependent RyR2 regulation (44, 46, 47). Stimulation of β-adrenergic receptors results in PKA activation and phosphorylation of RyR2 at S2808, which causes a transient decrease in the binding affinity of calstabin2 and an increase in Ca^{2+}-dependent activation of the channel (38, 48, 49). This mechanism allows for increased SR Ca^{2+} release upon Ca^{2+} influx via $Ca_v1.2$ as a part of the "fight-or-flight" mechanism. By contrast, chronic PKA hyperphosphorylation of RyR2 can result in incomplete channel closure and a Ca^{2+} "leak" during diastole, which causes depletion of the SR Ca^{2+} store and reduced Ca^{2+} release upon receptor activation (38, 48, 49). The majority of studies have found that PKA phosphorylation increases and dephosphorylation decreases RyR channel activity, as reviewed in detail previously (71). Moreover, the cAMP-specific PDE4D3 associates with RyR through mAKAP, thereby creating a localized negative feedback loop, ensuring tight regulation of RyR2 phosphorylation and channel activity (46).

The RyR complex also associates with CamKII by an unidentified targeting mechanism. CamKII phosphorylation increases single-channel RyR2 open probability (50), but to a smaller extent than PKA phosphorylation (51). Mutagenesis study of the full-length RyR2 has confirmed S2814 as the CamKII and S2808 as the PKA phosphorylation sites, respectively (44, 51). A second RyR2 PKA phosphorylation site has been suggested (52). However, single-channel experiments and the analysis of the RyR2-S2808A knock-in mouse have confirmed S2808 as the sole functional PKA phosphorylation site (53). Unlike CamKII phosphorylation, RyR2 PKA phosphorylation transiently dissociates calstabin2 from the channel complex, which induces subconductance states and may contribute to the greater increase in open probability (51). Although calstabin2 dissociation was not observed by Chen and colleagues (52), the majority of studies have confirmed calstabin2 dissociation during physiologic conditions or chronic depletion in heart failure. Both PKA and CamKII are thought to play important roles in the regulation of EC coupling in the heart. CamKII activity in the heart increases at higher heart rates and phosphorylates RyR2 at S2814 to enhance SR Ca^{2+} release to maintain the positive force-frequency relationship (51). Increased CamKII activity also phosphorylates phospholamban to help accelerate diastolic filling of the ventricles at higher heart rates (54). Catecholaminergic stimulation of the heart as part of sympathetic nervous system activation during stress (fight or flight) has been linked to RyR2-S2808 PKA phosphorylation and may play an important role during rapid increases of intracellular Ca^{2+} release also referred to as EC coupling gain (53, 55).

Other RyR-Associated Proteins

Sorcin is a cytosolic 22-kDa Ca^{2+}-binding protein associated with both RyR2 and the L-type Ca^{2+} channel. Sorcin contains two high-affinity EF Ca^{2+}-binding domains and inhibits RyR2 presumably in the Ca^{2+}-bound conformation (56). Direct PKA phosphorylation of sorcin relieves the stabilizing RyR2 effects (56). Sorcin overexpression in cardiomyocytes decreased Ca^{2+}-transient amplitude and Ca^{2+} "spark" frequency (57). Sorcin may undergo Ca^{2+}-dependent conformational changes and transiently bind to the cytosolic RyR2 domain during systolic Ca^{2+} release to facilitate channel closure.

RyR2 may be modulated by a luminal Ca^{2+} sensor complex comprised of triadin, junctin, and calsequestrin (CSQ). Ca^{2+}-dependent conformational changes in CSQ may modulate RyR channel activity directly (58) or through interactions with triadin and/or junctin (59, 60).

The SR represents the primary Ca^{2+} storage organelle in striated muscles. Acidic Ca^{2+}-binding proteins provide luminal Ca^{2+} buffering mechanisms maintaining intra-SR free [Ca^{2+}] around 1 mM. CSQ is the major luminal SR Ca^{2+}-binding protein providing low-affinity, high-capacity intracellular Ca^{2+} buffering particularly at the terminal SR Ca^{2+} release site (61). Multimers of the histidine-rich Ca^{2+}-binding protein (HRC) function as a luminal Ca^{2+} storage protein at lower concentrations than CSQ (62) and interact with triadin at low [Ca^{2+}] (63). Junctate, a transmembrane luminal Ca^{2+}-binding protein homologous to junctin, is localized to the junctional SR and contributes to high-capacity Ca^{2+} buffering at the sites of SR Ca^{2+} release together with CSQ and HRC (64). Junctophilins1–3 form junctional complexes between the plasma membrane and the SR membrane and may thereby regulate cross talk between plasma membrane components and RyR channels (65).

INTRACELLULAR Ca^{2+} RELEASE

There are two known modes for RyR activation. The first is conformational Ca^{2+} release whereby skeletal RyR1 Ca^{2+} release is directly activated by the voltage activation of four associated $Ca_v1.1$s, by direct protein-protein interaction. A second mode of cardiac RyR2 activation occurs by CICR, also promoting recruitment of RyRs not located in close proximity to the activating $Ca_v1.2$s (66). In cardiac muscle RyR2 is gated by CICR through the cardiac $Ca_v1.2$s isoform ($Ca_v1.2$) located on the T-tubule membrane (67).

Ca^{2+} spark: the smallest readily detected local Ca^{2+} release event

Ca^{2+} Leaks, Sparks, and Transients

Ca^{2+} sparks have been documented in isolated cardiomyocytes (68), in smooth muscles (69), and in skeletal muscles (70). A silent efflux of Ca^{2+} from the SR through RyRs under resting conditions, or a Ca^{2+} leak, affects the SR Ca^{2+} content and subsequently EC-coupling gain (55). In cardiac and skeletal muscle cells, at least 4–6 RyR channels cluster into dense arrays (71), allowing for the generation of Ca^{2+} sparks, which are short lived, local release events that can either be spontaneous or triggered by $Ca_v1.2$ activation (68, 70, 72). A Ca^{2+} spark may last for about 50 ms and spreads to a size of about 2 μm in diameter, arising from a point source of Ca^{2+} in a ryanodine-sensitive manner (68). These important observations, complemented with biochemical and electrophysiological data, are the basis of our current understanding of the pathophysiology of heart failure. A result of this was the hypothesis that Ca^{2+} sparks are the smallest form of Ca^{2+} release and that, upon membrane action potential, the simultaneous generation of a large number of sparks causes the formation of the Ca^{2+} transient (see the model in **Figure 2**). Most of the Ca^{2+} that causes cardiac muscle contraction is released from the SR [70% to 92%, depending on the cell type and species (73)]. This Ca^{2+} release would be expected to cause a positive feedback that would release even greater amounts

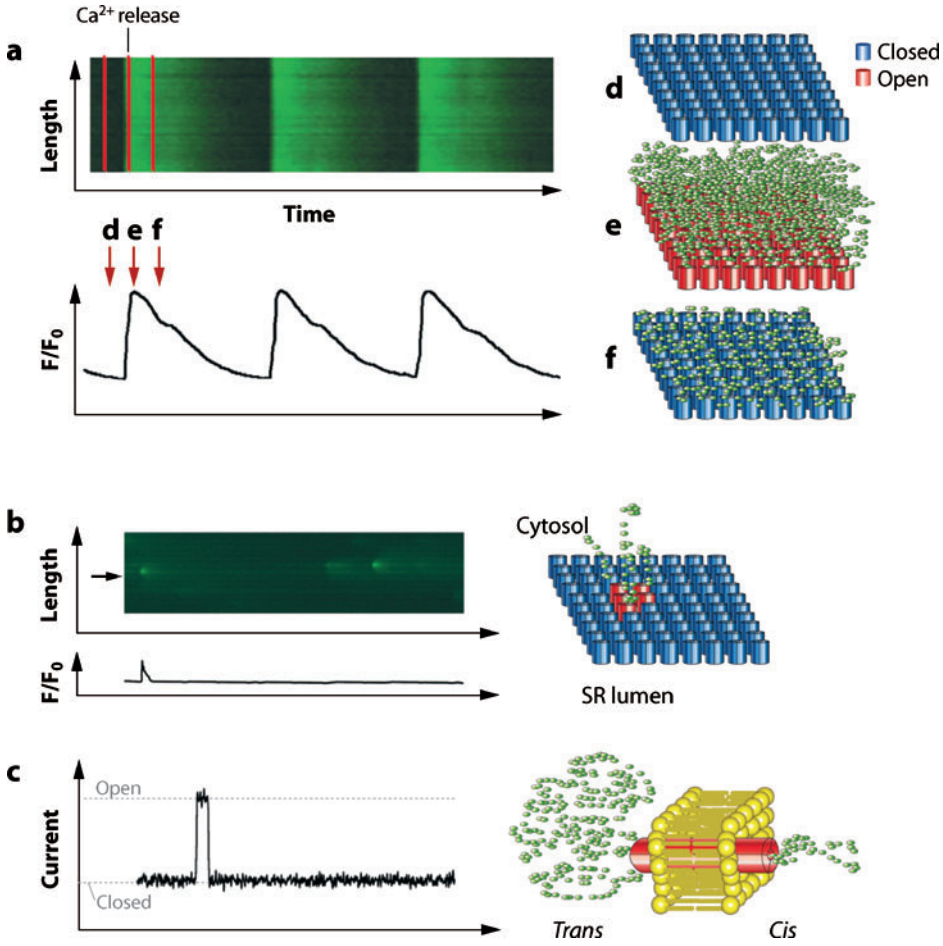

Figure 2

Measuring and imaging RyR-related Ca^{2+} release events. (*a*) Confocal line scan image of electrically evoked (1 Hz) Ca^{2+} transients in cardiac myocyte. Ca^{2+} release is indicated by the red lines. (*b*) Line scan imaging of calcium sparks. (*c*) Current trace of a RyR single-channel recording. Panels *d–f* are the schematic view of the RyRs at open (*red*) and closed (*blue*) states for each form of Ca^{2+} release.

of Ca^{2+}. However, both the time and the amplitude of SR Ca^{2+} release are tightly regulated such that sparks terminate within milliseconds to promote muscle relaxation. It was shown that discrete Ca^{2+} release sites are activated by local Ca^{2+} currents. However, the termination of SR Ca^{2+} release occurs by an unidentified mechanism within the RyR channel clusters (72) together with a decrease in luminal $[Ca^{2+}]$, which enhances the termination of the release unit (74). Despite the clear experimental evidence for the increased Ca^{2+} sensitivity of RyR upon PKA and CAMKII phosphorylation, the effect of phosphorylation on spark generation in vivo is still unclear because some groups could not detect an increased Ca^{2+} spark rate upon adrenergic stimulation (55, 75), even when a Ca^{2+} leak upon adrenergic stimulation is evident (38, 76, 77).

Although Ca^{2+} spark formation is a well-established phenomenon in cardiac muscle, these local Ca^{2+} release events are considered rare under physiological conditions in the adult mammalian skeletal muscle. As opposed to cardiac RyR2, which is activated by $[Ca^{2+}]$ elevation (CICR), skeletal muscle RyR1 is activated via direct protein-protein interaction with the $Ca_v1.1$. Concerted release of Ca^{2+} sparks are observed upon depolarization of the plasma membrane as the $Ca_v1.1$ is activated by the action potential. Spontaneous Ca^{2+} sparks could be found in skeletal muscle primary cultures in regions of cells lacking T tubules. In myocytes cultured from dysgenic mice lacking

$Ca_v1.1$, sparks could be detected near the T-tubule regions, suggesting that $Ca_v1.1$s play a critical role in suppressing the production of Ca^{2+} sparks in the mammalian skeletal muscle (78). Under conditions of fatigue or osmotic shock and in dystrophic muscle, increased Ca^{2+} spark activity can be observed also in the mature mammalian skeletal muscle (79), supporting the role of $Ca_v1.1$ in suppressing Ca^{2+} leak because these conditions may disrupt the RyR1-$Ca_v1.1$ interaction and allow spark formation.

The $Ca_v1.2$-RyR architecture may allow for the formation of Ca^{2+} microdomains as revealed by spatially restricted, increased cytoplasmic Ca^{2+} concentration in regions of the cell close to the channel. The tubular morphology of the SR with its specific distribution within the muscle cell allows the localized release of Ca^{2+} close to the T tubules and to the myofibers (19). RyRs are localized mainly to the junctional SR close to the sarcolemmal T tubules forming the "feet" structures (4). The high Ca^{2+} buffering capacity, the fast activity of SERCA, and the slow Ca^{2+} diffusion rate all contribute to the localized nature of the Ca^{2+} signal.

Coupled Gating

Modeling has predicted that a receptor, bound to its ligand, propagates a change in activity in neighboring receptors in a cluster, lowers the activation threshold, and increases the range of responses to ligand concentration (80). We have shown that physical and functional association between RyR channels results in coordinated gating behavior termed coupled gating (16, 17). Functional coupled gating of RyR requires the binding of RyR to calstabin, thereby identifying an additional role for calstabin in the functional coordination of RyR channel complexes and allowing clusters of channels to function as "Ca^{2+} release units."

Modulation by Small Molecules

Physiologic modulators of RyR function include ATP, Ca^{2+}, and Mg^{2+}, posttranslational modifications (e.g., phosphorylation, oxidation), and pharmacologic substances (e.g., ryanodine, caffeine).

Ca^{2+} microdomains: increased cytoplasmic Ca^{2+} concentration in localized regions of the cell close to the Ca^{2+} release channels

Modulation by ions. Force production in cardiomyocytes is activated by about a 10-fold increase in intracellular [Ca^{2+}] by the process of CICR. Cardiac RyR2 functions as a ligand-activated ion channel, which upon Ca^{2+} binding releases Ca^{2+} from the terminal SR (6). Single-channel experiments have documented RyR2 activation by high-nanomolar to low-micromolar free *cis* [Ca^{2+}], corresponding to the cytosolic channel side. Skeletal RyR1 channels are inhibited by approximately 1 mM *cis* [Ca^{2+}], implicating a physiological role for the bell-shaped Ca^{2+} dependence, whereas cardiac RyR2 channels require significantly higher *cis* [Ca^{2+}] for Ca^{2+}-dependent inactivation to occur. The exact molecular mechanism of RyR Ca^{2+} regulation, however, has remained elusive. RyR2 is also modulated by putative low-affinity Ca^{2+} sensors from inside the SR lumen (81). A decrease of SR [Ca^{2+}] on the luminal side inactivates RyR2 and contributes to termination of CICR (74).

The cytosol of most cells contains approximately 1 mM free [Mg^{2+}] and approximately 5 mM ATP, most of which is bound to Mg^{2+}. Cytosolic Mg^{2+} is a powerful RyR channel inhibitor (82). Regulation of RyR channels by Mg^{2+} decreases the Ca^{2+} sensitivity, and functional assays have indicated two distinct metal-binding sites (82). Moreover, RyR post-translational modifications (PKA phosphorylation, S-nitrosylation) or missense mutations may decrease the Mg^{2+} sensitivity of the channel (83), and hormonal regulation of intracellular [Mg^{2+}] may further influence RyR Ca^{2+} release.

Oxidation and nitrosylation. RyRs contain 80–100 cysteines per monomer with approximately 25–50 in the reduced state. An additional six to eight are considered hyperreactive, making them suitable for modification by oxidation (84). Oxidation of critical

CPVT: catecholaminergic polymorphic ventricular tachycardia

sulfhydryls located on the cytoplasmic side of RyR1 affect both the gating properties of the channel and responsiveness to channel modulators, such as adenine nucleotides, caffeine, and Ca^{2+} and Mg^{2+} sensitivity; the channel's ability to bind calmodulin (85) and calstabin were also affected (86). Reactive oxygen increases Ca^{2+} efflux from the cardiac SR vesicles, and calmodulin was found to function as a mediator of reactive oxygen-triggered Ca^{2+} release through the RyR (87). Nitric oxide (NO) was suggested as the physiological modulator of the RyR redox state because its endogenous nitrosylation was described as leading to alteration in channel activity (84). The C3635 or other cystein residues of RyR1 may undergo selective covalent modification by NO (88), thereby contributing to redox regulation of the RyR1 complex (89).

Nucleotides, ATP, and cADPR. Adenine nucleotides stimulate the [^{3}H]-ryanodine-binding affinity and enhance the open probability of the channel in a Ca^{2+}-independent manner in RyR1 with a K_a of about 1 mM. Thus, under physiological conditions in the skeletal muscle, ATP may contribute to RyR channel activation. Different effects of distinct purines on RyR activity suggest a separate binding site for each of these purines (90, 91). ATP increases single-channel RyR2 activity in the presence of Ca^{2+}. AMP-PCP, a nonhydrolyzable analogue of ATP, shows a synergistic effect together with Ca^{2+} in the induction of major structural changes in the clamp regions and the pore region of RyR1 (92).

cADPR acts as a RyR activator in nonmuscle cells (93), but other reports showed no effect of cADPR on RyR activity. It is possible that cADPR-mediated Ca^{2+} signaling of the RyR occurs via upstream events and not by directly modulating the channel. It was recently demonstrated that cADPR-mediated Ca^{2+} release plays an important role in the regulation of NO production (94), and hypoxic conditions were also shown to activate cADPR-mediated RyR activation (95), which may suggest that cADPR-mediated RyR activation could be a redox state-related phenomenon.

Pharmacology

Ryanodine is an alkaloid that binds the channel with high affinity in a Ca^{2+}-dependent manner, making it an important tool for biochemical characterization of the channel and a specific blocker of single channels, inducing characteristic subconductance states (96). Two ryanodine-binding sites, a high-affinity and a low-affinity binding site, have been described at the C terminus of the receptor (97).

Caffeine. Caffeine increases both the RyR mean open time and open probability. It acts in a cooperative manner with both Ca^{2+} and ATP and therefore increases the affinity of RyR for these physiological activators (98). Additionally, caffeine is used to measure cellular SR Ca^{2+} content indirectly because its application causes emptying of the SR Ca^{2+} store.

JTV-519. JTV-519, also known as K201 (a 1,4-benzothiazepine), is a member of a class of drugs known as Ca^{2+} channel stabilizers, shown to increase calstabin binding to RyR and to prevent ventricular arrhythmias (49, 99, 100). JTV519 inhibits diastolic SR Ca^{2+} leak and prevents arrhythmias by increasing the binding affinity of calstabin2 for RyR2 (100, 101). However, in a R4496C knock-in mouse model of catecholaminergic polymorphic ventricular tachycardia (CPVT) treated with caffeine and catecholamines, JTV519 showed no antiarrhythmogenic effect (102).

RYR DYSFUNCTION AND DISEASES

Skeletal Muscle

Skeletal muscle disease (myopathy) is a potentially devastating condition occuring from genetic or acquired muscle defects. RyR1

mutations are a frequent cause of inhalation anesthesia complications and also occur in other forms of myopathy. Muscle fatigue may involve RyR1 dysfunction, a syndrome that occurs concomitantly with many other diseases and has important implications for life quality and disease outcome.

Malignant hyperthermia. Malignant hyperthermia (MH) is a pharmacogenetic shock syndrome, triggered by halogenated inhalation anesthetics (e.g., halothane) and/or depolarizing muscle relaxants (e.g., succinylcholine) typically during surgery (103). Genetically susceptible patients exposed to these drugs develop skeletal muscle rigidity, rapidly rising body temperature, fast heart rates, and ultimately a metabolic crisis including acidosis. Triggering agents initiate uncontrolled intracellular Ca^{2+} release in skeletal muscle contraction, resulting in excessive ATP hydrolysis, acidosis, cyanosis, and heat generation (104). In vitro contracture tests of muscle biopsies obtained from MH-susceptible patients confirm abnormal sensitivity to caffeine and halothane (104). In pigs susceptible to MH, a single R615C mutation in the *RyR1* gene has been identified (105). In MH-susceptible patients, three mutation clusters have been identified in the RyR1 gene: the N-terminal region C35-R614, the central region D2129-R2458, and the C-terminal region I3916-A4942. It has been proposed that MH mutations in the N-terminal and central regions of the RyR1 protein disrupt a critical interdomain interaction, destabilizing the channels' closed state (106). As a result of the closed-state destabilization, RyR1 MH mutations contribute to the abnormal sensitivity to channel activation by agents, including caffeine, halothane, 4-chloro-*m*-cresol, or membrane depolarization (107, 108). Dantrolene is thought to function as a RyR1 channel blocker, application of which is used as an antidote to prevent intracellular Ca^{2+} leak and to counter development of an MH crisis in susceptible mutation carriers (109). Although RyR1 mutations account for over 50% of all MH cases, other MH susceptibility loci exist, including $Ca_v1.1$ missense mutations of a conserved arginine residue in the $Ca_v1.1$ III-IV linker region (R1086H and R1086C) (110). Analogous to RyR1 MH mutations, the $Ca_v1.1$ R1086H mutation increases the sensitivity of the SR Ca^{2+} release to activation (111).

MH: malignant hyperthermia

CCD: central core disease

Central core disease and multiminicore disease. Central core disease (CCD) characteristically presents as a congenital myopathy at infancy with proximal muscle weakness and delayed attainment of motor milestones (112). Although variable, skeletal muscle biopsies reveal amorphous cores or regions devoid of mitochondria and oxidative enzyme activity (113). Multiminicore disease also manifests as a myopathy with muscle weakness and has histologic areas that lack oxidative enzyme activity (114). CCD has been linked to mutations in the RyR1 gene in the same regions of the RyR1 protein as MH (115–117). CCD patients are frequently susceptible to MH (118). The majority of RyR1 CCD mutations are localized in the C terminus (118, 119). RyR1 mutations linked to CCD alter RyR1-dependent Ca^{2+} release mechanisms (120), cause an SR Ca^{2+} leak (108), or result in EC uncoupling (121). In the later case, muscle weakness in CCD may result from reduced SR Ca^{2+} release during EC coupling owing to mutations in the C-terminal RyR1 pore region, which result in reduced sensitivity to voltage-dependent Ca^{2+} release (121, 122).

Skeletal muscle fatigue. A reduced capacity for muscle force production after prolonged activity from exercise is commonly referred to as muscle fatigue and has been attributed to accumulation of lactic acid (123). Studies performed under more physiologic conditions have shown that tetanic contractions do not result in significant intracellular pH changes and even protect from fatigue (124). Acute fatigue development has been linked to a reversible depression of intracellular Ca^{2+} transients (125) and to specific

intracellular Ca^{2+} transport mechanisms (126). RyR1-dependent SR Ca^{2+} leak mechanisms have recently been shown during sustained exercise and implicated in dystrophic muscle remodeling (79). Chronic fatigue is also a common symptom in a variety of disease forms. The clinical severity of heart failure is classified according to fatigue symptoms. We have recently documented an intracellular Ca^{2+} leak in skeletal muscles from animals with heart failure (HF), which was attributed to chronic RyR1 PKA hyperphosphorylation, resulting in a gain-of-function defect (127). Moreover, we have shown that a drug that fixes the RyR1 Ca^{2+} leak in heart failure also improves fatigability in skeletal muscle (99). Thus a chronic hyperadrenergic state during HF may cause intrinsic skeletal muscle fatigue and contribute to a debilitating syndrome in patients.

Cardiac Muscle

Heart disease is a leading cause of death worldwide, and the incidence of heart failure increases in an epidemic fashion. RyR2 dysfunction has been linked to heart failure and arrhythmias. Although considered rare at present, inherited arrhythmias have been linked to RyR2 mutations and provide a paradigm to understand channel function in physiology and disease.

Catecholaminergic polymorphic ventricular tachycardia and arrhythmogenic right ventricular dysplasia. Missense mutations in the cardiac RyR2 have been associated with CPVT (128–130) and possibly with a form of arrhythmogenic right ventricular dysplasia (ARVD2) (131), although the linkage of RyR2 mutations to ARVD2 has recently been called into question and the phenotype of patients with RyR2 mutations likely does not match the criteria for diagnosis of ARVD. Analogous to the MH/CCD mutations in RyR1, the RyR2 mutations cluster in three regions: in the N terminus (176–420), the central region (2246–2504), and the C-terminal region (3778–4950) that includes the transmembrane pore region (132). CPVT has a high risk of stress-induced juvenile sudden death, and no specific therapeutic treatment exists (83, 129). Unlike ARVD2, patients with CPVT are thought to have functionally and structurally normal hearts, apart from minor changes reported in a few patients (129, 133). We have found a gain-of-function defect, which occurs specifically following PKA phosphorylation of RyR2 at Ser2808 and coincides with significantly decreased calstabin2-binding affinity (38, 83), in a group of CPVT-mutant RyR2 channels. Moreover, increasing calstabin2 binding to RyR2 by mutagenesis (38) or with the drug JTV519 normalized CPVT-mutant RyR2 single-channel function (83). An intracellular Ca^{2+} leak has been confirmed in a different CPVT-mutant RyR2, expressed in atrial tumor cells (134). Stress testing in calstabin2-deficient mice reproduced CPVTs (38), which could be prevented by pretreatment with the RyR2 channel stabilizer JTV519 in calstabin2$^{+/-}$, but not in calstabin2$^{-/-}$ mice (49). Moreover, we have documented an intracellular Ca^{2+} leak in calstabin2$^{+/-}$ cardiomyocytes, activating an arrhythmogenic transient inward current (I_{ti}) and delayed afterdepolarizations (DADs) consistent with triggered arrhythmias (38, 101). Bidirectional ventricular arrhythmias, I_{ti}, and DADs have been demonstrated in a RyR2-R4496C knock-in mouse (102). However, in one study, JTV519 treatment did not prevent arrhythmias or DADs in the R4496C mouse model, following epinephrine and caffeine, but the more disease-relevant exercise testing was not performed (102).

Stress-induced CPVT has also been linked to missense and nonsense mutations in cardiac CSQ (135, 136). One mutation results in substitution of an aspartic acid for a histidine at position 307 in the negatively charged C-terminal region involved in Ca^{2+} binding. This may cause an increased SR Ca^{2+} leak by as yet undefined molecular mechanisms (137). Indeed, overexpression or knockout studies

using the CPVT-mutant CSQ in heterologous cells or cardiomyocytes have confirmed a luminal SR Ca^{2+} leak (138).

Heart failure. Heart failure (HF) is a leading cause of mortality and is characterized by secondary activation of neuroendocrine pathways in a futile attempt to overcome depressed cardiac function, typically following myocardial infarction, viral myocarditis, toxic cardiomyopathy, or other insults. Over weeks to months, a chronic hyperadrenergic state ensues with elevated plasma catecholamine levels, which contribute to a progressive, maladaptive response, including cardiac chamber remodeling, progressive pump dysfunction, and deadly arrhythmias. In HF, RyR2 is PKA hyperphosphorylated, contributing to an intracellular SR Ca^{2+} leak (44, 100, 139, 140). PKA hyperphosphorylation has been reported for other proteins, including the plasma membrane Na^+/Ca^{2+} exchanger (141), the L-type Ca^{2+} channel (142), and sorcin (143). Importantly, although HF results in a decreased SR Ca^{2+} load, a RyR2-dependent SR Ca^{2+} leak can be maintained despite a reduced Ca^{2+} gradient (144), potentially contributing to maladaptive remodeling and triggered arrhythmias.

Which molecular mechanisms may contribute to RyR2 PKA hyperphosphorylation? During HF, PKA levels are unchanged (44, 127), and phosphatase (PP1, PP2A) levels in the RyR2 protein complex are decreased, contributing to a reduced rate of S2808 dephosphorylation (44, 127). In parallel, because of the chronic hyperadrenergic state, desensitization of β1-adrenoceptors and reduced global intracellular cAMP synthesis occur (145–147). Decreased phosphodiesterase activity in the RyR2 complex could result in increased local cAMP concentration, directly contributing to chronic PKA hyperphosphorylation of RyR2 and an intracellular Ca^{2+} leak.

A splice variant of the PDE4 family, PDE4D3, contains an N-terminal targeting motif for mAKAP, forming a PKA-mAKAP-PDE4D3 signaling module (148).We demonstrated a specific association of PDE4D3 with the cardiac RyR2 complex (46). In human HF, PDE4D3 levels in the RyR2 complex were decreased by 43%, and the cAMP hydrolyzing activity of RyR2-bound PDE4D3 was decreased by 42% (46). Thus, RyR2 PKA hyperphosphorylation and calstabin2 depletion from the RyR2 complex may result directly from reduced PDE4D3 activity in the RyR2 complex (46). For that reason, we investigated whether PDE4D3 deficiency in the RyR2 complex contributes to the development of HF using a mouse model of *PDE4D* gene inactivation made by Jin et al. (149). *PDE4D*$^{-/-}$ mice showed an age-dependent increase in left ventricular dimensions and a concomitant decrease in cardiac function that was significant by 9 months of age, consistent with a slowly progressive form of heart failure (46). Because PKA and phosphatase (PP1, PP2A) protein levels in the RyR2 complex were unchanged, the PKA hyperphosphorylation in *PDE4D*$^{-/-}$ mouse hearts was likely caused by decreased PDE4D3 protein levels in the channel complex (46). To demonstrate that the cardiomyopathy and arrhythmias in PDE4D-deficient mice are due to PKA phosphorylation of RyR2, we crossed these mice with the RyR2-S2808A knock-in mice that harbor RyR2 channels that cannot be PKA phosphorylated and showed that these mice are protected against HF progression and arrhythmias (46). Thus, the RyR2 channel is the critical mediator of cardiovascular pathology in *PDE4D*$^{-/-}$-deficient mice.

CONCLUSIONS

The RyR channels are increasingly recognized as potential therapeutic targets for diverse human disorders, including those affecting cardiac and skeletal muscles and the central nervous system. Greater understanding of RyR structure/function relationships is critical to advance the therapeutic potential for this target.

SUMMARY POINTS

1. Altered Ca^{2+} release/handling contributes to impaired muscle contractility, leading to pathological conditions of skeletal and cardiac muscles.
2. Chronic β-adrenergic stimulation results in PKA hyperphosphorylation of both cardiac and skeletal muscle RyRs, causing the dissociation of the channel-stabilizing protein calstabin and leading to an intracellular Ca^{2+} leak.
3. The RyR channels are potential therapeutic targets for a wide range of human cardiac and skeletal muscle diseases and perhaps also for central nervous system disorders.
4. RyR structure/function relationships are critical to advance the therapeutic potential of this target. Great progress has been made in recent years in revealing some of the functional effects of alterations in channel structure on its biophysical properties.

FUTURE ISSUES

1. Ryanodine-related diseases are still being discovered, and most of them are not fully characterized at the functional and physiological levels. Analysis of animal models with knocked-in mutations of RyRs and their modulatory proteins as well as drugs designed for and targeted to the RyRs will be of great benefit to the field.
2. The molecular mechanism by which the RyR channel is gated is still far from resolved. A high-resolution structure of the receptor, backed with a detailed biochemical characterization, is still the key for understanding, at the molecular level, the structure-function relationship of the RyR channels.

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DISCLOSURE STATEMENT

A.R. Marks has a commercial interest in and S.E. Lehnart is a consultant for a company targeting RyR channels for heart failure.

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