Ryanodine Receptor Calcium Release Channels

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RyR-mediated Ca$^{2+}$ release have taken many forms and have steadily advanced our knowledge. This robust field, however, is not without controversial ideas and contradictory results. Controversies surrounding the complex Ca$^{2+}$ regulation of single RyR channels receive particular attention here. In addition, a large body of information is synthesized into a focused perspective of single RyR channel function. The present status of the single RyR channel field and its likely future directions are also discussed.

I. INTRODUCTION

Calcium is a common second messenger (38, 42, 123) that regulates many processes in cells (e.g., contraction, secretion, synaptic transmission, fertilization, nuclear pore regulation, transcription). Although cells are typically bathed in solutions containing a relatively high Ca$^{2+}$ concentration (i.e., in the millimolar range), the cytoplasm of most cells contains much lower resting Ca$^{2+}$ concentrations (i.e., in the 100 nM range). Thus Ca$^{2+}$ entry across the surface membrane can substantially elevate cytosolic Ca$^{2+}$ levels providing the Ca$^{2+}$ trigger signals for a large number of physiological processes. The surface membrane, however, is not the only source of triggering Ca$^{2+}$ signals. Most cells have developed an additional pathway to generate localized and fast trigger Ca$^{2+}$ signals deep inside the cell. This other pathway involves specialized intracellular Ca$^{2+}$ storage/release organelles.

Cells have many different types of Ca$^{2+}$ transporters (channels, exchangers, pumps) that modulate cytosolic Ca$^{2+}$ levels. The rich array of Ca$^{2+}$ transport proteins in the surface and intracellular membranes provides numerous potential Ca$^{2+}$ signaling pathways that can respond to many different types of stimuli. Specificity of any one Ca$^{2+}$ signal pathway to a particular process is often made possible by assembling the particular signaling proteins into a local complex. Localizing the Ca$^{2+}$ signaling effectively allows Ca$^{2+}$ to be effectively directed to a particular physiological function. Certain vascular smooth muscles are a good example of this. In these smooth muscles, large global intracellular Ca$^{2+}$ signals activate the contractile machinery causing the cell to contract. However, small very localized Ca$^{2+}$ signals (i.e., Ca$^{2+}$ sparks) that arise from intracellular Ca$^{2+}$ stores activate certain K$^+$ channels in the surface membrane promoting relaxation of the cell (219).

The primary intracellular Ca$^{2+}$ storage/release organelle in most cells is the endoplasmic reticulum (ER). In striated muscles, it is the sarcoplasmic reticulum (SR). The ER and SR contain specialized Ca$^{2+}$ release channels. There are two families of Ca$^{2+}$ release channels: the ryanodine receptors (RyRs) and inositol trisphosphate receptors (IP$_3$Rs). Each family of Ca$^{2+}$ release channels appears to contain three different isoforms (300, 309). However, there may be a fourth type of RyR channel in fish (i.e., RyR-slow; Refs. 90, 210). The Ca$^{2+}$ release channels are large oligomeric structures formed by association of either four RyR proteins or four IP$_3$R proteins. The RyR and IP$_3$R proteins share significant homology, and this homology is most marked in the sequences that may form the channel’s pore (204, 271, 299, 315a, 367).

Calcium regulates all the RyR and IP$_3$R channels. However, each Ca$^{2+}$ release channel has its own distinguishing functional attributes. The IP$_3$R channels require the presence of inositol 1,4,5-trisphosphate. The activity of one type of RyR channel is coupled to a “voltage sensor” in the plasma membrane. The Ca$^{2+}$-induced Ca$^{2+}$ release process governs the activity of the other types of RyR channel. The distinguishing functional attributes of each RyR or IP$_3$R channels likely underlie the spatiotemporal complexity of intracellular Ca$^{2+}$ signaling in cells.

During the last 10 years, there have been numerous reviews on several aspects of regulation of RyR-mediated Ca$^{2+}$ signaling and/or related topics (e.g., Refs. 18, 43, 60, 69, 76, 84, 87, 92, 112, 179, 195, 199, 202, 225, 245, 252, 253, 255, 260, 265, 281, 287, 300, 301, 307, 315a, 335, 346, 354). Here, we focus on the RyR Ca$^{2+}$ release channels found in striated muscle. The controversial and complex Ca$^{2+}$ regulation of single RyR channels is given particular attention. An attempt has been made to cover this large rapidly growing and dynamic body of information. Regrettably, it is impossible to describe all of the quality studies that characterize this field. It is hoped that the single-channel perspective of this review will be useful to readers seeking to understand the complex dynamics of single RyR channel regulation.

II. HISTORY

The significance of SR Ca$^{2+}$ release during skeletal muscle contraction was recognized many decades ago (for review, see Refs. 38, 42, 260). Electrophysiological experiments and $^{45}$Ca autoradiography studies provided evidence in intact muscle that Ca$^{2+}$ is released from the terminal cisternae of the SR in response to depolarization of surface membrane invaginations called transverse tubules (t tubules) (reviewed in Ref. 260). A number of studies followed, and a putative mechanism was proposed (43, 251–255, 265–267, 296), namely, that there is a physical coupling between an integral t-tubule protein and the SR Ca$^{2+}$ release channel. The idea was that voltage-dependent movements of charged particles in the integral t-tubule protein activate the SR Ca$^{2+}$ release channel through this physical coupling. This mechanism is generally referred to as depolarization-induced Ca$^{2+}$
release (DICR). Parallel work in other experimental preparations (i.e., SR vesicles and skinned muscle fibers) confirmed the existence and importance of the DICR mechanism in skeletal muscle (38, 94, 121, 127, 138, 150, 151, 183, 260, 296, 321).

In other early works, it was realized that a small increase in cytosolic Ca\(^{2+}\) concentration could induce massive intracellular SR Ca\(^{2+}\) release events in both skeletal and cardiac muscle (75, 89). This phenomenon is often called Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR). Later, meticulous studies by Fabiato (80, 81) established that the CICR process was sensitive to both the speed and amplitude of the applied Ca\(^{2+}\) trigger. These studies and others helped reveal the complexity of the regulatory mechanisms that control SR Ca\(^{2+}\) release.

A major advancement was the identification of the SR Ca\(^{2+}\) release channel using an alkaloid called ryanodine. Ryanodine is found naturally in the stem and roots of the plant *Ryania speciosa*. The ryanodine alkaloid was first isolated as a potential insecticide (73, 133), and its potent paralytic action on skeletal and cardiac muscles was immediately evident (73, 82, 93, 218, 311, 312). However, its action was difficult to understand. Specifically, it was difficult to understand how ryanodine could induce rigid paralysis in skeletal muscle but flaccid paralysis in cardiac muscle.

The ryanodine alkaloid was shown to inhibit SR Ca\(^{2+}\) release by binding with high affinity to a protein present in the SR membrane (41, 80, 88, 93, 309–312, 345, 347). The ryanodine binding protein was purified (39, 88, 124, 147, 240, 241) and existed in a tetrameric complex (149) whose shape was subsequently visualized using electron microscopy (131, 147). Interestingly, the size and shape of the ryanodine binding protein complex was similar to that of the “feet” structures, which appear to physically link the tubule and SR membranes (83, 94). Incorporation of the ryanodine binding protein complex into artificial planar lipid bilayers revealed that this structure was an ion channel (124, 130, 147, 294). The RyR channel is a poorly selective Ca\(^{2+}\) channel (selectivity Ca\(^{2+}\)/K\(^{+}\) ~6) with very high conductance (~700 pS when K\(^{+}\) is charge carrier and ~100 pS when Ca\(^{2+}\) is charge carrier). The channel is regulated by Ca\(^{2+}\), Mg\(^{2+}\), ATP, and caffeine. Interestingly, application of the ryanodine alkaloid locks the channel in a slow-gating subconductance state (124, 147, 294, 337). Openings of the ryanodine-bound channel are long-lived and have a smaller unit current (~1/3 or ~1/2 of control amplitude). This action of ryanodine on single RyR channel function is quite distinctive and is now often used to functionally identify the channel.

These early single-channel studies unambiguously identified the RyR channel as the SR Ca\(^{2+}\) release channel in striated muscle (84, 87). It also became evident how the ryanodine alkaloid can induce rigid paralysis in skeletal muscle but flaccid paralysis in cardiac muscle. In cardiac muscle, ryanodine promotes Ca\(^{2+}\) leak from intracellular stores because it locks the RyR channels in the long-lived subconduction state. This “leaked” Ca\(^{2+}\) is rapidly removed from the cell by the relatively strong surface membrane Ca\(^{2+}\) extrusion mechanisms present in cardiac muscle (17, 20). Intracellular Ca\(^{2+}\) stores eventually deplete, yielding the observed flaccid cardiac paralysis. In contrast, skeletal muscle lacks the strong surface membrane Ca\(^{2+}\) extrusion mechanisms present in cardiac muscle. The ryanodine-induced Ca\(^{2+}\) leak from intracellular stores causes Ca\(^{2+}\) to accumulate in the cytoplasm. The result is abnormally high cytoplasmic Ca\(^{2+}\) levels that induce sustained contraction (i.e., the rigid skeletal muscle paralysis).

### III. STRUCTURE-FUNCTION OVERVIEW

Molecular cloning studies have defined three different RyR isoforms in fish, amphibians, birds, and mammals (6, 60, 205, 215, 225, 245, 301, 307, 315a). Although the RyR proteins share significant homology with the IP\(_3\)R receptors, they have little homology with the more widely studied voltage-dependent Ca\(^{2+}\) channels found in the surface membrane (204, 315a, 321). In mammals, the three RyR isoforms (RyR1, RyR2, and RyR3) are encoded by three different genes on different chromosomes (186, 205, 318, 315a, 369). The RyR proteins are in a variety of tissues, but the highest densities are in striated muscles (5, 6, 148, 205, 212, 225, 227, 231, 233, 299, 308). There may also be structurally, and perhaps functionally, distinct splice variants of these channels (102, 221, 370). However, the functional significance and distribution among fiber types of RyR splice variants are currently unknown.

In mammalian striated muscles, the expression of the different RyR protein isoforms is tissue specific. The predominant RyR isoform in skeletal muscle is the RyR1 protein, commonly referred to as the skeletal RyR isoform (60, 195, 225, 245, 299). The RyR2 protein is the most abundant isoform in cardiac muscle, and the RyR2 protein is commonly referred to as the cardiac RyR isoform. The RyR3 protein is also found in mammalian striated muscles, but at relatively low levels (98, 308, 325). For example, the RyR3 protein represents <5% of the overall RyR population in diaphragm (135, 213).

What is the physiological role of the RyR3 channel in striated muscle or elsewhere? Mice missing the RyR1 and RyR2 gene products (i.e., RyR1 or RyR2 knock-outs) die early during embryonic development (317, 320). In contrast, mice missing the RyR3 gene product (i.e., RyR3 knock-out) lead relatively normal lives and have quite normal striated muscles (55, 317). Interestingly, the CICR process in neonate skeletal muscle fibers from RyR3-knockout mice appears to be impaired (357). The impli-
cations of this observation are not yet clear. In any event, it appears that the physiological role of the RyR3 channel in mammals, unlike that of the RyR1 and RyR2 channels, can be readily compensated for both during development and in the adult.

The RyR nomenclature and distribution are somewhat different in nonmammalian striated muscle (35, 148, 212, 232, 307). Nonmammalian skeletal muscle (e.g., frog, fish, and chicken) contains nearly equal amounts of two different RyR proteins, named the α-RyR and β-RyR. These isoforms are homologs of mammalian RyR1 and RyR3 forms, respectively (5, 148, 212, 223, 227, 233, 308, 307). It is known that embryonic chicken skeletal muscle cells fail to develop normal excitation-contraction coupling in the absence of α-RyR (132), the RyR1 analog. Although there have been no β-RyR knock-out studies, all indications are that the β-RyR must play some fundamentally important role in the operation of nonmammalian skeletal muscle (307). One likely possibility is that the β-RyR isoform participates in the CICR process, effectively amplifying the initial DICR mediated by the α-RyR isoform.

The RyR proteins are also expressed in smooth muscle and many nonmuscle tissues (e.g., neurons). These other tissues generally contain multiple RyR isoforms. For example, expression of all three RyR isoforms has been reported in aortic smooth muscle (162, 186), cerebrum (101, 115, 220), and cerebellum (101, 115, 187). These tissues also express multiple IP₃R isoforms, and thus one cell may contain many types of RyR and IP₃R channels. The significance of this remarkable redundancy in intracellular Ca²⁺ release channel expression in nonmuscle systems is not clearly understood. One might speculate that the rich morphological diversity implies equally rich functional heterogeneity and that the multiple functionally distinct Ca²⁺ release channels may govern different Ca²⁺ signaling tasks. Consequently, Ca²⁺ release channel diversity may represent one factor that allows cells to simultaneously carry out the myriad of Ca²⁺ signaling tasks they need to do to survive. Even striated muscles, which are highly specialized for the Ca²⁺ signaling that governs contraction, must carry out other types of Ca²⁺ signaling tasks to survive. Perhaps the RyR3 channel or the low density of IP₃Rs expressed in striated muscles (239, 276) carry out this “other” Ca²⁺ signaling.

At the amino acid level, the three mammalian RyR isoforms share ~70% identity (115, 215, 318, 315a). Analysis of the primary amino acid sequences suggests that the membrane-spanning domains of the RyR are clustered near its COOH terminus (271, 299, 315a, 367). Truncation mutants, containing only these putative transmembrane regions (TMRs), are sufficient to form Ca²⁺-selective channels when incorporated into planar lipid bilayers (23, 24). Mutation of certain regions in and around the putative TMRs generates channels with altered ion selectivity (23, 103, 367). Thus there is reasonable certainty that this region of the protein contains the determinants that form the Ca²⁺-selective pore of the RyR channel.

Analysis of the RyR’s primary amino acid sequence also has revealed several consensus ligand binding (e.g., ATP, Ca²⁺, caffeine, and calmodulin) and phosphorylation motifs. Experimental verification of these sequences as potential regulatory sites (e.g., Refs. 23, 45, 47) is ongoing. For example, substitution of alanine-3882 by a glutamate (E3882A) in the RyR3 protein results in RyR3 channels with altered Ca²⁺ sensitivity (45). This implies that this region of the protein may contain the “Ca²⁺ sensor” of the channel. A gross deletion of amino acids 183–4006 in the RyR1 protein results in channels that lack caffeine sensitivity and do not inactivate at high Ca²⁺ concentrations (23). Exchange of amino acids 4187–4628 of RyR1 for the corresponding cardiac RyR2 sequence results in channels with increased caffeine sensitivity and altered Ca²⁺ sensitivity (66, 67).

Two skeletal muscle diseases, malignant hyperthermia (MH) and central core disease (CCD), have also provided useful insights into the RyR1 structure-function relationship (180, 366). These diseases are marked by pathologies like hypercarbia, rhabdomyolysis, and generalized muscle contraction. A defect in the RyR1 channel function was suspected to be involved in these pathologies (85, 277, 338). It is now known that specific point mutations in the RyR1 protein are associated with the MH and CCD diseases (180, 243, 277, 366). Recent reports describe more than 20 different point mutations in the human RyR1 isoform that are associated with the MH and/or CCD syndromes (140, 193). The majority of the mutations are clustered in two regions of RyR1 sequence, residues 35–614 (near the NH₂ terminal) and 2163–2458 (a central location). These data are important because these mutations may reveal important points in the RyR1 structure that govern its function.

It is clear that the exploration of the RyR structure-function relationship by mutagenesis is still in its infancy. This is in stark contrast to the molecular study of other types of ion channels. There are several reasons why we have not progressed further or faster in this area. First, the RyR channels are expressed in intracellular organelles (ER or SR), and thus single RyR channel function cannot be directly and easily accessed. For surface membrane channels, function is often efficiently assessed by the classical patch-clamp method on a few tagged expressing cells. For the RyR case, function is generally defined in vitro after the channels have been biochemically isolated from millions of expressing cells. Second, the RyR is a huge protein (~1/10 the size of a ribosome). This large size complicates its genetic manipulation as well as the selection of sites to mutate. Third, recent studies suggest...
that RyR1 channel function may involve inter-RyR domain interactions (128, 279, 355), and thus some functional attributes may be controlled by multiple and very complex structural determinants. Despite these and other technical challenges, future mutagenesis studies promise to provide some very interesting insights into RyR structure-function in the not too distant future.

Usually, ion channel structure-function studies are interpreted in the absence of information concerning the channel’s detailed three-dimensional (3-D) structure. There are some exceptions where channels or channel segments have been crystallized. However, the RyR channel is not one of these exceptions. Intriguingly, some gross details of the 3-D RyR structure (i.e., general shape) have been predicted using cryoelectron microscopy and 3-D reconstruction techniques. The RyR1 channel has fourfold symmetry, presumably reflecting its formation by four RyR protein monomers (249, 270, 342). The RyR channel has two distinct domains (249, 270, 342). One is a large cytoplasmic assembly (~29 × 29 × 12 nm), consisting of loosely packed protein densities. The other is a small transmembrane assembly that protrudes (~7 nm) from the center of the cytoplasmic assembly (270, 342). This transmembrane assembly appears to have a central hole that can be occluded by a pluglike mass. This hole and plug may correspond to the Ca\(^{2+}\)-selective pore and its gate (230). In general, the shapes of the RyR1 and RyR2 channels are quite similar (275). There are, however, specific points of structural heterogeneity. These points may contribute to the isoform-specific functional attributes of the RyR channels. Cryoelectron microscopy studies have also revealed the sites where certain peptides (calmodulin, FK binding proteins, and imperatoxin) interact with the RyR channels (263, 343). Recently, antibodies have been used to map the location, on the 3-D structure, of a specific domain (amino acid residues 4425–4621) that may be implicated in the Ca\(^{2+}\)-dependent regulation of the RyR channel (15).

Certain structural details of the RyR channel have also been obtained using certain biophysical approaches. For example, the width of the RyR2 pore has been estimated from molecular sieving experiments (332). In these studies, it was found that only cations with a predicted circular radius smaller than 3.5 Å could permeate through the channel. In another study, streaming potential experiments indicated that the RyR2 channel contains a narrow region in which H\(_2\)O (radius 1.5 Å) and Cs\(^+\) (radius 2 Å) must pass in a single-file fashion (337). Combined, these results suggest that the RyR2 channel pore is ~3 Å wide. Electrical distance measurements, using trimethylammonium derivatives of varying length, indicated that the physical length of the voltage drop along the RyR2 channel pore is ~10.4 Å (331). This is consistent with steaming potential measurements that suggest that three H\(_2\)O molecules (~9 Å) occupy the single-file region of the RyR2 channel pore (337). Thus the RyR channels appear to have a relatively short permeation pathway.

### IV. EXCITATION-CONTRACTION COUPLING

A role in striated muscle excitation-contraction (E-C) coupling is probably the RyR channel’s most notable claim to fame. There is clear evidence that the RyR channels interact with voltage-dependent Ca\(^{2+}\) channels (i.e., dihydropyridine receptors; DHPRs) in the nearby t-tubule membrane (246, 247, 321, 322). This functional interaction between the DHPR and RyR is commonly referred to as E-C coupling (Figs. 1 and 2). Depolarization of the t-tubule membrane (i.e., excitation) induces conformational changes in DHPR that ultimately lead to activation of RyR channel in the SR membrane. The activation of RyR channels leads to massive Ca\(^{2+}\) release from the SR, which in turn initiates contraction. The bulk of information addressing RyR channel regulation has been obtained from studies focused on the process of E-C coupling in striated muscle.

More than a century ago, Ringer demonstrated that the cardiac E-C coupling process required the presence of extracellular Ca\(^{2+}\) (reviewed in Ref. 38). In cardiac muscle, the DHPR receptor (an L-type Ca\(^{2+}\) channel) carries a small Ca\(^{2+}\) influx that activates the RyR2 channel (16, 208, 250, 270, 272). A similar process governs the RyR channels in some invertebrate skeletal muscles (177, 260). However, this is not the case in most vertebrate skeletal muscle, where extracellular Ca\(^{2+}\) is not required for E-C coupling (9, 38, 91, 129, 177, 260). The primary role of the DHPR in vertebrate skeletal muscles is acting as a voltage sensor that directly (perhaps physically) modulates the activation gate of nearby RyR1 channels.

#### A. DHPR-RyR Interaction

In skeletal muscle (Fig. 1), the DHPR and RyR1 are thought to communicate via some sort of physical protein-protein linkage (39, 246, 247, 251, 253, 265). The membranes of the t tubule and SR are juxtaposed and separated by a small 10-nm gap. The cytosolic domain of the RyR1 channel spans this narrow gap (28, 29, 94–97). Electron microscopy (EM) studies show that the skeletal DHPR in the t tubules are arranged in clusters of four (tetrads). These tetrads are organized into distinct arrays. The RyR1 channels in the SR membrane are arranged in a corresponding fashion (28, 29, 94–97, 247). The arrays of DHPR and RyR align in certain fast-twitch skeletal muscle such that every other RyR1 channel is associated with a DHPR tetrad (28, 29, 247).
It is thought that the skeletal DHPR physically contacts the large cytoplasmic domain of the juxtaposed RyR1 channel to form a linkage through which t-tubule voltage-sensing information is transduced (246, 251–255, 265–267). A distinctive feature of this transduction mechanism is its speed. Signal transmission between the skeletal DHPR and RyR1 channel must occur during the very brief (~2 ms) skeletal muscle action potential. It may be
this “need for speed” that has converted the physiological role of the DHPR from Ca\(^{2+}\) channel to voltage sensor in mammalian skeletal muscle.

In cardiac muscle (Fig. 2), there is about 1 DHPR for every 5–10 RyR2 channels (29, 247, 313), and the DHPR and RyR2 channels are not aligned in such a highly ordered fashion (29, 95, 247, 313). During the long cardiac action potential (~100 ms), the DHPR Ca\(^{2+}\) channel has ample time to open and mediate a substantial Ca\(^{2+}\) action potential (100 ms), the DHPR Ca\(^{2+}\)/H11011 dered fashion (29, 95, 247, 313). During the long cardiac and RyR2 channels are not aligned in such a highly or-

every 5–200). These RyR1 channels are not coupled to DHPRs (7, 183, 197, 272). The involvement of a diffusible second messenger (i.e., Ca\(^{2+}\)) makes the DHPR-RyR2 signaling considerably slower than the DHPR-RYR1 signaling. This lack of speed, however, may provide a greater opportunity for regulating DHPR-RyR2 signaling. In fact, pharmacological regulation of DHPR-RyR2 signaling is fundamental to normal cardiac function (18).

The expression of mutant DHPRs in mouse skeletal muscle myotubes that lack the endogenous DHPR has provided insights into which regions of the DHPR are involved in DHPR-RyR1 interaction (1, 2, 106, 321). A region within the cytosolic II-III loop of the DHPR’s α-subunit (residues 720–765) appears to be critical. Furthermore, studies with myotubes lacking endogenous RyR1 channels showed that RyR2 or RyR3 could not substitute for RyR1 in the DHPR-RyR interaction (216, 320, 356). The expression of chimeric RyR channels (RyR1/RyR2) indicates that regions R9 (2659–3720) and R10 (1635–2636) of RyR1 contain sequences involved in the DHPR-RyR1 interaction (217, 246). Thus specific regions of the DHPR and RyR1 channels have been implicated in the DHPR-RyR1 interaction. How these regions mediate the required signal transduction remains to be determined.

The DHPR is not the only regulator of RyR1 channel function. Like the RyR2 channel, the RyR1 channel (in the absence of the DHPR) can be activated by cytosolic Ca\(^{2+}\) signals. This is important because more than half of the RyR1 channels are not coupled to DHPRs (7, 183, 197, 200). These “uncoupled” RyR1 channels are thought to be available to participate in the CICR process. It is generally believed that CICR acts to amplify the DICR signal (i.e., that generated by the DHPR-RyR1 interaction). Interestingly, the presence of “coupled” and uncoupled RyR1 channels implies that RyR1 function is inherently heterogeneous “in vivo.” The amount of uncoupled RyR1 channels is not the same in all skeletal muscles. Slow-twitch skeletal muscles may have three or more uncoupled RyR1 channels for each DHPR-linked RyR1 channel (7, 183, 197, 200). This may mean that these muscles have a greater CICR component. This may be related to the substantially slower rate of E-C coupling in slow-twitch muscle (104, 223, 227, 260, 370).

V. STUDY OF INTRACELLULAR CALCIUM RELEASE

The first methods used to study Ca\(^{2+}\) transport across cell membranes monitored radioisotope (\(^{45}\)Ca) influx/efflux between two defined compartments (e.g., bath and cytosol; Ref. 141). From these initial studies, it became clear that Ca\(^{2+}\) was sequestered into intracellular organelles and that the cytosol represented an intermediary compartment with variable Ca\(^{2+}\) buffer properties. The next generation of studies examined Ca\(^{2+}\) influx/efflux across the SR of mechanically or chemically skinned muscle fibers. Here, the barrier of the plasma membrane was eliminated and the cytosolic solution could be controlled. The importance of ATP-mediated Ca\(^{2+}\) uptake by the SR and some of the attributes of CICR and even DICR were defined (80, 81, 150, 153, 151). Almost concurrently, methods for generating subcellular fractionation of functional SR vesicles were developing. Studies of Ca\(^{2+}\) loading and release from suspensions of SR vesicles provided improved temporal resolution and further revealed the intricacies of the SR Ca\(^{2+}\) release process (121, 127, 138, 196, 198, 199, 234–236).

It was noticed by several investigators that the binding of the ryanodine alkaloid appeared to depend on the activity level of the SR Ca\(^{2+}\) release channel (e.g., Refs. 53, 122). Experimental conditions that promoted SR Ca\(^{2+}\) release increased ryanodine binding. Experimental conditions that inhibited SR Ca\(^{2+}\) release decreased ryanodine binding. Subsequently, “nonequilibrium” ryanodine binding became a commonly used tool to assess RyR channel activity (discussed in detail in Ref. 225). Thus a number of methods have been developed to study the SR Ca\(^{2+}\) release phenomenon in populations of RyR channels.

The development of the patch-clamp technique revolutionized the study of ionic channels, including the study of the RyR channel. In this classic method, a small glass pipette is placed on the surface of a cell to physically isolate a small patch of membrane in which the function of an individual ion channel can be measured. This method has been widely applied to define the function of surface membrane ion channels. The patch-clamp technique, however, cannot be easily applied to study ion channels in intracellular organelles. Instead, microsomes isolated from cellular homogenates are fused into artificial planar lipid bilayers (206). The same instrumentation used to record across the membrane patch is then used to record across the artificial bilayer. Thus studies of single RyR channel behavior became possible (detailed in sect. vi).

The recent development of laser scanning confocal microscopy opened yet another chapter in the study of intracellular Ca\(^{2+}\) release. Confocal imaging has made it possible to visualize very localized intracellular Ca\(^{2+}\) release events (Ca\(^{2+}\) sparks; Refs. 50, 336). These local events may arise from the opening of an individual chan-
VI. STUDY OF SINGLE RYANODINE RECEPTOR CHANNELS

Defining the characteristics of single RyR channels is fundamental to understand the origin of many intracellular Ca\(^{2+}\) signaling phenomena. The amplitude of single RyR channel current reveals the number of Ca\(^{2+}\) passing through the channel per second. The open probability \(P_o\) of single RyR channels allows us to predict how certain agonists and antagonists regulate its activity. Dwell-time analysis of single RyR channel activity reveals the durations of the open and closed events. Kinetic studies of single RyR channel activity reveal how fast it can respond to a ligand stimulus (i.e., time constants and first latencies) as well as its specific patterns of opening (i.e., modal gating or bursting behavior). Additionally, single RyR channel studies can reveal the extent of homogeneity or heterogeneity of individual channel function in the overall RyR channel population (56, 60, 178, 238). These types of information are generally not available when Ca\(^{2+}\) signaling phenomena are studied in a population of RyR channels. Thus single RyR channel studies provide detailed information about the origin of intracellular Ca\(^{2+}\) signaling phenomena that simply cannot be obtained by other means.

The study of single RyR channel behavior requires the incorporation of native SR vesicles (enriched in RyR channel) or purified RyR channel proteins into an artificial planar lipid bilayer. The artificial planar bilayer is generally formed across a small aperture that separates two solutions. A patch-clamp amplifier is used to monitor electrical signals that arise from the bilayer. Single RyR channel activity is evident as a sudden appearance of single-channel activity in the bilayer. The sudden appearance of channel activity marks the moment a channel protein has been incorporated into the bilayer. This methodology is now widely used and has been described in detail elsewhere (206).

The environment in which the single RyR channel operates in the cell is complex (163). The resting cytosolic free Ca\(^{2+}\) concentration is near 100 nM, and the free Ca\(^{2+}\) concentration inside the SR is thought to be near 1 mM (196, 274). The cytosol contains a complex mixture of salts (~140 mM K\(^+\), ~1 mM Mg\(^{2+}\), 5–10 mM ATP, and various other nucleotides; Refs. 234, 371). The cell also contains several proteins that are known to interact with the RyR channel (e.g., DHPR, calmodulin, FK binding proteins, triadin, kinases-phosphatases, calsequestrin, annexin, etc.). Reconstructing or mimicking, at least in part, this complex situation in vitro (i.e., during a planar bilayer experiment) still needs to be done. Nearly all the single RyR channel studies have been done under very simple experimental conditions that do not reflect the channel’s physiological reality (e.g., Refs. 54, 56, 116, 145, 147, 291). This fact should always be taken into consideration when interpreting single RyR channel data.

How well does single RyR channel function in the bilayer reproduce the single RyR channel behavior in the cell? The correlation between single RyR channel function in bilayers and SR Ca\(^{2+}\) release phenomena observed in cells is surprisingly strong. For example, the primary regulatory features of SR Ca\(^{2+}\) release in cells (e.g., its Ca\(^{2+}\), Mg\(^{2+}\), and ATP sensitivity) are clearly evident at the single RyR channel level in bilayers. The actions of many secondary pharmacological regulatory agents like caffeine, ruthenium red, and ryanodine in the cell are mimicked at the single RyR channel level in bilayers. The fast gating and high conductance of single RyR channels in bilayers is consistent with the speed and large amplitude of intracellular Ca\(^{2+}\) release phenomena in cells (173). There is growing evidence that many regulatory factors and closely bound regulatory proteins are actually retained when single RyR channels are isolated in native membranes (e.g., Refs. 30, 189, 287, 300, 346, 348, 365). Thus defining single RyR channel behavior in bilayers is likely to provide insights into the origins of RyR-mediated Ca\(^{2+}\) signaling in cells.

The RyR channel is usually isolated by differential centrifugation of muscle homogenates. The RyR channel is found in heavy microsomal fractions. The RyR-containing vesicles (microsomes) can then be fused into planar lipid bilayers (36, 54, 56, 258, 291, 298). Alternatively, the RyR-containing vesicles can be detergent-solubilized (CHAPS plus phospholipids mixtures) and the resulting “purified” RyR channels can then be directly fused in a lipid bilayer (124, 135, 238) or reconstituted into liposomes (145, 147). These proteoliposomes can then fuse into planar lipid bilayers. Single RyR channel function has been examined using all these approaches. An advantage of using the SR vesicle approach is that the RyR channel is present in its native membrane, increasing the likelihood that closely associated regulatory proteins/factors may still be present (e.g., Refs. 189, 327). A disadvantage of using the SR vesicle approach is that the vesicles may contain other types of ion channels, specifically K\(^+\) and/or Cl\(^-\) channels. The presence of other channels complicates things considerably. Potential interference from other channels is minimal if Ca\(^{2+}\) (or Ba\(^{2+}\)) is used as the charge carrier (with no other permeant ions present). Many single RyR channel studies, however, chose to use a monovalent charge carrier to boost signal-to-noise characteristics. In this case, carefully selected ionic substitutions are usually made to eliminate potential contamination from other channel types.
In our experience, fusion of SR vesicles into planar bilayers results in more stable/consistent single RyR channel behavior compared with that obtained using CHAPS-solubilized RyR channel preparations. As mentioned above, the use of SR vesicles requires that certain specific steps be taken to avoid contaminating currents from other ion channels. The first unitary Ca\(^{2+}\) current recordings associated with the RyR channel were made after heavy SR vesicles were fused in a bilayer in the presence of \(\sim 50\) mM Ca\(^{2+}\) and little else (291). The two most common single RyR channel recording conditions are listed below. One consists of a Ca-HEPES solution (50 mM Ca\(^{2+}\)) on the luminal side with an isosmotic Tris-HEPES solution on the cytosolic side of the channel. The second consists of a Cs-methanesulfonate solution on both sides of the channel. In both cases, contaminating anion currents are avoided by the use of impermeable or poorly permeable anions. In the first case, contaminating cation currents are avoided by the presence of a nonpermeable organic monovalent cation (Tris\(^{+}\)). In the second case, contaminating K\(^{+}\) currents are avoided by using Cs\(^{+}\) as a permeant monovalent cation. The use of Cs\(^{+}\) works because SR K\(^{-}\) channels are either poorly permeable to Cs\(^{+}\) or blocked by this ion, while RyR channels are highly permeable to Cs\(^{+}\) (as well as other monovalent cations).

VII. PERMEATION PROPERTIES

The ability of the RyR channel to mobilize intracellular Ca\(^{2+}\) depends on both its permeation and gating properties. Its permeation properties include its unitary conductance and ion selectivity. Its gating properties include its \(P_o\) and the duration of individual open/closed events. A single RyR channel with optimal permeation properties would be ineffective at mobilizing Ca\(^{2+}\) if it never opened (gated). Conversely, a single RyR channel with optimal gating behavior would be worthless if it did not allow Ca\(^{2+}\) to pass efficiently. The opening and closing of the RyR channel presumably involves the physical motion of the protein. Ions are able to traverse its pore only when the channel is in the open configuration. It is generally assumed that movement of the gate does not alter the nature of the pore (i.e., its diameter, length, charge, etc.). It is in the pore where the channel discrimination between different ions occurs. The pore also contains determinants that define how quickly particular ions will pass. Specific RyR channel permeation properties are described below. Specific features of single RyR channel gating are discussed in later sections.

The permeation properties of the different RyR channel isoforms are quite similar. The RyR channels are permeable to many different divalent and monovalent cations (e.g., Refs. 167, 294, 330). In symmetrical solutions containing a monovalent cation (e.g., \(\sim 200\) mM K\(^{+}\), Na\(^{+}\), or Cs\(^{+}\)) as the main permeant species, the unitary RyR channel current-voltage relationship is linear with a slope conductance usually \(\geq 500\) pS (167, 294). In asymmetric solutions containing a divalent cation (e.g., \(\sim 50\) mM Ca\(^{2+}\), Ba\(^{2+}\), or Sr\(^{2+}\)) as the main permeant species, unitary RyR channel current-voltage relationships have a slope conductance of \(\sim 100\) pS (330). These are very large conductance values. For comparison, high-conductance ion channels of the plasma membrane such as the Ca\(^{2+}\)-activated K\(^{+}\) channels (i.e., maxi-K\(_{ca}\) channels) have a maximal conductance of near 250 pS (157). The unitary Ca\(^{2+}\) conductance of the L type Ca\(^{2+}\) channel (i.e., DHPR) in the surface membrane is no more than \(\sim 20\) pS (119).

Thus the RyR channels are very-large-conductance Ca\(^{2+}\) channels. This feature is probably fundamental to its physiological role in cells, passing a large number of Ca\(^{2+}\) quickly.

The relative permeability of the RyR channels to different monovalent and divalent cations has been defined under bi-ionic conditions (e.g., Refs. 167, 294, 330). The RyR channels show very little selectivity between different monovalent cations. The RyR channels also show very little selectivity between different divalent cations. However, the RyR channels are selective, albeit poorly, between mono- and divalent cations (\(P_{Ca}/P_{K}\) \(\sim 6\); more permeable to Ca\(^{2+}\) than K\(^{+}\)). This modest selectivity for divalents is also unusual for a Ca\(^{2+}\) channel. The typical surface membrane Ca\(^{2+}\) channel (e.g., DHPR) displays a much higher degree of discrimination between monovalent and divalent cations (\(P_{Ca}/P_{K}\) \(> 20\)). The point is that the RyR channel is not a highly selective Ca\(^{2+}\) channel. Intuitively, the apparent deficiency in Ca\(^{2+}\) selectivity may be related to its high conductance. A high-conductance channel (i.e., ions passing rapidly) would have little time per ion to perform the steps needed to discriminate between ions.

The relatively poor Ca\(^{2+}\) selectivity of the RyR channels does have important physiological ramifications. In cells, Ca\(^{2+}\) is not the only ion that can permeate through the open RyR channel pore. Other permeable cations (Mg\(^{2+}\), Na\(^{+}\), and K\(^{+}\)) are also present at substantial levels (i.e., \(-1, 10,\) and \(140\) mM, respectively). Thus Ca\(^{2+}\) competes with Mg\(^{2+}\), Na\(^{+}\), and K\(^{+}\) for occupancy of the RyR channel pore. The consequence is that the unitary Ca\(^{2+}\) current carried by a single RyR channel in the cell (i.e., in the presence of multiple permeant ions) will be considerably less than measured under the traditional simple recording conditions used in bilayer studies (e.g., Refs. 256, 259, 295, 330).

Tinker et al. (329) were the first to examine how unitary Ca\(^{2+}\) current through the RyR2 channel is affected by the presence of other permeant ions. They estimated that unitary Ca\(^{2+}\) current (at 0 mV) under physiological
conditions would be ~1.4 pA. This still quite large current was explained using a Eyring-rate model (328) assuming that some mechanism allows the association rate of Ca$^{2+}$ at the mouth of the pore to exceed, by about an order of magnitude, the normal diffusional limit. A more recent examination of unitary Ca$^{2+}$ current through the single RyR2 channel suggests that unitary Ca$^{2+}$ current (at 0 mV) under physiological conditions may be closer to 0.5 pA (201). This is important because accurately defining the amplitude of the unitary Ca$^{2+}$ current through a single RyR channel is fundamental to understanding the origin of local intracellular Ca$^{2+}$ signaling events (e.g., Ca$^{2+}$ sparks) in cells. The unitary RyR Ca$^{2+}$ current value is central to the debate whether Ca$^{2+}$ sparks arise from the opening of one RyR channel or the concerted opening of many RyR channels.

The larger unitary RyR channel Ca$^{2+}$ current prediction suggests that a mechanism to enhance permeation may possibly exist (328). The smaller unitary Ca$^{2+}$ current prediction indicates that perhaps there is no need for any permeation enhancement mechanism (201). What might the permeation enhancement mechanism be? Several possible permeability enhancement mechanisms have been proposed. It has been argued that the RyR2 channel may contain a large charged vestibule that dielectrically focuses ions near the pore’s mouth (328). Alternatively, dielectric focusing by fixed surface charges could occur near the RyR channel’s mouth in the absence of a large vestibule structure (337). It has also been suggested that a single RyR channel contains multiple parallel conduction pathways (i.e., a multibarrel pore). The later possibility was supported by the initial 3-D RyR channel reconstructions that suggested the presence of multiple conduction pathways (342). Furthermore, Ondrias et al. (228) suggested that the four commonly observed subconductance states of the RyR channel might correspond to uncoordinated opening of pores in the individual RyR monomers of the tetrameric channel complex. They suggested that the presence of the FK-binding protein synchronizes the openings of these separate permeation pathways. However, recent and more detailed 3-D reconstruction data suggest that the RyR channel structure contains a single central pore (i.e., the RyR is not a multibarrel channel; Refs. 230, 270, 275). However, the possibility that the channel contains a large vestibule and/or surface charges that impact ion permeation still exists and should not be discounted.

VIII. ACTION OF RYANODINE

The alkaloid ryanodine binds with high affinity to the RyR proteins (225). Its binding induces a complex change in single RyR channel function. This change is similar in all three RyR channel isoforms. Ryanodine binding induces very-long-duration open events and simultaneously reduces ion conductance through the pore (e.g., Refs. 259, 294). This dual impact on single-channel function (i.e., slower gating, reduced conductance) suggests a complicated mode of action. This is also evidenced by the rather complicated ryanodine dose dependency. Low doses of ryanodine (~10 nM) are reported to increase the frequency of single RyR channel openings to the normal conductance level (34). Intermediate ryanodine doses (~1 μM) are reported to induce the classical ryanodine action described above (34, 259, 294). High doses of ryanodine (~100 μM) are reported to lock the channel in a closed configuration (368).

The fact that ryanodine binding alters single-channel conductance suggests that ryanodine binds in or near the RyR channel pore. In fact, the ryanodine-binding site has been experimentally localized to the COOH terminus of the protein (37), the region of the RyR protein thought to contain the structural determinants of the pore. Altered conductance suggests that ryanodine binding somehow changes the nature of the permeation pathway. This idea has also been experimentally confirmed. Two independent studies have indicated that ryanodine binding actually changes the effective diameter of the RyR channel pore (332, 337).

The complex action of ryanodine may be related to how the alkaloid binds to the channel. It is generally assumed that each RyR monomer contains one high-affinity ryanodine binding site ($K_D <50$ nM; Refs. 147, 225). It is possible that cooperative interaction between these different ryanodine binding sites is what generates the observed complexity (53, 242). Some ryanodine binding studies suggest the existence of additional lower ($K_D >1$ μM) affinity ryanodine binding sites (53, 147, 225, 242). Thus the RyR channel complex may contain multiple ryanodine binding sites. One interpretation suggests that ryanodine binding to the high-affinity site depends on whether or not the RyR channel is in the open configuration (53). It may be that conformational changes related to the opening of the RyR channel exposes (or improves access to) the high-affinity ryanodine binding site. This would impart a use dependence to the kinetics of ryanodine binding, allowing “nonequilibrium” ryanodine binding to be used as a monitor of RyR channel function (as described previously).

IX. OVERVIEW OF RYANODINE RECEPTOR REGULATION

The RyR channels are modulated by numerous factors, including a number of physiological agents (e.g., Ca$^{2+}$, ATP, and Mg$^{2+}$), various cellular processes (e.g., phosphorylation, oxidation, etc.), and several pharmacological agents (e.g., ryanodine, caffeine, and ruthenium
red). Some of these modulatory factors are discussed below.

A. Cytosolic Ca\(^{2+}\)

The action of Ca\(^{2+}\) on the RyR channel is complex. The Ca\(^{2+}\) turns on, turns off, and conducts through this channel. Steady-state single RyR channel activity is generally a bell-shaped function of cytosolic Ca\(^{2+}\) concentration (35, 46, 54, 56, 60, 135, 161, 224, 238, 285, 294, 333). The RyR channels are activated by low Ca\(^{2+}\) concentrations (1–10 \(\mu\)M) and inhibited by high Ca\(^{2+}\) concentrations (1–10 mM). It has been reported that individual RyR1 channels do not all respond in the same way (i.e., they are functionally heterogeneous) to activating cytosolic Ca\(^{2+}\) levels (56, 161, 178, 238). In contrast, individual RyR2 and RyR3 channels respond much more homogeneously to cytosolic Ca\(^{2+}\) (46, 54, 56, 135, 161). Inhibition at high cytosolic Ca\(^{2+}\) concentrations of the RyR1 is also different from that of RyR2 and RyR3 channels. The RyR1 channel is almost entirely inhibited by 1 mM Ca\(^{2+}\), whereas the RyR2 and RyR3 channels are not (35, 46, 54, 56, 60, 135, 161, 224, 225, 238, 264). Substantially higher Ca\(^{2+}\) levels are required to inhibit the RyR2 and RyR3 channels (46, 54, 56, 135, 161). The reported half-maximal inhibiting concentrations for the RyR1 channel is also different from that of RyR2 and RyR3 channels. The RyR1 channel is almost entirely inhibited by 1 mM Ca\(^{2+}\), whereas the RyR2 and RyR3 channels are not (35, 46, 54, 56, 60, 135, 161, 224, 225, 238, 264). Substantially higher Ca\(^{2+}\) levels are required to inhibit the RyR2 and RyR3 channels (46, 54, 56, 135, 161). The reported half-maximal inhibiting concentrations for the RyR2 and RyR3 channels range from 2 to >10 mM. It is not clear that such high cytoplasmic Ca\(^{2+}\) concentrations are ever reached in the cells. Thus the physiological role of this very high Ca\(^{2+}\) inhibition is not yet clear. Nevertheless, high Ca\(^{2+}\) inhibition of the RyR2 channels remains a controversial topic. This is further discussed in section xv.

B. Luminal Ca\(^{2+}\)

Early Ca\(^{2+}\) release studies suggested that Ca\(^{2+}\) binding to the luminal surface of the RyR channel may regulate the channel (64, 65). Single RyR channel studies have confirmed the existence of luminal Ca\(^{2+}\) regulation (51, 110, 286, 285, 333, 341). It appears that the sensitivity of RyR channel to certain cytosolic agonists increases at high luminal Ca\(^{2+}\) levels (110, 286). Trypsin digestion of the luminal side of the RyR channel suggested the presence of both activating and inactivating divergent sites (51). It is possible that the luminal Ca\(^{2+}\) effects are mediated, at least in part, by associated proteins like calsequestrin (14, 314) or junctin (364).

It was also reported that luminal Ca\(^{2+}\) moving through the channel feeds back and interacts with cytosolic Ca\(^{2+}\) binding sites (333). Thus luminal Ca\(^{2+}\) levels may affect single RyR channel function by interacting with luminal binding sites, with associated luminal proteins, or with cytosolic binding sites (i.e., feed-through regulation). Distinguishing between these possibilities is the challenge.

C. ATP and Mg\(^{2+}\)

Cytosolic ATP is an effective RyR channel activator (57, 60, 291, 297, 298, 353). Cytosolic Mg\(^{2+}\) is a potent RyR channel inhibitor (57, 60, 158, 291, 353). The cytosol of most cells contains ~5 mM total ATP and ~1 mM free Mg\(^{2+}\). This means that most ATP is in its Mg\(^{2+}\)-bound form. Free ATP (~300 \(\mu\)M in the cytosol) is the species that binds to and activates the RyR channel (297, 298). The action of ATP and Mg\(^{2+}\) on single RyR channel function is isoform specific. Free ATP is a much more effective activator of the RyR1 channel than the RyR2 channel (57, 60, 291, 297, 353). The RyR3 channel is also less ATP sensitive than the RyR1 channel (46, 135, 297). The Mg\(^{2+}\) action on single RyR channels is complicated (57, 158). First, Mg\(^{2+}\) may compete with Ca\(^{2+}\) at the Ca\(^{2+}\) activation site, shifting the Ca\(^{2+}\) sensitivity of the channel (57, 60, 158, 353). Second, Mg\(^{2+}\) competes with Ca\(^{2+}\) at the high Ca\(^{2+}\) inhibition site (57, 158). The high Ca\(^{2+}\) inhibition site does not discriminate between different divalent cations. The RyR1 channel is substantially more sensitive to this action of Mg\(^{2+}\) than the RyR2 or RyR3 channels (57, 135). In the presence of physiological levels of Mg\(^{2+}\) and ATP, the RyR1 channel requires less Ca\(^{2+}\) to activate than the RyR2 or RyR3 channel (57, 257, 320, 353).

D. Redox Status

Various redox processes may also modulate single RyR channels. The RyR monomers contain 80–100 cysteine residues (315a, 318). Many of these are suitable for modification by oxidants. There is evidence that oxidant and reducing agents do indeed impact single RyR channel function (182, 222, 349), including the channel’s Mg\(^{2+}\) sensitivity. There is also evidence that oxidants may prevent certain closely associated regulatory molecules, like calmodulin, to bind to the RyR channel (363). Recently, it has been suggested that nitric oxide (NO) may modulate the redox regulation of the RyR channels (79, 118, 352). It was proposed that in vivo initial NO release may activate RyR channels and subsequent increases in NO levels inhibit RyR channel activity (118). Thus there is a growing interest in identifying the cysteine residues that may be involved in RyR channel oxidation and/or S-nitrosylation (352). It is quite possible that redox modulation of single RyR channels represents an important regulatory mechanism. It is clear that additional information is needed in this area.
E. Cyclic ADP-ribose

Cyclic ADP-ribose (cADPR) appears to play an important role in many nonmuscle systems (164). The cADPR molecule, a metabolite of NADP, has been found to act as a powerful intracellular Ca\(^{2+}\) release agent in sea urchin eggs, and its action is thought to be mediated by RyR or RyR-like channels (164). The direct action of cADPR on RyR channels from muscle systems is controversial. An early report indicated that cADPR dramatically activates single mammalian RyR channels (203). However, subsequent studies reported only minor effects of cADPR (284) or no impact of cADPR at all (58, 100, 209). The controversial reports may suggest a requirement of an unrecognized cofactor and/or a potential interaction with some closely associated regulatory factor (i.e., calmodulin, FK-506 binding protein; Refs. 58, 164). When studied in intact cellular systems (that contain endogenous calmodulin and FK-binding proteins), cADPR had only indirect action on RyR channel function (107, 126, 175). It appeared that cADPR activated the Ca\(^{2+}\)-ATPase (175), which indirectly activated the RyR channel by increasing luminal SR Ca\(^{2+}\) levels.

F. Phosphorylation/dephosphorylation

Analysis of the RyR protein sequence reveals that it contains many consensus phosphorylation sites (315a, 318). For more than a decade, the effects of exogenously applied kinases and phosphatases on single RyR channel behavior have been examined (60, 114, 170, 189, 298, 315, 339, 348). The capacity of protein kinase A (PKA) to activate the RyR1 channel has been reported (60, 298). This is consistent with the observation that PKA significantly increases depolarization-induced Ca\(^{2+}\) release from skeletal muscle SR vesicles (125). However, studies in skinned and intact skeletal muscle fibers have not supported this idea (27). The capacity of calmodulin kinase and PKA to activate the RyR2 channel has also been studied. Contradictory results have been reported for calmodulin kinase (70, 114, 170). Similarly, some reports on PKA action find activation of RyR2 channels (114), whereas others report a slight decrease in the steady-state P\(_o\) of RyR2 channels (339). Still others suggest PKA destabilizes RyR2 channel openings (i.e., promote substation) via phosphorylation-induced dissociation of FKBP12.6 (189). Yet another report shows that PKA phosphorylation does not impact the RyR-mediated Ca\(^{2+}\) spark in intact cardiac myocytes (166). The reason for the appearance of such contradictory and confusing results is unclear. It is possible that different experimental conditions are responsible. It is safe to conclude that the impact of phosphorylation and/or dephosphorylation on single RyR channel behavior will be debated for some time.

X. REGULATION BY CLOSELY ASSOCIATED PROTEINS

A number of different proteins may be associated with and modulate the RyR channels (60, 63, 136, 142, 144, 169, 181, 183, 185, 196, 231, 257, 287, 334, 343). However, only a few of these proteins have been thoroughly studied at the single-channel level. These few “well-studied” proteins include calmodulin, calsequestrin, FK-506 binding protein, and certain fragments of the DHPR. A brief survey of how these proteins may interact with the RyR channel is provided below.

A. Calmodulin

Calmodulin (CaM) was the first peptide found to interact with single RyR channels in lipid bilayers (295). CaM appears to bind the RyR in stoichiometric proportions (11, 207, 334). It binds to sites in the RyR channel cytoplasmic assembly that are ~10 nm from the putative entrance to the transmembrane pore (343). Application of CaM can either activate (at low Ca\(^{2+}\) levels) or inhibit (at high Ca\(^{2+}\) levels) the RyR1 and RyR3 channels (46, 99, 295, 334). For the RyR2 channel, only inhibitory effects of CaM have been reported (99, 295). It was reported that oxidation modifies RyR1 channel behavior and perhaps its interaction with CaM (117, 363). It has also been suggested that CaM may somehow protect the RyR1 channel from oxidative modifications during periods of oxidative stress (363). CaM also appears to bind to the DHPR and consequently has also been implicated in the DHPR-RyR1 interaction (117, 269). A potential role of CaM in modulation of the RyR2 channel during E-C coupling is not yet clear.

B. Calsequestrin

Calsequestrin is the main Ca\(^{2+}\) binding protein inside the SR. Calsequestrin has a large number of acidic amino acids that permit it to coordinately bind 40–50 Ca\(^{2+}\)/molecule (181, 358). Calsequestrin is also known to aggregate in the presence of divalent cations (190). Electron microscopy studies suggest that calsequestrin aggregates are present in the regions of the SR (i.e., terminal cisternae) known to contain RyR channels (96). It has been suggested that Ca\(^{2+}\)- and pH-dependent conformational changes in calsequestrin may modulate RyR channel activity (61, 120). It also has been suggested that calsequestrin action on the RyR channel may require the presence of triadin, another RyR-associated protein (365). Thus there is disagreement concerning the nature of the calsequestrin-RyR interaction.

Some studies in lipid bilayers suggest that calsequestrin activates the RyR channel (139, 226, 314). However,
one study suggests calsequestrin inhibits the RyR channel (14). Thus the physiological ramifications of the calsequestrin-RyR interaction require clarification. Similarly, the impact of calsequestrin in E-C coupling is unclear. Studies in calsequestrin-deficient Caenorhabditis elegans found no defects in body wall formation or locomotion, suggesting calsequestrin is not essential for muscle contraction (52). On the other hand, overexpression of calsequestrin in mouse heart results in the development of cardiac hypertrophy and altered Ca$^{2+}$ signaling (143).

C. FK-506 Binding Protein

The FKBP12 and FKBP12.6 proteins (185) associate with the RyR1, RyR2, and RyR3 proteins in apparently stoichiometric proportions (326, 327). Thus there are four FKBP s bound to each RyR channel complex. The RyR1 and RyR3 channels bind both the FKBP12 and FKBP12.6 forms. In cells, these RyR channels will have FKBP12 bound because of its high abundance in the cytosol (185, 326, 327). The RyR2 channel in dog heart appears to preferentially bind FKBP12.6 (327), and thus RyR2 channels will have FKBP12.6 bound to them. The RyR2 channels in other species have slightly less specificity for FKBP12.6 and thus may be bound to both FKBP12.6 and FKBP12 (134).

How does FKBP binding impact single RyR channel function? There is a good consensus that removal of FKBP12 from the RyR1 channel activates the channel (3, 12, 32, 48, 191, 326). The FKBP impact on RyR2 channel function is not so clear. Some groups report that removal of FKBP12.6 from the RyR2 channel activates the channel (137, 350). However, other groups report that FKBP12.6 removal has no impact on RyR2 channel function (12, 50, 327, 351). Thus there is disagreement concerning the action of the FKBPs on RyR2 channel function. Some points of this controversy are described below.

Several groups have shown that FKBP removal promotes the appearance of subconductance states (3, 4, 32, 137, 189). Subconductance substates are open events with less than normal unit current amplitude. This observation led to the suggestion that FKBP binding stabilizes the RyR channel complex and in doing so stabilizes the structure of the permeation pathway. Some groups, however, do not report the appearance of subconductance substates in FKBP12.6-depleted RyR2 channels or RyR2 channels isolated from FKBP12.6-deficient mice (12, 50, 327). Subconductance states are also only rarely observed in “purified” RyR1, RyR2, and RyR3 channels (135, 211, 352) known to be largely devoid of FKBP (211, 343). More recently, it has been suggested that FKBP may synchronize the single-channel function neighboring RyRs (188). The suggestion is that this synchronization permits several neighboring RyR channels to open and close simultaneously. It is not yet understood how the same protein (FKBP) could both stabilize RyR monomer interactions within the RyR channel complex and synchronize the activity of neighboring RyR channel complexes.

FKBP modulation of the RyR1 channel is generally accepted (248), yet there is some uncertainty as to the extent of this modulatory action. Studies in mechanically skinned skeletal muscle fibers reported that FKBP12 removal reduces DCR or “uncouples” the DHPR-RyR1 interaction (155). The suggestion is that FKBP12 may be the physical coupler between the DHPR voltage sensor and the RyR1 channel. One study in human skeletal muscle strips found that FKBP increases the contractile sensitivity to halothane and/or caffeine (33). Interestingly, animals deficient in FKBP12 die at embryonic stages (278), attesting to the importance of FKBP. These animals die of severe structural and functional abnormalities of the brain and heart, but the characteristics of their skeletal muscle are normal (278). This may imply that FKBP may not be central to skeletal muscle structure-function. Furthermore, no apparent major defects in ambulation, or other motor functions, were reported in animals with a skeletal muscle-specific FKBP12 knockout (324). These latter animals, however, had abnormal muscle contraction (decrease in contractility). Thus it appears that FKBP12 does have some role in modulating RyR1-mediated Ca$^{2+}$ release or muscle structure integrity (324). This role, however, needs further clarification.

There are reports that suggest that FKBP12.6 is critical to normal RyR2 channel operation in heart (137, 192, 306). It has even been suggested that abnormal FKBP12.6-RyR2 interactions may be implicated in certain types of heart failure (184, 189, 229, 359). However, other studies suggest that FKBP12.6 does not modulate the activity of single RyR2 channels (12, 50, 327) or Ca$^{2+}$ release in isolated cardiac myocytes (68). Local RyR-mediated Ca$^{2+}$ sparks in FKBP12.6 knockout mice have slightly increased amplitude and duration compared with those in wild-type animals (351). In contrast, a previous report showed that FK-506 or rapamycin (drugs that dissociate FKBP12.6 from the RyR2) reduced Ca$^{2+}$ spark amplitude and increased spark duration by ~500% (350). The hearts of male, not female, FKBP12.6 knockout mice develop mild cardiac hypertrophy over time (351). However, the hearts of these male FKBP12.6 knockout mice do not progress to more severe myopathies or heart failure. This implies that decreased cytosolic FKBP12.6 level, per se, may not be the primary culprit in generation of these pathologies. Thus the impact of FKBP12.6 (or FKBP12) on single RyR2 function is not yet clearly established.

D. DHPR-Loop Peptide

Another protein that interacts with and regulates the function of single RyR channels is the DHPR (323). As
described previously, the DHPR-RyR interaction is clearly involved in the signaling that links surface membrane excitation to SR Ca\(^{2+}\) release in striated muscle. The nature of the DHPR-RyR interaction is tissue specific. In skeletal muscle, the interaction is thought to involve a direct physical link between the two proteins. In cardiac muscle, the DHPR Ca\(^{2+}\) channel mediates a small Ca\(^{2+}\) influx that activates the RyR2 channel. Our attention here will be focused on the physical DHPR-RyR1 link in skeletal muscle.

Tanabe et al. (321) showed that the cytoplasmic II-III loop of the skeletal DHPR \(\alpha_1\)-subunit is required for the functional DHPR-RyR1 interaction. Although other regions of the DHPR may be involved (62, 165, 289), the potential role of the II-III loop has been studied most to date. Peptide fragments that correspond to the II-III loop activate single RyR1 channels in planar lipid bilayers (171, 172). Interestingly, different regions of the peptide appear to have different actions on RyR1 channel function. One region called peptide A activates single RyR1 channel (71, 171, 172). Another region called peptide C blocks the RyR1 channel but at a site that appears to be quite distant from the RyR1 regions thought to interact with the DHPR (263). If peptide A and imperatoxin A bind to the same site, then the distant location of this site is difficult to reconcile with our current understanding of the DHPR-RyR1 interaction. Recently, the interpretation of the physiological role of peptide A on RyR1 channel function was further complicated. Grabner et al. (106) showed that a chimeric DHPR, lacking the peptide A region, supported normal DHPR-RyR1 signaling when expressed in dysgenic myotubes (i.e., myotubes lacking endogenous DHPR). Thus the role of peptide A in the DHPR-RyR1 interaction has yet to be clearly established.

### XI. CALCIUM REGULATION PARADOX

Although the RyR channels are modulated by many factors, Ca\(^{2+}\) regulation plays a central role in all RyR-mediated signaling cascades. In some cases (i.e., cardiac muscle), the regulating Ca\(^{2+}\) signal initiates RyR channel activity. In other cases (i.e., skeletal muscle), the regulating Ca\(^{2+}\) signal may amplify RyR channel activity. There is also evidence that regulating Ca\(^{2+}\) signals may terminate RyR channel activity. The importance of understanding how Ca\(^{2+}\) regulates the RyR channels cannot be overstated.

Our discussion of RyR2 channel Ca\(^{2+}\) regulation starts with the classical work of Fabiato (80, 81), which defined the Ca\(^{2+}\) regulation of the SR Ca\(^{2+}\) release process in a skinned cardiac muscle preparation. This work showed that the amplitude and duration of trigger Ca\(^{2+}\) stimulus finely grades the CICR process (i.e., Ca\(^{2+}\)-induced Ca\(^{2+}\) release). Specifically, the amount of Ca\(^{2+}\) released from the SR was a bell-shaped function of trigger Ca\(^{2+}\) stimulus amplitude, and that trigger Ca\(^{2+}\) stimulus speed scaled this relationship.

Intuitively, the CICR process should be self-regenerating because the Ca\(^{2+}\) released by a RyR2 channel should feedback and further activate the channel or its neighbors. However, self-regeneration of CICR is not observed in cells. This is the Ca\(^{2+}\) regulation paradox. The implication is that some sort of negative-feedback mechanism(s) must exist to counter the inherent positive feedback of CICR. Fabiato (81) proposed that the negative-feedback mechanism was Ca\(^{2+}\)-dependent inactivation. The idea was that two different Ca\(^{2+}\) binding sites (activating and inhibitory, respectively) regulate the Ca\(^{2+}\) release channel (i.e., RyR2). The Ca\(^{2+}\) activation process was thought to have a fast action and a relatively low affinity. The Ca\(^{2+}\) inactivation process had a slower action but a relatively high Ca\(^{2+}\) affinity (81). Consequently, a fast Ca\(^{2+}\) stimulus transiently activates the channel because the channel would be “turned on” as Ca\(^{2+}\) occupies the activation site and then “turned off” as Ca\(^{2+}\) more slowly acts at the inactivation site. A slow Ca\(^{2+}\) stimulus would not effectively activate the channel because inactivation would “keep pace” with the activation process. At very high Ca\(^{2+}\) levels (>100 \(\mu\)M), the inactivation sites would be saturated, leaving the channel in an inactivated state. The mechanism of Ca\(^{2+}\)-dependent inactivation as envisioned by Fabiato (81) implies that the channel becomes refractory (unable to respond to further Ca\(^{2+}\) stimulation). Recovery from the refractory state would require removal of the Ca\(^{2+}\) stimulus and enough time for some sort of recovery process (i.e., repriming) to occur. This scheme is still widely discussed and is often applied to explain the complex Ca\(^{2+}\) regulation of many RyR-mediated Ca\(^{2+}\) signaling events.

Despite its popularity, the existence of high-affinity Ca\(^{2+}\)-dependent inactivation has been challenged. Studies on patch-clamped cardiac myocytes have not conclusively confirmed the existence of the Ca\(^{2+}\)-dependent inactivation phenomenon (208, 214, 360). For example, the capacity of a second Ca\(^{2+}\) stimulus to trigger SR Ca\(^{2+}\) release was shown to be independent of the interval between the first and second stimuli (214). The argument here is that if the SR Ca\(^{2+}\) release channels were inactivated after the first stimulus, then the effectiveness of the second stimulus should be affected. In another study (360), a prolonged triggering Ca\(^{2+}\) signal was used to inactivate the Ca\(^{2+}\) release process. A subsequent trigger Ca\(^{2+}\) signal, however, reactivated the apparently inactivated release process (i.e., the process was not refractory).
Still, it is possible that the second Ca$^{2+}$ stimulus (in both cases above) was activating neighboring Ca$^{2+}$ release sites that did not participate in the first Ca$^{2+}$ stimulus. In any event, studies at the whole cell level have not conclusively confirmed or dismissed the existence of Ca$^{2+}$-dependent inactivation. Studies of RyR2 channel Ca$^{2+}$ regulation at the single-channel level promised to resolve this question.

XII. STEADY-STATE CALCIUM DEPENDENCE

As discussed above, several laboratories have defined the Ca$^{2+}$ dependence of single RyR1 and RyR2 channels under steady-state conditions (e.g., Refs. 54, 56, 161, 256). In the absence of other modulating agents, these studies show that there is little spontaneous channel activity at low cytosolic free Ca$^{2+}$ concentrations (<100 nM). This is consistent with the rarity of spontaneous Ca$^{2+}$ release events (i.e., Ca$^{2+}$ sparks) at resting intracellular Ca$^{2+}$ levels in cells (25, 50). There is substantial spontaneous activity of single RyR channels at steady-state Ca$^{2+}$ concentrations in the micromolar range. The degree of spontaneous activity peaks at ~10 μM. The apparent $K_D$ of single RyR channel Ca$^{2+}$ activation is typically between 0.5 and 5 μM. This is consistent with the Ca$^{2+}$ sensitivity of SR Ca$^{2+}$ release activation in skinned cardiac cells (81) and isolated cardiac and skeletal SR vesicle preparations (54, 199, 234–236, 355).

The activity of single RyR1 channel decreases to near zero if the steady-state Ca$^{2+}$ concentration is elevated to ~1 mM (54, 56, 161, 238). This is not true for the RyR2 or RyR3 channels. Substantial inhibition of these channels is only observed when the steady-state Ca$^{2+}$ concentration exceeds 5–10 mM (54, 56, 135, 161, 238). It is unlikely that the free Ca$^{2+}$ levels in the cytosol of a cell would ever exceed the 1 mM mark because the Ca$^{2+}$ sources used to elevate cytosolic Ca$^{2+}$ (i.e., extracellular solution and SR lumen) contain only ~1 mM Ca$^{2+}$. Thus the physiological relevance of such low-affinity Ca$^{2+}$ inhibition is questionable. It is unclear how such low affinity of Ca$^{2+}$ inhibition (as observed at the single-channel level) is related to the apparent high-affinity Ca$^{2+}$-dependent inactivation observed in cells (81). However, the RyR channels operate in a dynamic (not stationary) Ca$^{2+}$ signaling environment. Thus steady-state measurements of channel activity (over minutes) may not reveal all the intricacies of single RyR channel Ca$^{2+}$ regulation in cells (on a millisecond time scale).

XIII. CALCIUM ACTIVATION KINETICS

Several groups have defined the kinetics of single RyR2 channel Ca$^{2+}$ regulation in bilayers (e.g., Refs. 112, 113, 159, 264, 283, 339). There is considerably less data addressing the kinetics of single RyR1 channels (e.g., Ref. 340) and none concerning single RyR3 channel function. Consequently, the discussion here focuses mainly on the kinetics of RyR2 channel Ca$^{2+}$ regulation.

A. Rate of Ca$^{2+}$ Activation

Fast Ca$^{2+}$ stimuli were applied to single RyR2 channels using two different methodologies. One methodology generates fast Ca$^{2+}$ stimuli by liberating Ca$^{2+}$ from caged-Ca$^{2+}$ compounds using laser flash photolysis (112, 339, 340). The other methodology generates fast Ca$^{2+}$ stimuli by mechanically changing the solution near the RyR2 channel (159, 264, 283). The RyR2 channel activates with a time constant of ~1 ms when the Ca$^{2+}$ is very rapidly changed via flash photolysis of caged-Ca$^{2+}$ and slightly slower (τ = 2–20 ms) when Ca$^{2+}$ is changed by a mechanical solution change technique. These numbers are fairly consistent and are generally in agreement with whole cell studies that clearly show that Ca$^{2+}$ activation of SR Ca$^{2+}$ release occurs on a millisecond time scale.

Interestingly, the reported RyR2 channel Ca$^{2+}$ activation time constants vary with the speed of the applied Ca$^{2+}$ stimulus. For example, Laver and Curtis (159) applied relatively slow mechanical Ca$^{2+}$ changes and reported a time constant of <20 ms. Sitsapesan et al. (283) applied slightly faster mechanical Ca$^{2+}$ changes and reported a Ca$^{2+}$ activation time constant of <10 ms. Schiefer et al. (264) applied even faster mechanical Ca$^{2+}$ changes and reported a time constant between 2 and 5 ms. The fastest Ca$^{2+}$ changes, however, were applied using the flash photolysis method. These studies report a Ca$^{2+}$ activation time constant of ~1 ms (112, 113, 339).

The interpretation of the mechanical solution change data described above must consider the presence of unstirred layers immediately in front of the bilayer. In some of the mechanical solution change studies (159, 283), the size of the unstirred layers clearly impacted the rate of Ca$^{2+}$ change in the microenvironment of the channel. Unfortunately, concerns about the impact of unstirred layers have rarely been discussed. Unstirred layers would tend to slow the measured Ca$^{2+}$ activation time constant. Unstirred layers, however, do not complicate the interpretation of the flash photolysis data, but there is another concern here. The Ca$^{2+}$ waveform applied by flash photolysis is not a simple square step. Instead, there is a fast Ca$^{2+}$ overshoot at its leading edge (77, 78, 194, 372). This very brief Ca$^{2+}$ overshoot (lasting ~150 μs) likely accelerates the initial opening transition of the RyR2 channel. The overshoot in these studies would tend to speed up the measured Ca$^{2+}$ activation time constant. Thus there are studies (flash photolysis) that may overestimate the Ca$^{2+}$ activation kinetics as well as studies (mechanical solution change) that may underestimate it.
What is the real RyR2 channel Ca\(^{2+}\) activation kinetics? The single RyR2 channel Ca\(^{2+}\) activation time constant (\(\tau \sim 2–5\) ms) to a steplike Ca\(^{2+}\) stimulus is probably best represented by the data of Schiefer et al. (264). This study applied very fast mechanical Ca\(^{2+}\) changes on very small-diameter bilayers alleviating unstirred layer concerns. These types of experiments, however, have never been repeated. The physiological Ca\(^{2+}\) trigger in cells is clearly not a simple square Ca\(^{2+}\) step. The RyR2 channels in vivo are governed by the very fast transient Ca\(^{2+}\) changes that occur near DHPR (i.e., L-type) Ca\(^{2+}\) channels when they flicker open (302). It has been estimated that the free Ca\(^{2+}\) concentration near the open mouth of a DHPR Ca\(^{2+}\) channel can reach very high levels (\(\sim 100\) \(\mu\)M) when it is open and fall very rapidly (\(\sim 10\) \(\mu\)s) after the channel closes. Interestingly, the fast Ca\(^{2+}\) overshoot generated by flash photolysis has similar characteristics (78). The rise time of the fast Ca\(^{2+}\) overshoot has a time constant of \(\sim 30\) \(\mu\)s, and the Ca\(^{2+}\) overshoot can theoretically peak at \(50–100\) \(\mu\)M before it decays with a time constant of \(\sim 150\) \(\mu\)s. Thus the fast Ca\(^{2+}\) overshoot may be a reasonable reproduction of the free Ca\(^{2+}\) changes that initiate RyR2 channel activity in the cell. Therefore, the single RyR2 channel Ca\(^{2+}\) activation time constant to physiological Ca\(^{2+}\) stimuli may be best represented by the flash photolysis data (\(\tau \sim 1\) ms; Ref. 112).

B. Rate of Ca\(^{2+}\) Deactivation

Fast Ca\(^{2+}\) elevations clearly turn on single RyR2 channels rapidly (112, 264). An equally important question is, How quickly do single RyR2 channels turn off (deactivate) in response to fast Ca\(^{2+}\) reductions? Intuitively, it will take some time for Ca\(^{2+}\) to “fall off” the Ca\(^{2+}\) activation site and for the channel to move into a closed configuration. The Ca\(^{2+}\) deactivation kinetics of single RyR2 channels have been explored using both fast solution exchanges (264, 283) and flash photolysis (340). Sitsapesan et al. (283) reported that the time constant of RyR2 channel Ca\(^{2+}\) deactivation was \(\sim 10\) ms, about the measured rate of the Ca\(^{2+}\) change. Schiefer et al. (264) reported that RyR2 channel Ca\(^{2+}\) deactivation occurred with a time constant of \(\sim 6\) ms, \(>10\) times slower than measured Ca\(^{2+}\) change. Velez et al. (340) photolyzed Diazmo-2 (a caged Ca\(^{2+}\) chelator) to reduce the local free Ca\(^{2+}\) concentration near a single RyR2 channel in planar bilayers. They reported a deactivation time constant of \(5.3\) ms, \(>10\) times slower than measured Ca\(^{2+}\) change. Thus there is reasonable consensus that single RyR2 channels deactivate rapidly with a time constant of \(5–6\) ms in response to a fast Ca\(^{2+}\) reduction.

The point is that the Ca\(^{2+}\) activation and deactivation kinetics of single RyR2 channels in vitro are sufficient for the channel to respond to the very brief (<1 ms) trigger Ca\(^{2+}\) concentration changes, the kind of local trigger Ca\(^{2+}\) signals that are thought to govern RyR2 channel activity in vivo (302).

XIV. FEEDBACK CALCIUM REGULATION

One suggestion is that the Ca\(^{2+}\) interacting with the Ca\(^{2+}\) activation site is different from the Ca\(^{2+}\) that passes through the open RyR channel pore. Another hypothesis is that the Ca\(^{2+}\) activation site may “see” the Ca\(^{2+}\) flux that passes through its own open pore. In other words, the RyR channel may operate in a local “common Ca\(^{2+}\) pool” (302). There are several whole cell observations that are consistent with this latter common Ca\(^{2+}\) pool idea (25, 168, 237, 344).

Is this type of self-feedback Ca\(^{2+}\) regulation seen in single RyR channel studies? Most single RyR studies have been done under conditions that generate superphysiological unitary Ca\(^{2+}\) currents (285, 290–292, 306). The pertinent single RyR channel studies were done with huge Ca\(^{2+}\) driving forces that are more than 50 times greater than those thought to exist in cells. This would increase the possibility of feedback Ca\(^{2+}\) regulation in ways that do not exist in the cell. This fact must be considered here.

The steady-state cytosolic Ca\(^{2+}\) sensitivity of the RyR1 or RyR2 channels appears to be the same whether the charge carrier is Ca\(^{2+}\) or a monovalent cation (58, 161, 290, 294, 306). This observation implies, but does not prove, that charge carrier identity does not impact RyR channel Ca\(^{2+}\) regulation. Sitsapesan and Williams (285) have evaluated single RyR2 channel gating in the presence of different luminal Ca\(^{2+}\) concentrations. They concluded that \(P_o\) and open/closed lifetimes of Ca\(^{2+}\)-activated, Ca\(^{2+}\)-conducting RyR1 channels were independent of the Ca\(^{2+}\) flux through the channel. In contrast, Tripathy and Meissner (333) reported that the duration of single RyR1 channel openings was significantly longer in the presence of even a relatively small Ca\(^{2+}\) flux through the pore. They go on to predict that the RyR1 channel’s Ca\(^{2+}\) activation site must be \(\sim 75\) nm from the pore to explain their results. Interestingly, the RyR channel complex is only \(\sim 25\) nm square (230, 249, 342). This may indicate that the RyR1 channel’s Ca\(^{2+}\) activation site may be in some sort of protected pocket that shields the site from the Ca\(^{2+}\) coming through the pore. In any event, it is clear that additional data are required to establish whether or not feedback Ca\(^{2+}\) regulation occurs.

XV. POTENTIAL NEGATIVE CONTROL MECHANISMS

It is clear that some sort of RyR channel negative control mechanism must exist to counter the inherent positive feedback of the CICR process (81, 105, 214, 255,
There is a growing consensus that the negative control that counters the inherent positive feedback of CICR is actually a composite of factors/processes that act in concert to terminate local RyR-mediated Ca\(^{2+}\) release. This latter idea is consistent with the fact that no individual negative control mechanism appears sufficient by itself to explain the termination of local Ca\(^{2+}\) release. Several candidate RyR channel negative control mechanisms are discussed below.

### A. Ca\(^{2+}\)-Dependent Inactivation

The first negative-feedback mechanism considered was Ca\(^{2+}\)-dependent inactivation (81). It was reported that the SR Ca\(^{2+}\) release process is substantially inactivated at steady-state Ca\(^{2+}\) concentrations as low as 60 nM in skinned cardiac muscle fibers (81). However, single RyR2 channel studies in bilayers have forwarded no evidence of Ca\(^{2+}\)-dependent inactivation at such low Ca\(^{2+}\) concentrations (54, 161, 256). Also, subsequent studies on intact cells presented contradictory results (109, 208, 214, 280). For example, Lukyanenko and Györke et al. (173) imaged permeabilized ventricular myocytes using a confocal microscope and showed that elevation of resting Ca\(^{2+}\) levels resulted in an increase in RyR2-mediated Ca\(^{2+}\) spark activity, not the decrease expected if Ca\(^{2+}\)-dependent inactivation exists. Thus the existence of high-affinity Ca\(^{2+}\)-dependent inactivation is debated. The existence of low-affinity Ca\(^{2+}\)-dependent inactivation is not. Single RyR1 and RyR2 channel data clearly demonstrate the existence of low-affinity Ca\(^{2+}\)-dependent inactivation is not. Single RyR1 and RyR2 channel data clearly demonstrate the existence of low-affinity Ca\(^{2+}\)-dependent inactivation is not. Single RyR1 channels clearly turn off as the cytosolic free Ca\(^{2+}\) concentration is elevated toward the 1 mM mark (196, 253, 307). This low-affinity Ca\(^{2+}\)-inhibition could come into play if local Ca\(^{2+}\) levels in the cell reach the 0.6–1 mM range (156). For the RyR2 channel, the situation is different. Single RyR2 channels turn off only after free Ca\(^{2+}\) concentrations reach much higher levels (5–10 mM;Refs. 12, 54, 56, 161, 199, 256). These levels are probably never realized in a cell. Thus Ca\(^{2+}\)-dependent inactivation as a potential negative control mechanism that may account for (or contribute to) the termination of local RyR-mediated Ca\(^{2+}\) release events is still not clearly established.

### B. Stochastic Attrition

Stochastic attrition refers to the inherent random closing of an individual ion channel. Stern (302) demonstrated using mathematical modeling that a SR Ca\(^{2+}\) release unit composed of one or relatively small number of RyR2 channels will turn off spontaneously as a result of reduced local Ca\(^{2+}\) levels resulting from the stochastic closures of individual RyR2 channels in the cluster. Thus stochastic attrition of single RyR2 channel activity could contribute to terminating a local Ca\(^{2+}\) release event. This process, however, would be very sensitive to the number of channels involved and the positive feedback gain of CICR in the channel cluster. Stern (302) showed that stochastic attrition was sufficient to terminate local Ca\(^{2+}\) release only if there were <10 RyR2 channels per cluster. If release units are composed of 10–30 or more channels (31, 95, 174), then the likelihood of termination solely by stochastic attrition is low. Experimentally, Lukyanenko et al. (176) showed that the rate of Ca\(^{2+}\) release termination is proportional to the magnitude of the release event (i.e., large events terminate faster than small events). Additionally, Sham et al. (273) demonstrated that local Ca\(^{2+}\) release events terminate rapidly despite the presence of a sustained trigger Ca\(^{2+}\) signal. These results are inconsistent with stochastic attrition playing a major role in control of the CICR process. Nevertheless, stochastic attrition may still contribute to local Ca\(^{2+}\) release termination if it acts in combination with other negative control mechanisms. It should be noted that the action of stochastic attrition would be highly nonlinear because once the number of active channels falls below some critical level, the remaining channels would be increasingly impacted by the phenomenon.

### C. Adaptation

Most single RyR channels studies have focused on defining RyR behavior under steady-state conditions. Györke and Fill (112), using laser flash photolysis of caged Ca\(^{2+}\), were the first to apply fast trigger Ca\(^{2+}\) signals to single RyR2 channels in bilayers. They reported that single RyR2 channels activated rapidly reaching \(P_o\) values that were well above those predicted from previous steady-state studies. After an initial burst of single-channel activity, \(P_o\) spontaneously decayed with a time constant of \(~1\) s. Application of second Ca\(^{2+}\) stimulus reactivated the apparently “inactivated” channels. This suggested that the spontaneous decay observed after the first Ca\(^{2+}\) stimulus was not due to a conventional absorbing Ca\(^{2+}\) inactivation mechanism (81) because the channels were not in a refractory state. They proposed that the spontaneous decay may be mediated by a different process they termed adaptation (112, 230).

The RyR2 channel adaptation proposal spurred an active debate. One point of debate centered on the relatively slow time course of adaptation (~1 s). The point was that adaptation might be too slow to be a negative control mechanism that stabilizes the CICR process in heart. Somewhat lessening this concern, Valdivia et al. (339) demonstrated that the RyR2 channel adaptation phenomenon was ~10-fold faster in the presence of physiological Mg\(^{2+}\). Another point of debate was that not all...
investigators reported “adaptive behavior” of single RyR2 channels. Although several groups reported that fast Ca$^{2+}$ stimuli induced a transient burst of single RyR2 channel activity (112, 159, 264, 283, 339), different groups interpreted their results in different ways. Still another point of debate was centered on the nature of the Ca$^{2+}$ stimulus applied in the original adaptation study (112). It was suggested that the fast Ca$^{2+}$ overshoot at the leading edge of the applied Ca$^{2+}$ stimulus might generate the observed adaptation phenomenon (154). As a result, the single RyR2 channel data generated by the flash photolysis have undergone an unusual level of scrutiny. It has endured, has been frequently reviewed (86, 152, 288), and has generated new insights into single RyR2 channel Ca$^{2+}$ regulation. Details concerning the RyR2 adaptation debate are discussed in section XVI.

We now know that the adaptation phenomenon is due to a Ca$^{2+}$- and time-dependent shift in the modal gating behavior of single RyR2 channels (111). A fast-sustained Ca$^{2+}$ stimulus transiently drives the RyR2 channel into a high $P_o$ gating mode before it has time to equilibrate (i.e., adapt) between all its different gating modes. A specific kinetic scheme of RyR2 channel gating has been developed describing this behavior (86). It is now clear that Ca$^{2+}$-dependent inactivation and adaptation are not mutually exclusive mechanisms (as generally thought). These two phenomena may simply be different manifestations of a common underlying mechanism, Ca$^{2+}$- and time-dependent modal gating.

**D. Activation-Dependent or “Fateful” Inactivation**

Pizarro et al. (244) found that the global RyR1-mediated Ca$^{2+}$ release transient in frog skeletal muscle, triggered by a maximally depolarizing stimulus, was inhibited in proportion to the degree of Ca$^{2+}$ release generated during a prestimulation. This suggests that the RyR1 channels activated in the prestimulation were not available for activation during the main stimulus. Thus preactivated RyR1 channels appeared inactivated or refractory. These authors proposed that RyR1 channel inactivation is strictly and “fatefully” linked to their activation. In other words, RyR1 channel inactivation is use dependent. Certain single-channel studies are consistent with this idea. It is possible that a modal gating shift (i.e., high to low $P_o$ modes) may be a single-channel manifestation of fateful inactivation (i.e., use dependence) observed in situ. Several different groups have now reported modal gating behavior of single RyR channels (8, 86, 111, 361).

**E. Depletion/Luminal Ca$^{2+}$ Effects**

When the SR is overloaded with Ca$^{2+}$, periodic spontaneous Ca$^{2+}$ waves can occur in heart muscle (304). It appears that luminal Ca$^{2+}$ levels modulate these RyR2-mediated Ca$^{2+}$ waves. The presence of a luminal Ca$^{2+}$ regulation may also explain why a critical Ca$^{2+}$ load must be attained before Ca$^{2+}$ can trigger SR Ca$^{2+}$ release from SR vesicle preparations (234–236). Several single RyR channel studies have also addressed the possibility that a luminal Ca$^{2+}$ regulatory site exists (110, 341, 285, 333). Velez and Suarez-Isla (341) showed that RyR channel activity changed as a function of luminal Ca$^{2+}$ concentration and suggested this phenomenon was mediated by a low-affinity Ca$^{2+}$ binding site on the luminal side of the channel. Later, two groups (110, 285) systematically investigated luminal RyR2 Ca$^{2+}$ regulation and showed that its extent depended on how the RyR2 channel was activated. For example, ATP-activated RyR2 channels were more sensitive to luminal Ca$^{2+}$ levels than Ca$^{2+}$-activated RyR2 channels.

The pertinent low-affinity luminal Ca$^{2+}$ binding site(s) may reside on the RyR channel or possibly on another closely associated luminal protein. These sites would need to sense changes in intra-SR Ca$^{2+}$ levels in the appropriate Ca$^{2+}$ concentration range (0.2–20 mM). As SR Ca$^{2+}$ levels fall, the RyR channel would sense the reduced SR Ca$^{2+}$ level leading to a decrease in channel activity. This would then represent an interesting form of RyR negative control. There are caveats, however, in this luminal Ca$^{2+}$ story. Repetitive Ca$^{2+}$ sparks should reduce local SR Ca$^{2+}$ load and thus downregulate RyR2 channel activity. Yet, multiple Ca$^{2+}$ sparks can occur frequently at the same site in cells with little sign of progressive rundown. This may suggest that SR Ca$^{2+}$ levels either do not change dramatically and/or that luminal Ca$^{2+}$ may not impact RyR2 channel activity dramatically. The spark data, however, do not rule out the possibility that very brief and highly localized luminal Ca$^{2+}$ depletions govern RyR2 channel function.

**F. Allosteric or Coupled Gating**

Recently, Stern et al. (305) evaluated several published single RyR2 gating schemes and found that they all generated unacceptable instability when applied in a model of Ca$^{2+}$ release local control. One reason for the instability was the lack of a strong negative control mechanism to minimize local regenerative Ca$^{2+}$ release in clusters of RyR2 channels. Another reason for the instability was the relatively low cooperativity of the RyR2 channel Ca$^{2+}$ activation process. The low cooperativity does not allow the RyR2 channels to adequately discriminate between trigger and background Ca$^{2+}$ signals. Interestingly, Stern et al. (305) suggested that RyR2-RyR2 allosteric interactions could theoretically overcome these instability problems. Recently, it has been reported that neighboring RyR1 channels may be physically coupled by the
FK506-binding protein (FKBP12) and consequently gate in synchrony (188). Is this coupled RyR channel gating operative under physiological conditions? The answer to this question is not yet clear (19, 305). Other investigators have not reported coupled RyR channel gating. Additionally, certain thermodynamic considerations may also be a problem (i.e., multiple large macromolecules operating with microsecond synchrony). Furthermore, removal of FKBP12.6 from the RyR channel does not abolish Ca\(^{2+}\) sparks, but actually increases their frequency and duration (351). Although coupled RyR channel gating is an interesting and provocative hypothesis, its existence and nature still require verification.

**XVI. ADAPTATION DEBATE**

The Györke and Fill (112) RyR2 adaptation hypothesis was controversial. One concern was that the decrease in RyR2 channel activity, interpreted as adaptation, might reflect slow Ca\(^{2+}\) deactivation (154) following a short-lived Ca\(^{2+}\) transient (i.e., the fast Ca\(^{2+}\) overshoot) at the leading edge of the applied Ca\(^{2+}\) stimuli. This possibility was experimentally addressed, and the data suggest that the brief Ca\(^{2+}\) overshoot has little impact on the much slower (i.e., 1,000-fold slower) adaptation phenomenon. The data indicate that the impact of the fast Ca\(^{2+}\) overshoot is most likely limited to accelerating the initial closed to open transition of the RyR2 channel (i.e., it essentially supercharges the applied Ca\(^{2+}\) stimuli). A detailed discussion of the Ca\(^{2+}\) overshoot concern is presented below after a detailed description of the Ca\(^{2+}\) overshoot itself. Another concern regarding the original adaptation hypothesis (112) was that some investigators did not report adaptive behavior of single RyR2 channels but instead forwarded alternate interpretations (49, 112, 159, 264, 283, 339). Some of these different interpretations are also presented below.

**A. DM-nitrophen Ca\(^{2+}\) Overshoot**

DM-nitrophen is an ultraviolet-sensitive high-affinity Ca\(^{2+}\) buffer that changes its Ca\(^{2+}\) affinity from \(\sim 4.8\) nM to \(\sim 3\) mM upon flash photolysis (74, 78, 194). Ca\(^{2+}\) liberation by flash photolysis of DM-nitrophen (the caged Ca\(^{2+}\) compound used the RyR2 adaptation studies) generates a fast Ca\(^{2+}\) overshoot (78, 154, 194, 372). It arises because flash photolysis liberates Ca\(^{2+}\) faster than free DM-nitrophen can bind Ca\(^{2+}\). Its characteristics and how its kinetics vary with experimental conditions are not generally understood and often the basis of debate (112, 154, 372).

The fast Ca\(^{2+}\) overshoot is very fast compared with kinetics of commonly used fluorescent dyes (78). Consequently, the kinetic characteristics of the fast Ca\(^{2+}\) overshoot are necessarily based on numerical calculations from the much slower fluorescence data even in the case of the fastest fluorescent indicators (78). The parameters estimated for the fast Ca\(^{2+}\) overshoot may then reflect more the kinetic characteristics of the indicator than the Ca\(^{2+}\) liberation kinetics of the caged Ca\(^{2+}\) compound. There is no debate that DM-nitrophen photolysis by a brief flash (\(< 50\) ns long) liberates Ca\(^{2+}\) in \(\sim 30\) ns (74, 78, 194). However, there is disagreement about the Ca\(^{2+}\) association rate of DM-nitrophen. Zucker (372) suggested a relatively slow association rate (\(1.5 \times 10^8\) M\(^{-1}\)s\(^{-1}\)), and use of this value predicts that Ca\(^{2+}\) overshoots should last several milliseconds. More recently, Escobar et al. (78) showed that the association rate was substantially faster (\(3 \times 10^7\) M\(^{-1}\)s\(^{-1}\)). This means that the fast Ca\(^{2+}\) overshoot is more than an order of magnitude faster (\(\sim 100\) \(\mu\)s vs. \(\sim 2\) ms) than previously thought (154, 372).

The magnitude of the overshoot depends on the free DM-nitrophen levels. At pCa 9, only \(~18\)% of unphotolysed high-affinity DM-nitrophen will be bound to Ca\(^{2+}\). Flash photolysis will liberate Ca\(^{2+}\) from Ca\(^{2+}\)-bound DM-nitrophen, but much of this Ca\(^{2+}\) will rebind to Ca\(^{2+}\)-free DM-nitrophen. At pCa 7, nearly 96% of unphotolysed high-affinity DM-nitrophen will be bound to Ca\(^{2+}\). The same flash will liberate much more Ca\(^{2+}\) from Ca\(^{2+}\)-bound DM-nitrophen, but there will be less Ca\(^{2+}\)-free DM-nitrophen to participate in rebinding. As a result, the Ca\(^{2+}\) overshoot will be smaller and slower. At pCa 6, nearly all unphotolysed high-affinity DM-nitrophen will be bound to Ca\(^{2+}\). There will thus be no Ca\(^{2+}\) rebinding and consequently no Ca\(^{2+}\) overshoot.

We estimate, based on the calculations presented in previously works (78, 86), that the Ca\(^{2+}\) overshoot in the original Györke and Fill (112) study peaked at 30–60 \(\mu\)M and lasted \(~100–200\) \(\mu\)s. Györke and Fill (112) used a Ca\(^{2+}\) electrode to measure free Ca\(^{2+}\) concentrations before and after photolysis. Thus the properties of the fast Ca\(^{2+}\) overshoot could not be measured. These kinetic estimates predict that the fast Ca\(^{2+}\) overshoot was at least three orders of magnitude faster than the observed adaptation phenomenon (112, 372).

**B. Ca\(^{2+}\) Overshoot Concern**

The concern was that RyR2 adaptation might simply be deactivation following the fast Ca\(^{2+}\) overshoot (154). In other words, the Ca\(^{2+}\) activation sites will be rapidly occupied during the overshoot. After the overshoot, occupancy of the site will fall and deactivate the channel. The idea was that the RyR2 deactivation after the overshoot (lasting \(< 200\) \(\mu\)s) generates the adaptation (\(\tau = \sim 1\) s) phenomenon. If this were the case, then the deactivation of the RyR2 would need to be very slow. Is RyR2 Ca\(^{2+}\) deactivation so slow? The answer is no. The rate of RyR2 channel deactivation in response to a fast Ca\(^{2+}\)
reduction has been experimentally defined (264, 340, 362), and it is quite fast ($\tau$ values $\sim$5 ms). Does the fast Ca$^{2+}$ overshoot impact single RyR2 channel function? The answer is yes. Zahradnìková et al. (362) using improved recording conditions showed that the fast Ca$^{2+}$ overshoot, albeit slightly smaller than those applied by Györke and Fill (112), can occasionally induce one or two brief opening events. Application of Ca$^{2+}$ stimuli like those applied by Györke and Fill (112) (i.e., overshoot followed by a sustained elevation), induce an entirely different pattern of single RyR2 channel activity (including the classical adaptation phenomenon). These data and more recent single-channel modeling (86) suggest the impact of the fast Ca$^{2+}$ overshoot on single RyR2 channel function may simply be to accelerate the initial opening transition.

Does the fast Ca$^{2+}$ overshoot induce the adaptation phenomenon? Few questions concerning single RyR channel function have been debated more (86, 152, 288). In principle, adaptation represents a slow transition of the RyR channel from one state to another. There is circumstantial experimental evidence that suggests that this slow transition is not simple Ca$^{2+}$ deactivation following the Ca$^{2+}$ overshoot (154, 264, 340, 362). There are also theoretical arguments that suggest that the slow transition is not induced by the fast Ca$^{2+}$ overshoot. A specific Markovian gating scheme, albeit still imperfect, has been forwarded to describe RyR2 adaptation (see Fig. 3; Ref. 86). In this scheme, a fast Ca$^{2+}$ overshoot is not required to generate adaptation-like phenomenon. This scheme suggests that RyR2 adaptation is due to a transient Ca$^{2+}$-dependent modal RyR2 gating shift (see sect. xvii and Fig. 3). There is, however, no experimental evidence directly addressing the salient point (i.e., demonstration that the same channel response is elicited by a Ca$^{2+}$ step with or without the initial overshoot). Thus the question above has not been definitively answered yet.

C. Differing Interpretations

Several studies have now defined the kinetics of single RyR2 channel Ca$^{2+}$ regulation (112, 113, 159, 264, 282, 283, 339, 340, 362). Although the data are generally consistent, how the different data sets have been interpreted varied a great deal. Some investigators choose to interpret their data in terms of adaptation (112, 339, 340), whereas others choose to interpret their data in terms of classical Ca$^{2+}$-dependent inactivation (159, 264, 283). Several factors contributed to the different interpretations. The various studies used very different techniques and consequently applied very different types of Ca$^{2+}$ stimuli. This is important because even relatively small differences in the characteristics of the Ca$^{2+}$ stimulus can have a large impact on single RyR2 channel function (81). For years, the absence of adaptation following the mechanically induced Ca$^{2+}$ stimuli was construed as evidence against the adaptation phenomenon. A great deal of attention was focused on understanding how the Ca$^{2+}$ overshoot could generate “adaptation.” Attention is now more appropriately focused on understanding how the different types of Ca$^{2+}$ stimuli may impact channel function. It is now clear that the photolytic Ca$^{2+}$ stimuli have a fast component not present in the mechanical methods. Thus it is not surprising that RyR channel behavior is different in the different studies. The point is that the results of the flash photolysis studies (112, 339, 340, 362) are not inherently at odds with those reported in the mechanical solution change studies (159, 160, 264, 282, 283), and there is no reason a priori to favor one type of study over another. Each study should be evaluated on its own merits, for each represents a heroic effort and makes a substantive contribution. In our opinion, the differences in the Ca$^{2+}$ stimuli make the data more (not less) interesting. The fast photolytic Ca$^{2+}$ stimuli are particularly interesting because they may mimic the Ca$^{2+}$ changes that activate RyR channels in cells.
XVII. MODAL GATING  

Under steady-state conditions, single RyR2 channel activity occurs in bursts. This means that channel openings tend to be clustered in time instead of being randomly distributed in time. The $P_o$ during a burst can be either high or low (Fig. 4). The high $P_o$ and low $P_o$ bursts do not occur randomly. Instead, the bursts tend to be clustered as well. Trains of high $P_o$ bursts characterize a high $P_o$ gating mode. Trains of low $P_o$ bursts characterize a low $P_o$ gating mode. Extended periods of no openings characterize a zero $P_o$ gating mode. Several different groups have now reported the existence of modal RyR2 channel gating (8, 86, 261, 361).

How does modal RyR2 channel gating work? This is still an open question. The identities, transitions, and characteristics of the different RyR2 gating modes are currently being defined. Preliminary interpretations suggest that there is a dynamic equilibrium between at least three different gating modes under steady-state conditions (i.e., at a constant Ca$^{2+}$ level). High, low, and zero $P_o$ modes are clearly evident in the steady-state activity of single RyR2 channels (8, 86, 261, 361). There also appears to be clear spontaneous shifts between the different gating modes. Simulated single-channel data are presented in Figure 4 to illustrate some of these points. It is thought that the dynamic Ca$^{2+}$-dependent equilibrium between gating modes is what generates the classical bell-shaped steady-state Ca$^{2+}$ dependence of the RyR2 channel (54, 161, 353).

An interesting thing happens when the Ca$^{2+}$ level changes suddenly. Small fast Ca$^{2+}$ elevations (from a low Ca$^{2+}$ level) upset the existing dynamic equilibrium momentarily. The result is a transient change in channel activity until a new equilibrium is reached. Specifically, it has been argued that single RyR2 channel activity momentarily rises to a $P_o$ level above that predicted by steady-state measurements before it spontaneously decays (86). Channel activity decays as the channel reequilibrates between the gating modes at the new higher Ca$^{2+}$ level (Fig. 4). Subsequent small fast Ca$^{2+}$ elevations would induce further transient modal gating shifts, inducing additional transient episodes of high channel activity. This behavior is reminiscent of the adaptation phenomenon. The magnitude of the transient modal gating shift should depend on the speed and magnitude of the Ca$^{2+}$ stimulus. A slow Ca$^{2+}$ stimulus would not induce a dramatic modal gating shift because mode reequilibration is time dependent (i.e., modes will reequilibrate during the slow stimulus). Large Ca$^{2+}$ stimuli should be less effective because at high Ca$^{2+}$ levels the channel will begin to frequent the inactivated state. Consequently, a slow large Ca$^{2+}$ stimulus would not induce a substantial transient modal gating shift but may instead induce Ca$^{2+}$-dependent inactivation.

Is this consistent with the experimental record? Applications of fast Ca$^{2+}$ elevations do indeed induce super steady-state $P_o$ levels that are followed by a spontaneous decay in channel activity (112, 159, 339). Also, repeated episodes of transient channel activity in response to multiple incremental Ca$^{2+}$ elevations have been reported (112). Experiments have also defined the dependence of channel activation rate on stimulus speed (159, 264, 283).

![Modal gating of the RyR2 channel.](image-url)
and clear refractory behavior at very high Ca²⁺ levels (264). Thus the modal gating theory qualitatively presented above has some experimental support. It is clear, however, that additional experimental results are needed. For example, current modal gating theory does not yet consider the potential impact of certain physiologically important ligands (e.g., luminal Ca²⁺, cytosolic Mg²⁺, and ATP).

Is modal RyR channel gating physiological? Modal gating may reproduce and reconcile certain single RyR channel phenomena in bilayers, but its physiological relevance is questionable. In cells, the RyR channels generate intracellular Ca²⁺ signals that last less than ~25 ms (e.g., sparks). Studies of “steady-state” RyR behavior in bilayers report that intermodal gating transitions occur over seconds. From these data, it appears unlikely that modal RyR gating is a physiologically relevant phenomenon. Modal gating may simply be a biophysical ramification of RyR channel Ca²⁺ regulatory complexity. This does not make it less important because understanding the origin of modal gating may provide new insights into RyR channel Ca²⁺ regulation.

XVIII. CONCLUDING REMARKS

The advent of in vitro single RyR channel recording has provided an abundance of information. It has revealed key properties of the RyR channel’s permeation pathway and how (and if) particular ligands alter channel function. Despite some amazing advances, many fundamental questions about how the RyR channels are turned on and off in vivo remain unanswered. For example, in vitro single RyR2 channel recording has not definitively established the identity of the mechanism(s) that terminate the SR Ca²⁺ release process or revealed how movement of the DHPR voltage sensor governs RyR1 channel activity.

Our understanding of single RyR channel regulation is clearly incomplete. This impairs our understanding of many intracellular calcium signaling phenomena. Long-standing RyR channel mysteries remain and are certain to motivate research long into the future.

We thank the reviewers for their meticulous revision of the manuscript and for providing us with very constructive comments. We also thank Dr. J. Ramos-Franco and M. Porta for comments on the manuscript and A. Nani for technical help.

Research in the laboratory of M. Fill is supported by National Heart, Lung, and Blood Institute Grant HL-57832. J. A. Copello is supported by American Heart Association Grant 0330142N. Address for reprint requests and other correspondence: M. Fill, Dept. of Physiology, Loyola University Chicago, 2160 South First Ave., Maywood, IL 60153.

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