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Ryanodine Receptors of Striated Muscles: a Complex Channel Capable of Multiple Interactions

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Franzini-Armstrong, Clara, and Feliciano Protasi. Ryanodine Receptors of Striated Muscles: a Complex Channel Capable of Multiple Interactions. *Physiol. Rev.* 77: 699–729, 1997.—The ryanodine receptor (RyR) is a high-conductance Ca^{2+} channel of the sarcoplasmic reticulum in muscle and of the endoplasmic reticulum in other cells. In striated muscle fibers, RyRs are responsible for the rapid release of Ca^{2+} that activates contraction. Ryanodine receptors are complex molecules, with unusually large cytoplasmic domains containing numerous binding sites for agents that control the state of activity of the channel-forming domain of the molecule. Structural considerations indicate that long-range interactions between cytoplasmic and intramembrane domains control channel function. Ryanodine receptors are located in specialized regions of the SR, where they are structurally and functionally associated with other intrinsic proteins and, indirectly, also with the luminal Ca^{2+}-binding protein calsequestrin. Activation of RyRs during the early part of the excitation-contraction coupling cascade is initiated by the activity of surface-membrane Ca^{2+} channels, the dihydropyridine receptors (DHPRs). Skeletal and cardiac muscles contain different RyR and DHPR isoforms and both contribute to the diversity in cardiac and skeletal excitation-contraction coupling mechanisms. The architecture of the sarcoplasmic reticulum-surface junctions determines the types of RyR-DHPR interactions in the two muscle types.

I. INTRODUCTION

The ryanodine receptor (RyR) is a Ca^{2+} channel of the endoplasmic reticulum [or sarcoplasmic reticulum (SR) in the case of muscle cells] with a very large cytoplasmic domain, with high affinity for ryanodine, a neutral plant alkaloid. Ryanodine receptors have considerable sequence and general structure similarities with the other intracellular channels, the inositol 1,4,5-trisphosphate ($InsP_3$) receptors, with which they share the task of releasing Ca^{2+} from the internal stores (231). Ryanodine receptors, however, have higher conductivity than $InsP_3$ receptors, and thus they are employed in situations that need fast release of large quantities of Ca^{2+}, such as during excitation-contraction (e-c) coupling in muscle.

In striated muscles, RyRs interact with several other proteins, and thus both their structure and their function must be understood within this complex set of interactions. Ryanodine receptors are located at high density in a special domain of the SR membrane, the junctional face membrane, belonging to the junctional SR (jSR). Within this domain, RyRs are associated, either directly or indi-

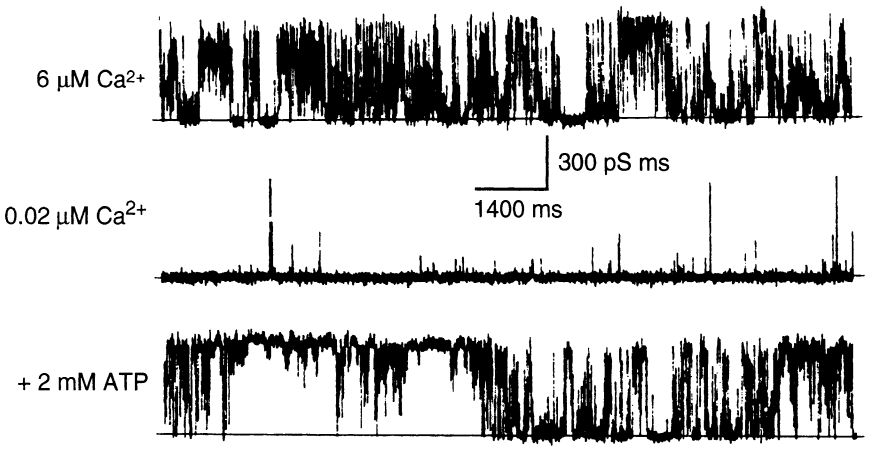

FIG. 1. Reconstitution of purified ryanodine receptor (RyR) isoform RyR1 into a planar lipid bilayer reveals a high-conductance channel with properties essentially identical to those of Ca^{2+} release channel of heavy sarcoplasmic reticulum (SR). Currents were recorded in presence of 6 μM Ca^{2+} (*top*), 0.02 μM Ca^{2+} (*middle*), and 0.02 μM Ca^{2+} plus 2 mM ATP (*bottom*). Ca^{2+} and adenine nucleotides greatly increase channel's open probability. [From Lai et al. (160).]

rectly, with other structural components of the jSR, calsequestrin, triadin, and junctin, with which they may functionally interact. The junctional face membrane is closely apposed to, and forms a specialized junction with, the external cell membranes [the surface membrane and its invaginations, the transverse (t) tubules]. The junctions between SR and external membranes allow interactions between RyRs and proteins of the surface membrane, the dihydropyridine receptors (DHPRs). Finally, RyRs are stably bound to one small protein, FKBP12 (FK506 binding protein, see sect. IIIA), and they are modulated by calmodulin (CaM) and cytoplasmic solutes. Several reviews (15, 59, 201, 206, 236, 304) thoroughly cover the properties and pharmacology of RyRs; a collection of monographs deals with properties and distribution of RyRs (298); and the proceedings of a recent symposium on channels include RyRs and $InsP_3$ receptors (56).

II. RYANODINE RECEPTOR: THE MOLECULE

A. Isolation and Characterization of Ryanodine Receptors

The way for the isolation of the RyR and its identification as the SR Ca^{2+} release channel was paved by several key observations on the properties of the heavy SR. The heavy SR is the higher density fraction of the reticulum containing the jSR and the internal Ca^{2+} binding protein calsequestrin (36, 203) and also intact triads (41). Ryanodine and doxorubicin, agents that have profound effects on muscle contraction by causing and/or inhibiting liberation of Ca^{2+} from the SR, bind with high affinity to a high-molecular-weight component of the heavy SR (85, 253, 255, 354, 356), which also binds CaM (279). Heavy SR, but not light SR, rapidly releases Ca^{2+} (203), due to the presence of channels with high permeability for monovalent and divalent cations (289) that are activated by adenine nucleotides and Ca^{2+} and inhibited by Mg^{2+} (274, 288; Fig. 1). These experiments established a direct relationship between a Ca^{2+} release channel of heavy SR and the fast Ca^{2+} release that plays a role in the activation of muscle contraction in skeletal and cardiac muscle.

Large structural components of the SR, the feet, are located in the junctional face membrane of skeletal (36, 60, 92, 93, 216) and cardiac (295, 296) muscle, facing the external membranes. A high-molecular-weight component of the jSR (or perhaps a doublet of proteins) was isolated and identified as the spanning protein by immunoelectron microscopy (33, 147, 148). Thus evidence for the location of Ca^{2+} release sites in the jSR and for a high-molecular-weight component playing a major role in the junction was already in place before RyRs were isolated.

Intact RyR molecules were first isolated and purified in skeletal (127, 130, 159, 160) and cardiac (9, 129, 131, 132, 267) muscles, after mild 3-[(3-cholamidopropyl)dimethylammonio]-propanesulfonate (CHAPS) solubilization of the heavy SR and using [^{3}H]ryanodine as a selective marker. They were subsequently found in smooth muscle (116, 170, 346; see Ref. 186 for a review) and are now known to be present in a large variety of cell types (see Refs. 297–299 for reviews). They are very large (30S) complexes constituted by homotetramers of ~560-kDa polypeptides. The large size of the molecule allows single-step purification by sucrose density gradient (160), but combined procedures have been used. These include sequential column chromatography on heparin-agarose and hydroxyapatite (130); immunoaffinity purification (127) and either ion-exchange chromatography or heparin-agarose column chromatography combined with density gradient centrifugation (114, 128) have also been used. A simple one-step procedure (286) and an affinity purification procedure based on the strong binding between RyR and FKBP12 have been devised (344).

The purified receptor, incorporated in a planar lipid bilayer functions as a Ca^{2+} channel (9, 20, 37, 121, 127, 160, 275, see 162 for review), with characteristics identical to the Ca^{2+} release channels previously identified in the heavy SR (288, 289) and in SR in situ (300). The full-length rabbit skeletal RyR cDNA has been functionally expressed

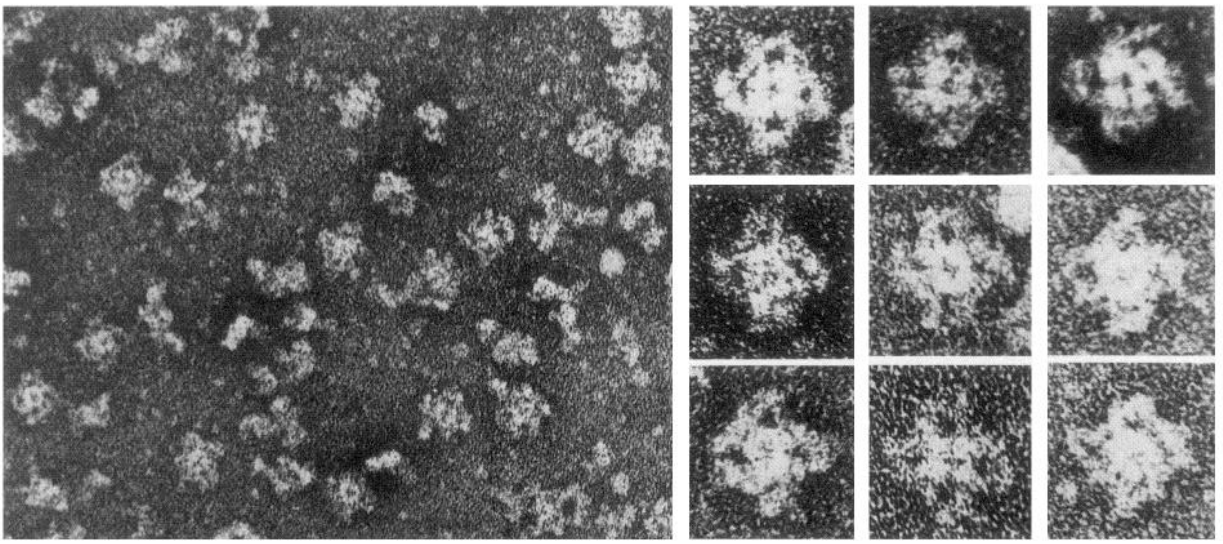

FIG. 2. Negatively stained purified RyR1 from rabbit skeletal muscle. Protein has 4 equal large subunits in form of a quatrefoil, identical in size and shape to feet of junctional SR. A smaller central region, also composed of 4 equal subunits, is intramembrane channel-forming domain. [From Lai et al. (160).]

in COS and Chinese hamster ovary (CHO) cells (48, 251, 310) and shown to form a channel with the appropriate properties (48). Identification of RyR as Ca^{2+} release channels relies on the assumption that the channel activity is due to the major protein composing the purified fraction (the RyR) and not to a minor contaminant. Reconstitution of the purified RyR into proteoliposomes allowed observation of channel function on a macroscopic scale, confirming the channel identity of RyRs (169). The presence of a single high-affinity site for ryanodine within a tetramer identifies the tetramer as a single functional entity or channel (160). The hereditary defect in malignant hyperthermia has been traced to the RyR (184, 213).

The isolated RyR is a large molecule, $\sim 29 \times 29 \times 12$ nm, with four equal subunits roughly approximated by four spheres that are closely associated to form a four-leaf clover or quatrefoil (21, 130, 131, 160, 332; Fig. 2). Structural comparison of the isolated channel and of the feet allowed direct identification. The overall size of the feet and their quatrefoil structure (82, 174, 216) are identical to those of the isolated RyR. In addition to the large domain, corresponding to the feet, the isolated molecule has a smaller central region constituted of four equal lobes, rotated by $\sim 45°$ relative to the larger domain. Because the small domain is not visible in the in situ molecule, it is logical to assume that it represents the intramembrane, channel-forming, portion of the RyR. Indeed, a small quatrefoil, with four equal lobes, is visible within the jSR membrane after freeze fracturing (21). Because ryanodine binds close to, but not within the presumed transmembrane domain, the molecule is often called RyR/Ca^{2+} release channel complex. Foot protein is another name that identifies the molecule based on the structure of its cytoplasmic domain.

With the use of the quatrefoil shape and the characteristic large size of the feet as unique identification markers for RyRs, the molecule has been identified in a large variety of muscles from vertebrates and invertebrates (174). A RyR has also been identified in two muscles from crustaceans (91, 239, 280), characterized in *Caenorhabditis elegans* (153), and identified and sequenced in *Drosophila* (113, 312). Ryanodine receptors have been detected in almost all cell types, although at variable levels of expression (see sect. II*B*).

One question remains unsolved: What is the relationship between the RyR and the 106-kDa protein that can be isolated by ryanodine-affinity chromatography and forms channels with conductance and pharmacology comparable to those of the RyR (118, 276, 349)?

Identification of the cytoplasmic domains of RyRs as feet has a very important implication. Feet are located in regions of the heavy SR that form junctions with surface membrane and t tubules. Therefore, the cytoplasmic domains of RyRs, constituting the feet, allow a direct connection between the Ca^{2+} release channel and the exterior membranes. The latter part of the review covers the role that this connection plays in e-c coupling, the series of steps that link depolarization of the surface membrane to contraction of the myofibrils, via release of Ca^{2+} by RyRs.

B. Ryanodine Receptor Isoforms: Tissue Distribution

1. *Three ryanodine receptor isoforms in a variety of tissues*

Three types of RyR with specific tissue distribution are now recognized (104; see Refs. 297, 299 for reviews).

The currently accepted terminology is based on the timing of purification of the RyRs from various tissues and the identification of the three isoforms by molecular probes. Thus RyR1, also called the skeletal type, is the isoform first detected (189) and then fully sequenced (313, 353) in skeletal muscle; RyR2, the dominant form in cardiac muscle, was subsequently sequenced (228, 244); and RyR3, sometimes called the brain isoform, was first detected (228) and later fully sequenced in brain (112) and epithelial cells (103). Sequence comparison of the tree isoforms in a single species reveals a homology of 67, 67, and 70% between RyR1/RyR2, RyR1/RyR3, and RyR2/RyR3 (112), respectively, but a phylogenetic tree generated from the optimal alignment of full-length RyR sequences available in 1996 indicates overall closer relationships between RyR1 and RyR3 than RyR1 and RyR2 (326).

The RyR1 isoform is primarily expressed in all skeletal muscles (5, 161, 237, 313, 326, 352) and in some parts of the brain, most prominently in Purkinje cells of the cerebellum (99, 104, 157, 168, 245), and is also present in some smooth muscle (230). The RyR2 isoform is the predominant form in cardiac muscle (228, 244) and is also the most widely distributed isoform in the brain (99, 104, 157, 158, 168). Indeed, the major form of RyR purified from brain is identified as RyR2 immunologically and by analysis of proteolytic products (200). Some expression of RyR2 in smooth muscle has also been found (230). The RyR3 isoform is a minor component of the brain, where it is the least widely distributed of the three isoforms. The RyR3 isoform is immunologically distinct from RyR1 and RyR2 (143). RyR3 is present in skeletal muscle, but it is particularly abundant in selected skeletal muscles from some species (see sect. II*B2*). Smooth muscle expresses RyR3 (230), but in minor amounts (189).

The dominant form of RyR in cardiac muscle is RyR2 (69, 228, 244). Both RyR1 and RyR3 are at such extremely low levels (112) that they can be truly detected only by ribonuclease protection assays (103) and reverse transcription-polymerase chain reaction amplification (168). Much of the RyR3 is attributable to smooth muscle cells of intracardiac coronary vessels, which are quite numerous, but weak hybridization of the working myocardium and of the conducting Purkinje system for cRNA probes for RyR3 indicates a minor presence of this isoform in myocardial cells (232). A detailed search for functional variations in RyR composition of SR isolated from various regions of the heart has shown channels with indistinguishable conductance and pharmacology, indicating that no region of the heart has a noticeably high presence of channels other than RyR2 (345). Thus myocardium is the muscle with the purest RyR composition, despite the fact that feet are located in two distinct regions of the SR: the jSR and corbular SR (see sect. IV*C*).

So far, few variations from the three isoforms have been found. First, a gene encoding a partial sequence of RyR3 expresses a protein that does not respond to caffeine (103). Second, a transcript from the COOH-terminal region of the RyR gene has been identified in brain (314). This shortened version of the molecule would lack the receptor regions for agents that affect channel activity. Third, alternative splicing has been found to introduce complexity in the RyR family. Two alternately spliced RyR transcripts were found in embryonic, slow- and fast-twitch rabbit skeletal muscle, regardless of the developmental stage (355). The difference between the two is very small: one of the transcripts lacks five amino acids, and it is not yet known if both mRNAs are expressed. Tissue-specific and developmentally regulated splicing have been detected for RyR3 (193, 219) and RyR1 (101). The latter involves modulatory segments with binding sites for Ca^{2+}, CaM, and ATP.

It is likely that further variations exist but have not yet been detected, due to the difficulty of fully sequencing this very large molecule. However, the positive results with hybridization analysis and immunolabeling across tissue types certainly indicate that RyR types are highly conserved. In this respect, it is interesting to contrast RyR with another large protein, myosin, which spans at least 11 families, each comprising a large number of isoforms.

2. One versus two ryanodine receptors in skeletal muscle

Muscles in nonmammalian vertebrate, bird, amphibian, reptile, and fish muscle contain two RyR isoforms, initially called α and β (5, 161, 221, 233, 240). Molecular approaches have shown that the skeletal muscle β-isoform of chicken and frog is actually RyR3, the isoform initially identified in the brain, whereas the α-isoform is recognized as homologous to RyR1 (58, 104, 237, 246). In frog and bullfrog, the α-isoform has 80% sequence similarity to rabbit skeletal RyR1, and the β-isoform has 85–86% similarity to rabbit brain RyR3, whereas they diverge considerably from cardiac RyR2 (237, 246). Use of the appropriate antibodies confirms that α- and β-isoforms of chicken differ from RyR2 (7), replacing initial evidence to the contrary. In fish, the homology of α-isoform with RyR1 has also been confirmed, but the β-isoform has not been characterized at the molecular level (234). The presence of RyR3 instead of RyR2 in skeletal muscle came as a surprise, in view of the fact that developing skeletal muscle transiently expresses cardiac type DHPR and myofibrillar proteins. Diversity of RyRs in muscle fibers and its significance are well covered in a recent review (304).

The RyR1 and RyR3 isoforms (α and β) are present in approximately equal amounts in nonmammalian skeletal muscle. The two isoforms are clearly located within the same muscle fiber in a position corresponding to that of the triads (5, 161, 240; Fig. 3), and both are equally enriched in the triad fraction (221, 233). However, although immunolabeling suggests a close proximity of the two

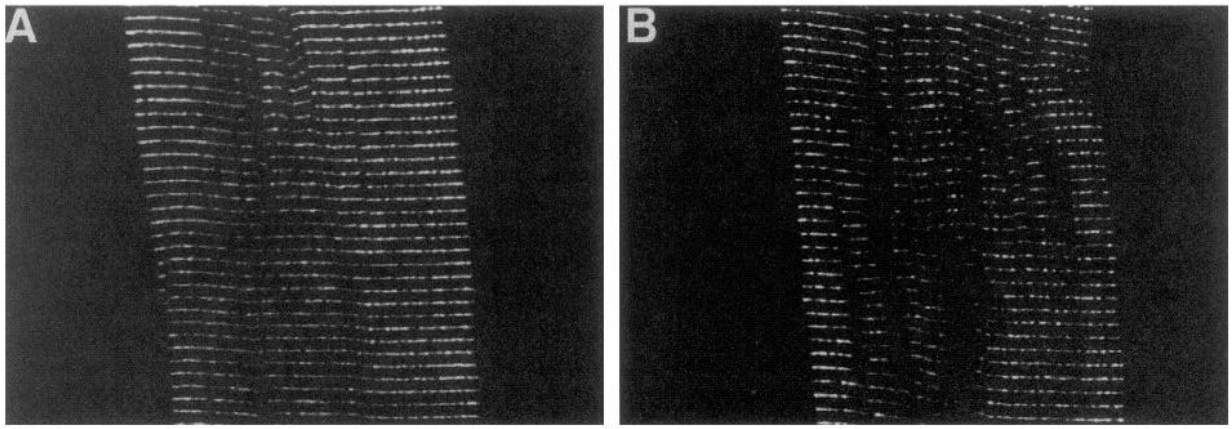

FIG. 3. Two ryanodine binding proteins coexist in same muscle fiber in bullfrog skeletal muscle. Consecutive, longitudinal section of muscle was stained with monoclonal antibodies (monoclonal antibody 32E in *A* and monoclonal antibody 26G in *B*) recognizing specific regions of α- and β-RyR isoforms, or RyR1 and RyR3. [From Olivares et al. (240).]

isoforms, the resolution is not sufficient to detect whether they are actually located within the same triad. Given the approximately equal amounts of the two proteins, and the fact that all triads appear structurally equal in these muscles, it is logical to assume that α and β coexist within the same junction. So far, no evidence for heterotetramers were found by immunoprecipitation studies with specific antibodies (see Ref. 304 for a review). Indeed, two types of channels with distinct properties are present in muscles that express the two isoforms (see sect. II*B3*), and this would not happen if the two isoforms were freely mixing.

Interestingly, although most muscles in fish, reptiles, and birds express RyR1 and RyR3, major groups of reptiles and some fast muscles in fish have a single isoform, the α or RyR1 (22; Fig. 4). The muscle-derived heater tissue has a single isoform, predicting a fast muscle origin for these cells (23).

Mammalian skeletal muscles also contain a second (RyR3) isoform in addition to the dominating RyR1 type, but at a 20- to 50-fold less concentration (104, 168, 315; see Ref. 297 for a review). It is not clear whether the two isoforms are present in the same muscle fibers, but location of both in the triadic microsomal fraction has been shown (58). For the moment, we assume that the two isoforms are intercalated in the junctions. However, it is clearly not excluded that RyR3 is segregated over some small regions of the SR. Indeed, a small percentage of RyRs are located at a small distance from the junctional face in mammalian muscle, although within the triad (68). The relative amounts of RyR3 vary widely between mammalian skeletal muscles, being higher in diaphragm and soleus and lower in the abdominal and tibialis anterior muscles of a variety of mammals (58). Interestingly, the fast-twitch extensor digitorum longus muscle has no

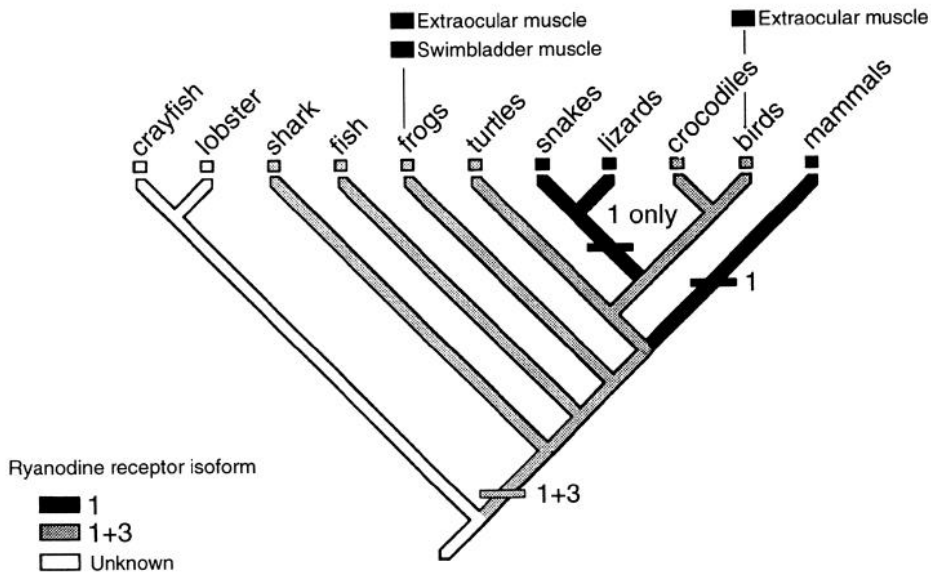

FIG. 4. Phylogenetic distribution of RyR expression patterns in skeletal muscle. Muscles with a predominant expression of RyR1 (or α) are present in more advanced phyla, but also in some very fast-acting muscles of fish. Isoform distribution in invertebrates has not been explored. (Courtesy of B. A. Block.)

RyR3, and this compares with the fact that in fish the fast muscles lack RyR3 (233). Immunologic cross-reactivities and mobility in sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels indicate more interspecies variations in the RyR1 than in the RyR3 isoforms. The latter indeed seems to be very highly conserved (222).

So far, only one type of RyR has been detected in muscles of invertebrates (91, 239, 280). The invertebrate RyR has properties closer to RyR2 and RyR3 than RyR1.

3. *Specific properties of ryanodine receptor isoforms*

Both RyR1 and RyR2 can be isolated in large quantities from skeletal and cardiac muscle, thus allowing studies of their individual and ensemble properties and a rigorous definition of their characteristics. Properties of RyR2 from different species are more similar to each other than those of RyR1 and RyR2 from the same species, indicating that general channel behavior is truly significant in the function of skeletal and cardiac muscle (see Ref. 304 for a review).

The RyR2 channels are more sensitive to activation by micromolar Ca^{2+} and less sensitive to inactivation by millimolar Ca^{2+} than RyR1 (54, 166, 209, 211, 258), and this also results in differential effect of Ca^{2+} on ryanodine binding to the two channels (see Ref. 206 for a review). Skeletal muscle channels, on the other hand, are more effectively activated by adenine nucleotides and more readily inhibited by Mg^{2+} (204, 207, 209). Responses to pharmacological agents also differ, with RyR1 being more easily blocked by ruthenium red whereas RyR2 is more readily activated by danorubicin and caffeine (211, 352). However, caffeine at low concentration activates Ca^{2+}-induced Ca^{2+} release (CICR) in all three types of RyRs (see Ref. 59 for a review). Overall, the cardiac channel is more readily activated and more reluctant to close than skeletal muscle. It is likely that RyR isolated from the brain (202) is RyR2, due to the low content of RyR1 and RyR3, relative to the RyR2 in this organ. It is interesting, however, that the channel purified from brain seems to have unusual properties, such as the requirement for caffeine and the single effect of ryanodine, which acts only as a blocker (202). Single channels mildly sensitive to $InsP_3$ have also been detected (11). These are not types of behavior that one would expect from a cardiac-type channel.

Because RyR3s are more highly expressed in some skeletal muscles than in the brain, most of their properties are deduced from muscle studies. Differences between RyR3 and RyR1 can be indirectly detected in studies of single-channel properties of muscle fibers that contain the α- and β-isoforms. Avian and amphibian skeletal muscles have two distinct sets of Ca^{2+} release channels that differ in Ca^{2+} sensitivity (31, 221, 252). One type, presumably the α or RyR1, has properties closely related to those of the rabbit RyR1; it is activated by relatively high Ca^{2+} concentrations and is inhibited at higher Ca^{2+} concentrations. The other type, presumably the β or RyR3, has a wider sensitivity to Ca^{2+}. Thus β or RyR3 channels of skeletal muscle are functionally more similar to RyR2 than RyR1, i.e., α- and β-RyRs in fish also differ in their Ca^{2+} sensitivities, indicating a similarity between β-RyR and RyR3 in these animals (234; see Ref. 22 for a review) (Fig. 5). Excitation-contraction coupling in *cn/cn* chick embryos, which lack RyR1 as a result of a null mutation but express low levels of RyR3, is dependent on extracellular Ca^{2+}, confirming the RyR1 is needed for skeletal type e-c coupling (133). An initial characterization of RyR3 isolated from brain, taking advantage of its cross-reactivity for antibodies against the skeletal β-isoform, indicates properties similar to those of the muscle RyR3 isoform (224).

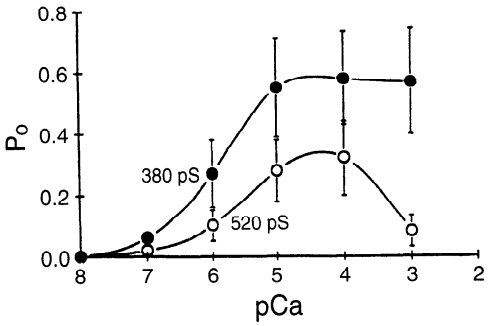

FIG. 5. Open probability (P_o) of RyR channels from toadfish white swimming muscle shows 2 types of channels with different conductance (520 and 380 pS) and Ca^{2+} dependence, as expected from a muscle containing both α- and β-RyR isoforms. Comparison with channels from fast extraocular muscles, containing only α-isoform, identifies this as a channel that has a higher conductance and a narrower bell-shaped response to Ca^{2+}. α-Isoform in fish has been identified with RyR1. [From O'Brien et al. (234).]

However, some recent data do not agree with the assessment of the differences between skeletal α- and β-isoforms given above. In bullfrog skeletal muscle, the two types of RyRs seem to have similar Ca^{2+} dependence, although sensitivity of the β-isoform was increased in the presence of 1 M NaCl (223). In dyspedic myotubes from mice carrying a null mutation of the gene for RyR1 (315), RyR3 channels respond to caffeine, Ca^{2+}, and adenine nucleotides, but contrary to the fish RyR3 (234), they have a much lower sensitivity to Ca^{2+} than RyR1. One possibility for the difference is that mouse muscle RyR3 were tested in situ, where their properties may be affected by intrinsic modulators.

4. *Why skeletal muscle has two ryanodine receptor isoforms*

What is the meaning of the coexistence of two RyR isoforms in approximately equal amounts in some skeletal muscle fibers? The phylogenetic distribution of RyR isoforms in vertebrates indicates that this condition is "primitive," whereas a dominant isoform is present in more ad-

vanced organisms and/or in the fast-acting muscles of some of the lower vertebrates (233; see Ref. 22 for a review) (Fig. 4). The RyR1 isoform is a channel readily inactivated by Ca^{2+} and thus more likely to turn itself off during a cycle of activity, whereas RyR3, with its extended sensitivity to Ca^{2+}, may be more readily activated and more capable of a sustained activity. On the basis of these observations and on the different sensitivity to Ca^{2+} of the α- and β-isoforms, it is proposed that the RyR1 isoform evolved to allow rapid cycles of muscle activity (234). It will be interesting to see whether a continued exploration of functional characteristics and phylogenetic relationships of RyR channels in invertebrate muscles dedicated to rapid activity will confirm this intriguing hypothesis.

C. Sequence and Primary Structure of Ryanodine Receptors

Primary sequences of the three known RyR isoforms predict molecules of ~5,000 amino acids and ~560 kDa (112, 228, 244, 313, 326, 353). This is slightly larger than the mass of the peptides constituting the purified molecule (127, 130, 131, 160), and definitely larger that the doublet of ~350 kDa that was obtained in early studies (148). The latter is clearly due to proteolysis, to which the molecule is particularly prone.

Hydropathy plots indicate a large hydrophilic NH_2-terminal region, thought to constitute the cytoplasmic domain of the molecule or "foot" and a smaller, mostly hydrophobic COOH-terminal region, predicted to form the intramembrane channel. This is in good agreement with the general structure of the molecule. The membrane-crossing region is the most highly conserved domain of the molecule (112, 228, 244, 313, 326, 353) and has strong similarity with the same region of the $InsP_3$ receptor (100, 214). In the foot region, four repeated motifs of ~120 residues, occurring in 2 tandem pairs, are located in corresponding positions in the 3 isoforms (112, 228, 244, 353). These four stretches are missing in the $InsP_3$ receptor, suggesting that their insertion (followed by duplication) in the RyR sequence contributed to the evolutionary divergence between the two molecules.

There is some disagreement on the number of membrane crossings of the COOH-terminal region of the molecule. *Model 1* (313) predicts a molecule with 4 intramembrane domains of ~20 amino acids each (M1-M4) located in the COOH-terminal "tenth" with the M1 between amino acids 4564 and 4580. *Model 2* (324, 353) proposes 10 membrane crossings in the COOH-terminal "fifth" (M1-M10 between amino acids 3978 and 4932) and 2 additional ones in the middle of the molecule (M′ and M″, respectively, at amino acids 3123–3143 and 3187–3205). *Model 3* (24) proposes six membrane crossings in the COOH-terminal regions and four more in the foot region, but at locations different from *model 2*. *Model 4* (326) supports the presence of at least six membrane-spanning regions, based on the alignment of the human RyR2 and on the cytoplasmic position of the epitope to a monoclonal antibody. All models generally agree on the position of the most hydrophobic domains closest to the COOH-terminal.

Predictions of the three models have been put to the test, and the results are more often in favor of *model 1*. Experimental evidence supports the commonly predicted cytoplasmic location of NH_2- and COOH-terminals (105, 192). Thus the molecule requires an even number of membrane crossings.

Extensive proteolysis of the isolated RyR shows numerous cleavage sites in the proposed foot region of the molecule, whereas the highly hydrophobic region of the molecule constitutes a large fragment (46, 55, 187). The M1-M2 and M3-M4 loops of *model 1* contain several negatively charged amino acids (187), consistent with their proposed luminal location in that model. It is suggested, however, that the M1 segment of *model 1* may need to be five amino acids longer (4559–4580) to fully cross the membrane (187). Binding of a hydrophobic probe after calpain hydrolysis confirms that the majority of the transmembrane segments are located in large fragments at the COOH-terminal, but also indicates a weak hydrophobic segment in the middle of the molecule, in partial support of *model 2*.

Antibodies against specific amino acids sequences indicate two luminal regions (amino acids 4581–4640 and 4860–4886) (105). The luminal position at amino acids 4860–4886 discriminates between *models 1* and *2*, favoring the former. In addition, an antibody assigns a luminal location to amino acids 4879–4898, which constitute one of the putative membrane-spanning regions in *model 2*. This also implies that a second putative membrane segment (amino acids 4789–4820) should not exist, since the total number of membrane crossings should be even and also because this crossing would result in a luminal location of Arg-4756, which is a cytoplasmic tryptic cleavage site (34).

D. Ryanodine Receptor Channel: Its Function and Modulation

1. *Agents affecting ryanodine receptor channel activity*

Activity of the SR Ca^{2+} release channel is modulated by a variety of agents (see Refs. 59, 206, 236 for reviews). Calcium in the micromolar range and adenine nucleotides at millimolar concentrations are activators, acting equally to increase the channel open probability and to induce rapid Ca^{2+} release from the SR, whereas Mg^{2+}, also in millimolar concentrations, is an inhibitor (124, 204, 205, 207, 209, 225, 257, 288, 289; see Ref. 84 for a review). These experiments provide a direct link between the activity of

the Ca^{2+} release channel and the role of the SR in rapid Ca^{2+} release. Under appropriate, not necessarily physiological, conditions, Ca^{2+} triggers a sudden, massive release of Ca^{2+} from the SR both in skinned fibers (see Refs. 75, 76 for reviews) and in the isolated SR (204, 238). This CICR is a property of the SR Ca^{2+} channels, as well as of $InsP_3$ receptors.

Several pharmacological agents have become tools for studying the function of RyR channels, and also for locating their presence in cells: caffeine, an activator of the channel; ruthenium red, a blocker; ryanodine and doxorubicin, agents with dual effects.

Caffeine at concentrations in the millimolar range induces muscle contracture and reduces the Ca^{2+} accumulation ability of the SR. The effect is stronger in the heavier SR fraction and is seen as a reduced coupling between ATP hydrolysis and Ca^{2+} accumulation, indicating leaky vesicles (338, 339). Caffeine action on the muscle fiber is also due to its effect on the SR, since caffeine does not change membrane polarization (65) and the drug can act directly on skinned muscle fibers. Indeed, caffeine allows CICR to occur in skinned skeletal fibers even at Mg^{2+} concentrations that would normally inhibit this phenomenon, by acting directly on the Ca^{2+} release channels of the heavy SR (124, 209, 274; see Refs. 75, 76 for reviews). This is confirmed by the Ca^{2+} dependence (272, 273) and ryanodine inhibition of caffeine action (78). However, although there is agreement that caffeine at low Ca^{2+} concentrations increases frequency of open-channel events (242, 272, 273, 287), there is some disagreement on whether the duration of the events is also affected. Figure 6 illustrates the effect of caffeine on the single-channel properties of the cloned expressed RyR, showing a definite increase in mean open time. The effect of caffeine on binding of ryanodine is related to its effect on channel opening (see sect. II*D2*). However, caffeine may have two modes of action (287). At relatively low concentrations (<2 mM), the effect of caffeine is dependent on the presence of Ca^{2+}, and it may simply be due to an increased sensitivity of the Ca^{2+} activation site. At higher caffeine levels, the channels open in the absence of Ca^{2+} and with different kinetics, while maintaining the same permeability, indicating a more direct effect of caffeine.

Ruthenium red is an agent that completely blocks CICR (178, 204, 218, 238, 289) and is often used as a tool to check for RyR-dependent Ca^{2+} leaks from the SR.

Calmodulin is a cytoplasmic, Ca^{2+}-dependent enzyme regulator. Millimolar CaM inhibits Ca^{2+} release from the heavy SR of cardiac and skeletal muscle (209), and it partially reduces single-channel open time without affecting the unitary conductance (291). The effect occurs in the absence of ATP, and thus it is not mediated by channel phosphorylation (98). Recent experiments, however, indicate a more complex response of the channels to CaM and a possible role of this protein in modulating channel activity during contraction. The effect of CaM on the channels is Ca^{2+} concentration dependent; at the concentrations prevalent in a relaxed muscle, CaM increases open probability of the RyR channel and SR Ca^{2+} release by severalfold, whereas at the higher Ca^{2+} concentrations to be expected during muscle activation, it has the opposite effect (32, 106, 325). This dual mode of action is confirmed in skinned muscle fibers, where CaM enhances CICR at low Ca^{2+} concentrations and inhibits it at high concentrations (126). Calmodulin binding to the channel is also Ca^{2+} dependent. It is estimated that at low Ca^{2+} concentration (<0.1 μM), 16 CaMs bind with high affinity to one tetramer, whereas at higher Ca^{2+} concentrations, 4 CaMs bind (325). However, under conditions similar to those during contraction and relaxation, the half time of CaM dissociation is very slow so that during a contraction-relaxation cycle most of the CaM remains bound (325). However, CaM rate of activity is not known, and thus it is not clear whether it may exert its inhibitory modulation during a short contraction. Thus the effect of CaM on possible positive or negative feedback of Ca^{2+} on the RyRs during contraction (see Ref. 277 for a review) cannot be predicted.

In addition to its direct action, CaM may affect channel activity through Ca^{2+}/CaM-dependent protein kinase (CaMKII). Phosphorylation by CaMKII affects channel activity and the effect of CaM on it (111, 117, 119, 175, 291, 335). Opposite effects of phosphorylation reported in these papers may have to do with the site of phosphorylation and/or ionic conditions of experiments. Both cardiac and skeletal channels need to be phosphorylated to be active under physiological Mg^{2+} concentrations (111, 196), but it is not known whether phosphorylation plays a modulatory role during e-c coupling in either muscle. Because phosphorylation of the cardiac channel seems to play a role opposite to the direct action of CaM at high Ca^{2+} concentration, the exact interplay of these effects during muscle activity remains to be ascertained.

Doxorubicin is a widely used chemotherapeutic agent, which can cause a cardiomyopathy, possibly due to sensitization of RyRs to activation by two physiological agents, Ca^{2+} and ATP (254, 256, 356), followed by an actual decline in RyR density (66). Doxorubicin has been used as a high-affinity label for the RyR (354). The effect on the isolated channel is an initial activation, followed by an irreversible inhibition, which occurs with a time delay, but not in a concentration-dependent manner (241). Dithiothreitol protects against the final inhibitory action, indicating importance of sulfhydryl groups for the functional integrity of the channel (241).

2. Complex action of ryanodine

Ryanodine is a neutral plant alkaloid derived from the stem and root of *Ryana speciosa*, a plant native of Trinidad. Its action is complex; in the whole animal and

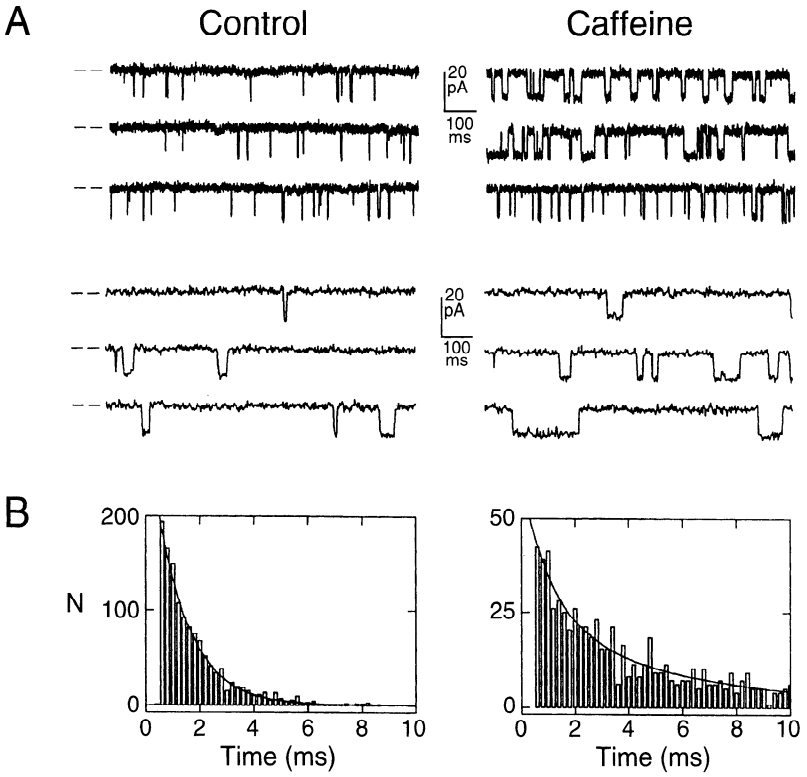

FIG. 6. *A*: single-channel properties of cloned expressed RyR1 (*left*) and its activation by 5 mM caffeine (*right*). Caffeine increases open probability as well as duration of events. *B*: mean open time before caffeine is 1.2 ms (*left*), and after caffeine is 9.0 ms (*right*). [From Ondrias et al. (242).]

when applied to an intact muscle, ryanodine can produce either flaccidity or an intense contracture, whose onset is accelerated by activity (see Ref. 136 for a review). The reason for these apparently contradictory effects is the direct, dual action of ryanodine on the heavy SR (85) and on the SR Ca^{2+} release channels (290). Low and high concentrations of ryanodine have opposite effects on Ca^{2+} retention by the heavy SR; at low concentrations, ryanodine results in Ca^{2+} loss, whereas at higher concentrations, it has a blocking action similar to that of ruthenium red (79, 85, 306, 351). Corresponding to these two actions, heavy SR has high-affinity (85, 163, 258) and low-affinity (198) sites for ryanodine. The above observations, and particularly the high affinity for ryanodine, provided the basis for the identification and purification of the RyR.

At low concentrations (<10 μM), ryanodine locks the channels in a partially open subconductance state (275, 290). This effect is strongly dependent on the channel condition at the time of ryanodine binding. Ligands known to open channels and stimulate Ca^{2+} release from the SR (μM Ca^{2+} or mM ATP, caffeine) stimulate ryanodine binding to the high-affinity site (53; see Ref. 59 for a review). Calcium, which has both activating and inactivating effect on RyRs, also dually affects ryanodine binding (162, 211, 258). This is thought to be due to better accessibility of the high-affinity ryanodine binding site in the open channel. Thus ryanodine binding can be used as a probe of channel conformation and properties (352; see Ref. 208 for a review).

As the concentration of ryanodine is increased, the affinity of RyRs for ryanodine decreases (198, 211, 258). The effect has been described as an allosteric negative interaction between four initially identical binding sites (one on each monomer). The first ryanodine molecule binds with high affinity to the open channel, blocking it into the partially open configuration (see above), and reducing binding to the other sites. Three more binding steps follow, each with increasingly lower affinity, indicating a different conformational state, until one ryanodine per monomer is bound and a long-lived state is reached, in which ryanodine is occluded and the channel is totally blocked (30, 39, 259). The effect occurs equally well after cross-linking with bifunctional reagents, indicating that the decreased ryanodine affinity is the result of interactions within the tetrameric molecule (39). However, the action of some cross-linkers results in a tetramer that is capable of binding ryanodine at high affinity and of occluding it, but has lost the low-affinity sites (284). Pretreatment with 100 mM ryanodine decreases maximum binding of high-affinity sites and induces loss of the low-affinity ones, perhaps by uncoupling the four negatively cooperative binding sites. Oxidation of critical receptor thiols is implicated in the process (351). A closer look at dissociation constants implies the presence of two distinct binding sites, which are allosterically or sterically coupled (336, 337).

3. Ryanodine receptors and inositol 1,4,5-trisphosphate receptors have different pharmacological profiles

Inositol 1,4,5-trisphosphate is a specific activator of a class of widely distributed intracellular Ca^{2+} release

channels closely related to the RyRs (16, 72, 100). The $InsP_3$ receptors are tetramers with a general configuration similar to that of RyRs, including the large cytoplasmic domains (43). Some cells (most notably smooth muscle and Purkinje cells) express both RyRs and $InsP_3$ receptors at high levels, but the two receptors are located in different areas of the cell and with different distributions (150, 334). Most cells have a prevalence of either one or the other of the two Ca^{2+} release channels (see Refs. 17, 122 for reviews).

The pharmacological profiles of RyRs and $InsP_3$ receptors are quite different (70, 71). For example, ruthenium red and relatively high concentrations of ryanodine totally block the RyR channel, but neither has an effect on the $InsP_3$ receptor (72, 247). Caffeine, the facilitator of CICR in the RyR, is an inhibitor of the $InsP_3$ receptor activity (19). Heparin is a competitive inhibitor of $InsP_3$ on the $InsP_3$ receptor (195), but it activates isolated RyRs in a Ca^{2+}-dependent manner (20).

After the observation that $InsP_3$ induces Ca^{2+} release from the isolated SR (329) and in skinned muscle fibers (328), it was proposed that $InsP_3$ may directly affect the SR Ca^{2+} release channels and play a role in e-c coupling. The evidence for and against this hypothesis has been well reviewed (see Ref. 134). The hypothesis, however, has fallen into disfavor, following inability of other laboratories to confirm a definite effect of $InsP_3$, particularly on frog fibers (167) and on single SR channels (72). However, large-conductance SR channels (presumably RyRs) of Chilean frogs seem to respond quite readily to $InsP_3$ (302). Heparin microinjected into single intact skeletal muscle fibers has no effect on depolarization-activated contractions (249), but it does block $InsP_3$-induced Ca^{2+} release in smooth muscle (156), a tissue rich in $InsP_3$ receptors (195). Some effect of heparin on e-c coupling may depend on its action on the t-tubule DHPR, rather than on the RyR (165).

E. Developmental Regulation

Ryanodine receptors are expressed early during muscle differentiation, and they are regulated by growth factors, similar to myofibrillar proteins (4, 189, 188). Ryanodine receptors are detected in a tetrameric form as early as embryonal *day 4* (E4) in chick myocardium (69), and feet are visible as early as E2.5 by electron microscopy (263).

Differential regulation of α- and β-RyR isoforms in skeletal muscle has been shown in the chicken (305). The α-isoform is expressed first in breast muscle, and it is the only form detected between E10 and E15. Around E15, the β-isoform first appears, and both are then synthesized at increasingly higher levels while the fibers mature. Note that the appearance of the β-isoform coincides approximately with the transition between myotubes and muscle fibers, when t tubules suddenly differentiate and the amount of jSR increases rapidly (309). This developmental regulation is important in understanding the phenotype of the crooked neck dwarf (*cn*) mutation in the chicken. *Cn/cn* is an autosomal recessive mutation, resulting in severe musculoskeletal developmental defects and eventual muscle degeneration (194). The mutation has been traced to a failure to make normal α-RyR (see Ref. 304 for a review). This in turn affects the synthesis of the β-isoform, which appears somewhat later than usual and at lower densities (3). Interestingly, the β-isoform is expressed at approximately normal levels in cultured *cn/cn* myotubes (6). This mutation offers the unique opportunity of finding out whether the β-isoform alone can substitute for the α/β-combination in the formation of junctions between SR and external membranes.

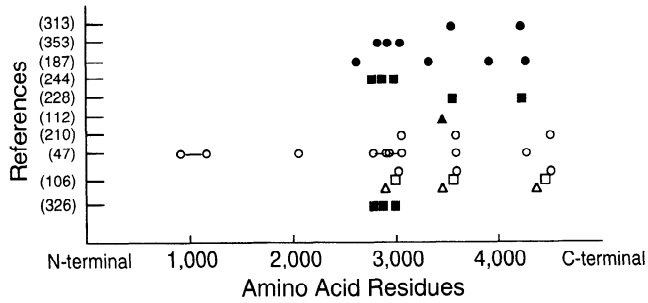

FIG. 7. Locations of proposed (solid symbols) and experimentally demonstrated (open symbols) calmodulin (CaM) binding sites. Circles, RyR1; squares, RyR2; triangles, RyR3. Consensus has been reached on general location of 3 CaM binding sites (CaM1-CaM3), located in region preceding most hydrophobic domain of molecule, which starts at amino acid 4564 for RyR1. Some disagreement exists on details, and also on total number of binding sites.

F. Functional Domains

Potential binding sites for solutes that modulate the RyR channel, such as CaM, Ca^{2+}, ruthenium red, ATP, and ryanodine, have been postulated on the basis of the primary structure and experimentally probed.

In the case of CaM, some agreement between predicted and demonstrated binding sites has been reached (Fig. 7). Three most likely CaM-binding stretches of the RyR have been proposed and experimentally identified in the hydrophilic region of the molecule (32, 47, 106, 112, 187, 210, 228, 244, 313, 353). However, there is no agreement on the total number of CaM binding sites; up to six per monomer have been proposed (47, 347), and four have been experimentally confirmed at low Ca^{2+} concentrations (325).

The first two CaM binding sites, CaM1 and CaM2, are located next to M′ and M″, the two putative membrane-spanning regions in the middle of the molecule (see *model 2*). This proximity of CaM1 and CaM2 to transmembrane regions is model dependent. A third CaM binding site,

CaM3, is in proximity of the highly hydrophobic region of the molecule, which seems to be very important in the modulation of the channel activity (see also the location of Ca^{2+}, ryanodine, and ATP binding sites, Figs. 8 and 9). The location of CaM3 implies that the effect of CaM on the channel activity may involve its action on putative transmembrane segments. Proximity of CaM and Ca^{2+} binding sites also implies that these regions are important for the control of channel activity.

Calcium binding sites span a long region of the primary structure (Fig. 8). Three possible high-affinity Ca^{2+} binding sites per monomer were proposed on the base of E-F hand-related sequences in the molecule in the region just preceding M1 in *model 1*, amino acids 4253–4499 (313), but disclaimed by other investigators (353; Fig. 8). A possible low-affinity site at amino acids 1872–1923 for RyR1 (353), two others between amino acids 1336 and 2021 for RyR2 (228), and one at amino acids 3934–3945 for RyR3 (112) have been proposed. Putative Ca^{2+} binding sites have been functionally probed with peptide-derived antibodies. A small decrease in CICR was found with an antibody against a general region (amino acids 4380–4625) (83, 324). A stronger and more specific effect was found by carefully focusing on shorter segments, and a short stretch (amino acids 4489–4499) was identified by the strong effect of its antibody on Ca^{2+} and caffeine sensitivity of the channel (49, 50). This stretch coincides exactly with one of the predicted E-F handlike segments (313). The channel-antibody complex is still responsive to ruthenium red, ryanodine, Mg^{2+}, and ATP, indicating that the sites of action of these compounds are not identical to the Ca^{2+} binding site. This, however, may be incompatible with the Mg^{2+} and Ca^{2+} competition at the activation site (see Ref. 206). Interestingly, this stretch contains a proline-glutamate repeat sequence only in skeletal muscle and constitutes one of the sites of variability between RyR1 and RyR2 (326).

The additional Ca^{2+} binding sites, which also bind ruthenium red, were localized between residues 1861–2094 and 3657–3776 (47). Two Ca^{2+} binding sites are closely related to consensus CaM binding sites CaM2 and CaM3, and one is closely related to an experimentally demonstrated CaM binding site (compare Figs. 7 and 8).

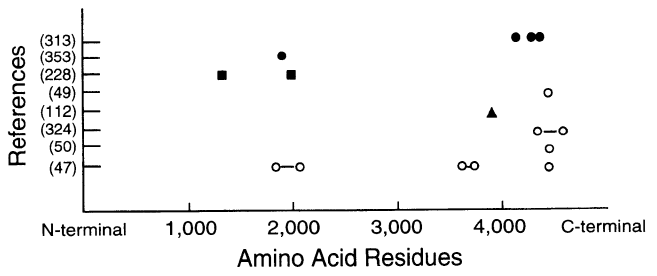

FIG. 8. Locations of proposed (solid symbols) and experimentally demonstrated (open symbols) Ca^{2+} binding sites. Symbols are as in Fig. 7. Site closest to COOH-terminal has been proposed on basis of some similarity to an E-F hand and thus is the most likely candidate for a high-affinity site involved in Ca^{2+}-induced Ca^{2+} release (CICR). Indeed, this is site recognized by antibodies that affect CICR.

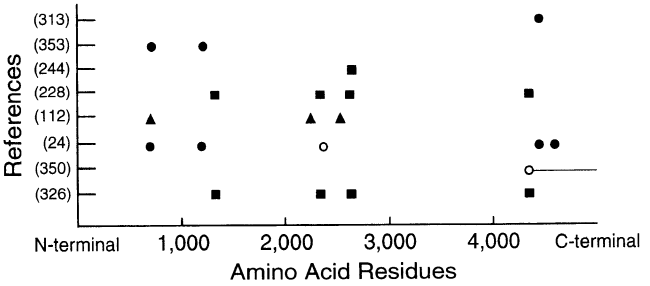

FIG. 9. Locations of proposed (solid symbols) and experimentally demonstrated (open symbols) ATP binding sites. Symbols are as in Fig. 7. Although there is some agreement on proposed sites, experimental confirmation is still scarse. Comparison with Figs. 7 and 8 clearly emphasizes a major modulatory site between residues 3614 and 4457 (47, 313).

Predictions of ATP binding sites (Fig. 9) cluster in three areas of the molecule, but little consensus exists on the actual location (24, 112, 228, 244, 313, 353). Experimentally, an ATP binding site is found in the COOH-terminal 76-kDa tryptic fragment (350).

Not much is known about the location of the ryanodine-binding sites. With one exception (46), there is general agreement that ryanodine binds to the 76-kDa COOH-terminal portion of the molecule (266, 285, 342). More specifically, both high- and low-affinity binding sites were found in the 14S region obtained by trypsin digestion of the receptor, which is contained within the 76-kDa fragment, constituting the COOH-terminal region of the molecule, after Arg-4475 (343). This stretch of the molecule contains the strongly hydrophobic region and the "modulator-binding region" immediately preceding it. Strong tryptic digestion of SR vesicles results in complete loss of ryanodine binding, without affecting Ca^{2+} accumulation activity (285), indicating a location close to the channel but not within a membrane-spanning segment. The binding site for neomycin, a potent inhibitor of skeletal muscle SR Ca^{2+} release, is also in the 14S fragment (337).

In cardiac RyR, a phosphorylation site was detected in correspondence to amino acid Ser-2809 (342), as predicted (244). The skeletal isoform is not significantly phosphorylated at that site. In fact, the sequence surrounding residue Ser-2809 in the skeletal muscle receptor is quite different from the cardiac sequence so that antibodies raised against the cardiac amino acid 2805–2819 sequence do not react with the skeletal isoform (342). The phosphorylation of the partially purified RyR activates the channel, suggesting that the phosphorylation by CaMKII may represent an important mechanism for regulating intracellular Ca^{2+} release in heart and brain. It is not clear, however, if the activation of the channel requires the phosphorylation of just one or all four RyR subunits (342).

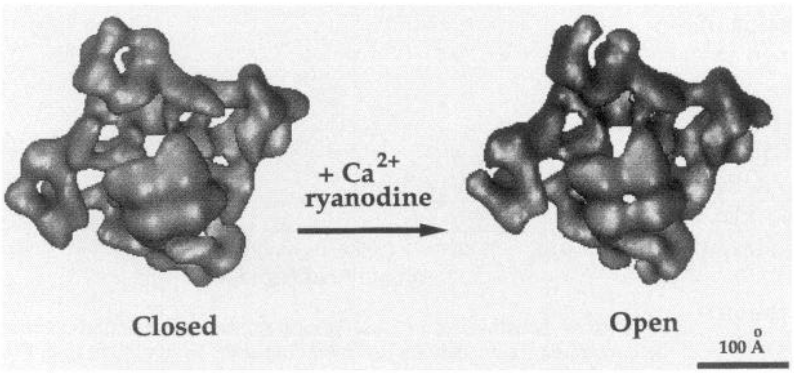

FIG. 10. Closed and open states of RyR are visualized after 3-dimensional reconstruction from electron micrographs of frozen hydrated molecules. In presence of Ca^{2+} and ryanodine, open channel shows a pore that is not seen in closed state. Also, 4 corners of molecule are slightly rearranged, indicating long-range interactions within molecule. [Modified from Orlova et al. (243).]

G. Three-Dimensional Structure of Ryanodine Receptors

Isolated RyRs are appropriate objects for single particle computer imaging techniques that allow complete three-dimensional structural reconstructions. This was initially done on negatively stained RyRs (332) and then on frozen hydrated molecules, thus avoiding drying artifacts (243, 264, 265, 281, 331, 333; Fig. 10). Two powerful approaches, conical tilt and angular reconstruction, have been used to obtain three-dimensional images at a fairly high level of resolution (~3 nm), which provide excellent details of the cytoplasmic domains and some features of the intramembrane region (243, 264, 265, 281, 330; see Ref. 333 for a review). The cytoplasmic quatrefoils are highly hydrated structures, with numerous canals and pathways cursing between loosely assembled protein domains. The molecule has the architecture of a scaffolding, providing a mechanical linkage between SR and surface membranes, while allowing excellent opportunity for flow of solutes (Ca^{2+}). Radially arranged canals might provide direct routes for Ca^{2+} exiting from the central channel to the periphery of the molecule. Protein domains located in proximity of the corners of the molecule are the farthest, in the *Z* direction, from the SR membranes and thus are most likely to interact with external membrane proteins (264). Interestingly, this region of the molecule is at a considerable distance from the channel region, suggesting that molecular interactions between external membranes and the channels may require long-range events (332). The four corners of the molecule have a complex outline with several grooves that seem appropriate for interdigitating with neighboring molecules, an interaction presumably needed for grouping of RyRs into the ordered arrays, as seen in situ (see Ref. 94 for a review). The height of the cytoplasmic domain, ~12 nm, is sufficient to span the distance between SR and exterior membranes, thus establishing direct physical continuity between the two.

The intramembrane domain is clearly delineated from the cytoplasmic assembly but shows fewer details. The proposed intramembrane domain is ~7 nm tall, and it is expected to have a small luminal region. It is estimated that the 12 membrane crossings proposed in *model 2* of the RyR (324) would fit into the observed membrane domain. A solvent-filled channel at the end farthest from the cytoplasmic domain is thought to represent the luminal mouth of the channel pore (264).

Low concentrations of ryanodine in the presence of Ca^{2+} block the channel in a partially open configuration. Differences between the resting and partially open channels embedded in ice have been detected (243; Fig. 10). The open channel has a visible opening on the luminal side of the intramembrane assembly, where none was visible in the closed configuration. In addition, the membrane assembly is rotated by 4° in relation to the cytoplasmic domain, and the four corners of the cytoplasmic domain farthest from the channel are slightly modified (243). This is a further indication of long-range molecular interactions between the cytoplasmic assembly and the channel domain of RyRs.

The first step in assigning the position of functional sites to the quaternary structure has been taken with the localization of a CaM binding site using labeling with small gold clusters (330). The gold clusters are located near the corners of the cytoplasmic assembly, at a position that is at considerable distance both in the *X-Y* and *Z* directions from the channel region of the molecule. Because CaM affects channel activity, these results also indicate that distant portions of the molecule may interact with each other. Unfortunately, it is not at the moment possible to know which of the CaM binding sites were occupied in the structural experiments.

Finally, the power of these imaging techniques is demonstrated by visualizing the position of the small FKBP12 protein, which has a stabilizing action on the channel (331; see sect. IIIA).

III. PROTEIN-PROTEIN INTERACTIONS OF RYANODINE RECEPTORS

A. FKBP12: a Stabilizing Factor

The channel behavior of isolated RyRs is not always consistent with the behavior of the in situ Ca^{2+} release

channel. Ryanodine receptors purified from rabbit muscle (121, 160, 173) or cloned and expressed in nonmuscle cells (48) may show up to four equal subconductance states. In the early observations it was questioned whether this may be the result of the isolation procedure inducing some denaturation of the molecule. It is likely, instead, that this behavior is due to the loss of a small molecule associated with the foot, the FK506 binding protein or FKBP12. FKBP12, a ~12-kDa protein, is a member of the immunophilin family, ubiquitously expressed proteins that act as cytosolic repressors for immunosuppressing drugs (FK506, rapamycin). FKBP12 is associated with the RyR (57), with which it copurifies coimmunoprecipitates, and colocalizes in the jSR (135). This tight binding has been used to devise a new purification procedure for RyRs (344). The molar ratio of FKBP to RyR is 1:1, i.e., one FKBP protein is bound to each of the four subunits of the RyR-channel complex. A cardiac-specific form of FKBP12, with a slightly higher molecular weight, is associated with RyR2 in cardiac muscle (164, 321).

FKBP12 can be dissociated from the RyR and reconstituted by titration with FK506 (322, 323). In the absence of FKBP12, the channels show an increased open probability and extended opening events at subconductance levels, resulting in increased mean ensemble current, leaky vesicles, and increased sensitivity to caffeine (2, 322), while maintaining their basic pharmacological properties (51, 197). All this indicates a stabilizing effect of FKBP12 on the channel. A key confirmation came from the expression of RyRs in Sf9 cells (28). The purified FKBP12-free channels readily open in response to caffeine, showing five discrete conductance levels: fully open, three-quarters open, one-half open, one-quarter open, and closed (Fig. 11). Coexpression of FKBP12, on the other hand, results in channels that are either fully open or fully closed, and the mean duration of fully open events (75 ms) is longer than in the FKBP12-free channel (4.4 ms). Sensitivity to caffeine is higher for the FKBP12-free channels, indicating that FKBP12 also regulates the closed state of the channel. Basically, in the absence of FKBP12, the channel is more easily opened, but it fails to stabilize in the fully open configuration. FKBP12, and its absence, helps to show that the four subunits, under experimental conditions, can behave semi-independently, while confirming that all four subunits contribute to the normal function of the channel, strongly supporting the evidence derived from ryanodine-binding studies.

A second action of FKBP12 is to make the channel into a rectifier, favoring lumen to cytoplasm currents and blocking the reverse (51, 180). The $InsP_3$ receptors are also associated with FKBP12 and are stabilized by its presence (35).

Rapamycin, a drug that inhibits prolyl isomerase activity of FKBP and dissociates it from cardiac RyR2, affects properties of these channels at the submicromolar

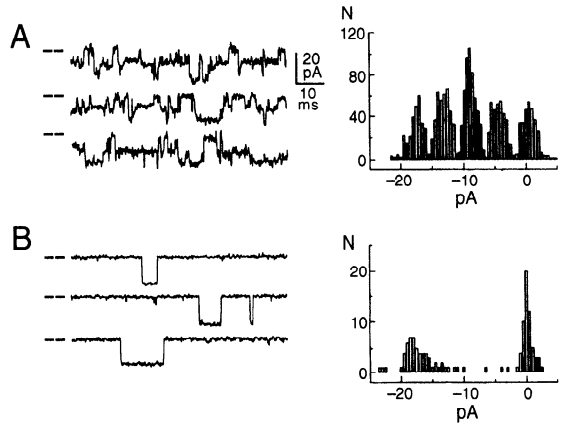

FIG. 11. Effect of FKBP12 on single-channel properties of purified recombinant RyR1, after activation by 5 mM caffeine. *A*: in absence of FKBP12, open events are frequent and fairly prolonged, but of variable amplitude (*left*). Amplitude histograms show 5 discrete conductance levels, from fully closed to fully open (*right*). *B*: after addition of recombinant FKBP12, channels show only 2 conductance levels (*left*), which correspond to closed and fully open configurations (*right*). Note that duration of fully open events is longer in RyR-FKBP12 complex. [From Brillantes et al. (28).]

level (145). Interestingly, the observed increase in open probability occurs earlier than a decrease in current amplitude, suggesting either two different actions of rapamycin or the effect of the gradual stripping of FKBP from the channels. Given the low concentration at which rapamycin affects RyR2, the question is whether immunosuppressant drugs may act on the RyR-regulated Ca^{2+} release of other cells.

A novel compound derived from a sponge (Bastadin 5, from *Ianthella basta*) stabilizes the channel in its high-affinity configuration for ryanodine, without affecting affinity of the activation site for Ca^{2+} or the response to caffeine (182). The effect of Bastadin is antagonized by FK506, and Bastadin enhances FKBP12 dissociation in the presence of FK506. This agent promises to reveal a novel modulatory site on FKBP12.

The location of FKBP12, directly visualized by cryoelectron microscopy, is just outside the boundary of the cytoplasmic domain of RyR, at a distance of ~10 nm from the transmembrane domain forming the channel (331).

B. Interactions of Ryanodine Receptors With Other Junctional Sarcoplasmic Reticulum Proteins

1. *Triadin*

Ryanodine receptors seem to be associated with DHPRs (Ca^{2+} channels of exterior membranes; see sect. IV*B*) on the cytoplasmic side and with calsequestrin (a Ca^{2+} binding protein; see sect. III*B3*) on the luminal side of the SR. In the search for proteins that may be involved in these interactions, a protein of 95 kDa, named triadin

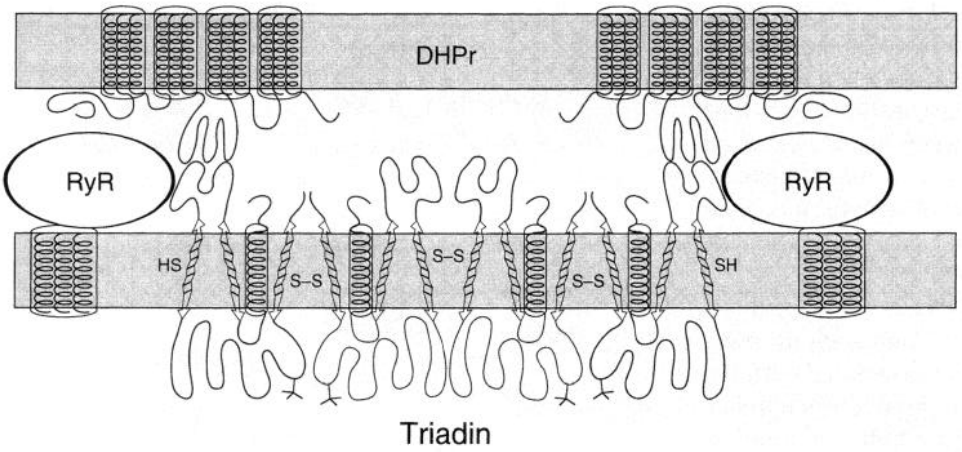

FIG. 12. Model for membrane topology of triadin and for its interactions with RyR and dihydropyridine receptor (DHPR). In this model, triadin crosses SR membrane 4 times (1 as an α-helix and 3 as a β-sheet). Interaction sites with RyR and with II-III loop of α_1-DHPR and S-S bonds creating triadin oligomers are indicated. NH_2- and COOH-terminals are cytoplasmic. [From Fan et al. (80).]

(40), has been closely examined. Triadin is greatly enriched in the jSR (27) and is present in skeletal as well as cardiac SR (26). This unusual intrinsic protein of the jSR tends to form large multimers through luminal disulfide bonds. For this reason, triadin does not dissolve well in a mild detergent (CHAPS) but is solubilized after reduction to the monomer by 2-mercaptoethanol (40). Triadin is a fairly abundant protein (1:1 molar ratio with RyRs), located in the jSR and absent from the longitudinal SR (26, 38, 87, 108, 110, 155). Three triadin isoforms have been detected in both skeletal and cardiac muscles, and the molecule is also present in other tissues (110, 250). The NH_2-terminals of different triadins have significant homology, but the COOH-terminals are quite different (250). Like RyRs, triadin is a substrate for CaM-dependent kinase (63).

Interest in triadin comes from the fact that it interacts with other junctional proteins, but a consensus on which of these interactions is significant has not been reached. Triadin was first found to form a complex with DHPRs (27) and then to bind to RyRs in overlay experiments (151). This led to the proposal that triadin might mediate the interaction between RyRs and DHPRs, thus allowing the two proteins to be linked within the junction despite their relatively low affinity (40, 152; Fig. 12). Data in support of this hypothesis are *1*) the immunoprecipitation of triadin with monoclonal antibodies for DHPR and RyRs (220); *2*) the effects of antitriadin antibodies on Ca^{2+} release from the SR (25); *3*) the positions of the intermolecular disulfide bonds, of binding sites for DHPRs; and *4*) the cytoplasmic location of tryptic and fusion peptides epitopes for various monoclonal antibodies (80, 81). Important is the observation that a cytoplasmic (II-III) loop of α_1-DHPRs, apparently critical in its functional interaction with RyRs, binds to various triadin fusion peptides (81). In a model accounting for these results, triadin repeatedly crosses the jSR membrane and cross-links to itself on the luminal side, whereas one of its cytoplasmic loops reaches out to connect DHPRs and RYRs on the cytoplasmic side (80, 81; Fig. 12).

A different interpretation of triadin location in the junctional membrane is based on its primary sequence and hydropathy plot (155). From these data the molecule is predicted to have a short cytoplasmic NH_2-terminal (47 amino acids), a single spanning membrane region (21 amino acids), and a long, highly charged COOH-terminal tail on the luminal side (109, 155), although some other short membrane-spanning segments may exist (80, 81; Fig. 13). The positively charged residues are considered optimal for a possible interaction with calsequestrin. The hypothesis is supported by the Ca^{2+} dependence of calsequestrin binding to affinity columns based on triadin fusion proteins for the luminal domain of the molecule (107). Conserved putative luminal regions of cardiac triadin also bind calsequestrin (110). In addition, the NH_2- and COOH-terminals of triadin are on opposite sides of the SR membrane (190), as predicted by Reference 155. The sequence homology observed between cardiac and skeletal triadin isoforms in the NH_2-terminal segment and in some portions of the COOH-terminal end are used to support the hypothesis that this molecule does not carry a skeletal muscle-specific function, as confirmed by binding of cardiac calsequestrin to skeletal triadin. This would imply that triadin does not participate in the DHPR-RyR link, since the link seems to exist in skeletal but not in cardiac muscle (107; see also sect. IV*C*).

There is general agreement that triadin binds to the RyR (40, 107, 110, 152), although two different interaction sites, one luminal and one cytoplasmic, are predicted. If the interaction is luminal, then the numerous positive charges of triadin (155) and the negative charges of two luminal RyR loops (M1-M2 and M3-M4) may acquire a significance (187). The interaction between these two molecules may depend on the state of activity of the channel and on the redox state of hyperactive sulfhydryl groups (171, 172).

2. Junctin

Junctin was first identified as a 26-kDa calsequestrin binding protein in cardiac and skeletal muscle (217). The

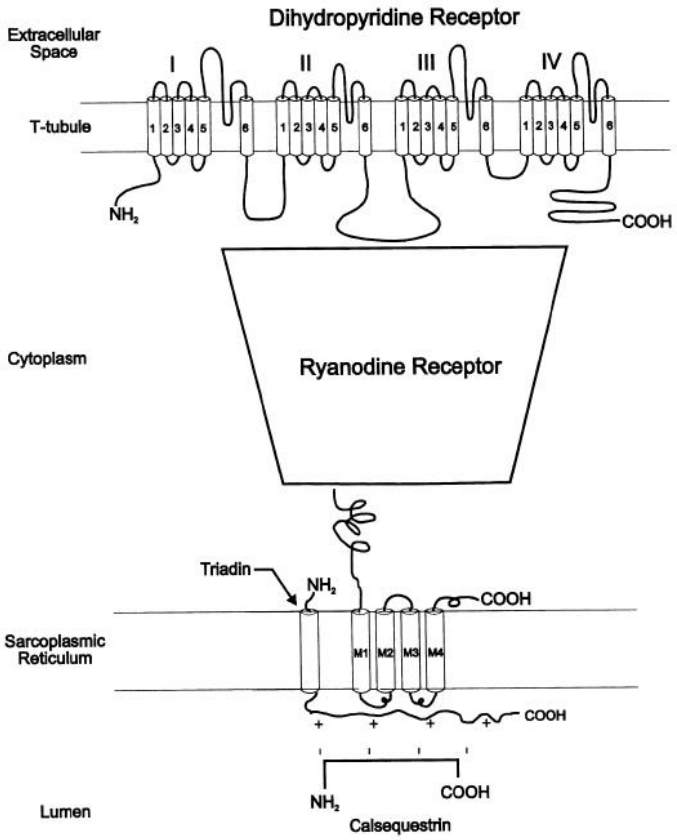

FIG. 13. Alternative model for relationship between triadin and other junctional proteins, proposing that triadin has a single transmembrane region, a short (47 amino acids) cytoplasmic NH_2-terminal, and a large positively charged luminal COOH-terminal region. The latter would interact with negatively charged calsequestrin and with a luminal loop of RyR. Note that RyR and its interaction with DHPR are illustrated essentially as in Reference 313. [From Guo et al. (109).]

protein is enriched in the jSR, and it binds ^{125}I-labeled calsequestrin intensely, particularly in the absence of Ca^{2+} (137). The predicted structure from the 210-amino acid sequence indicates a protein with a short cytoplasmic NH_2-terminal domain and a highly charged luminal domain. Junctin has sufficient homology with the transmembrane region of triadin and aspartyl β-hydroxylase to indicate belonging to the same family. Immunofluorescence indicates localization of junctin at the junctions of cardiac and skeletal muscle (hence the name), and it is proposed that the protein may be responsible for anchoring calsequestrin to the jSR membrane (137). It was not shown if junctin, like triadin, binds to RyRs. However, junctin is absent from corbular SR in cardiac muscle, even though this compartment contains both calsequestrin and RyRs (138).

3. *Calsequestrin*

Calsequestrin is an acidic luminal protein of the jSR (139–142, 199, 203) named for its high capacity for Ca^{2+} binding (see Ref. 183 for a review). The major function of calsequestrin and of its nonmuscle cell homolog, calreticulin, is to increase the endoplasmic reticulum-SR total capacity for Ca^{2+} (212). Although calsequestrin is an entirely luminal protein, it is clearly anchored to the jSR membrane (62, 95, 296; Fig. 14). This ensures the location of calsequestrin near the Ca^{2+} release channels, both in skeletal and cardiac muscle. However, association of calsequestrin to the junctional face membrane may be more structurally complex and have deeper functional implications than just a convenient proximity. In isolated heavy SR, calsequestrin and feet tend to cluster on the same side of the vesicles (29, 36). In addition, RyRs and calsequestrin remain associated with each other, and with other minor components of the junction, under conditions that extract other intrinsic proteins of the SR, such as the Ca^{2+}-ATPase (41, 60). This has led to the hypothesis that some relationship exists between calsequestrin and RyRs. The positively charged segments of triadin and junctin are now suggested as possible intermediaries for this association.

A functional interaction between calsequestrin and other proteins of the jSR with some role in Ca^{2+} release has been proposed. The fluorescence intensity of conformational probes associated with junctional face membrane proteins, but not with calsequestrin, vary with the Ca^{2+} concentration in a manner that is dependent on the presence of calsequestrin (125). In addition, a transient increase in free luminal Ca^{2+} concentration is observed when Ca^{2+} release is triggered by caffeine in isolated SR vesicles containing calsequestrin but not in those where calsequestrin was extracted (123), as if the activity of RyRs affects calsequestrin affinity for Ca^{2+}. Finally, a loss

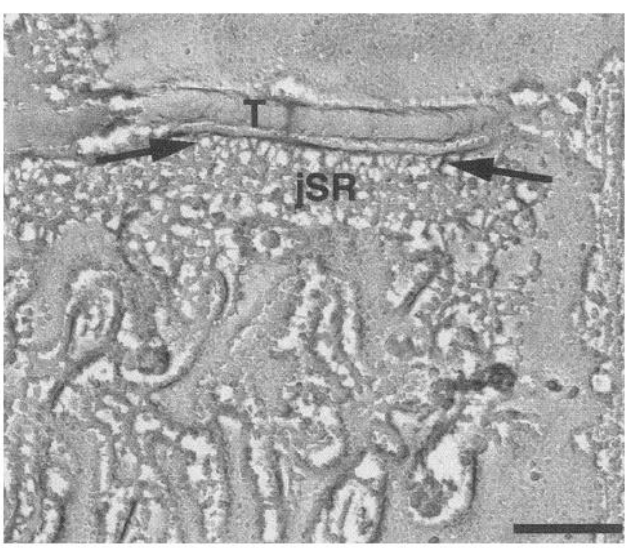

FIG. 14. Deep-etch rotary shadowed replica of junctional region from toadfish swimbladder muscle. A t tubule (T) runs from *left* to *right* in image. Immediately below t tubule is lumen of junctional SR (jSR), filled with a network of calsequestrin. Short strands join calsequestrin to jSR membrane (arrows). Bar, 0.2 μm. [From Franzini-Armstrong et al. (95).]

in responsiveness to Ca^{2+} of heavy SR vesicles that have been deprived of calsequestrin by treatment with EDTA or ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid has been interpreted as an evidence of calsequestrin interaction with the RyRs, even while acknowledging that the isolated RyRs do not need calsequestrin for their activity (149). Heterogeneity in RyR channel desensitization has been proposed to be due to interactions with other SR proteins (179).

Calsequestrin forms an early association with the jSR. The association is not dependent on the presence of feet, because it occurs normally in mouse muscle fibers lacking RyR1 and expressing extremely low levels of RyR3 (308). However, in the developing junctions of cardiac muscle, clustering of calsequestrin at the junctions follows with a small temporal delay the formation of assemblies of feet (263).

IV. RYANODINE RECEPTOR-DIHYDROPYRIDINE RECEPTOR INTERACTIONS IN EXCITATION-CONTRACTION COUPLING

During activation of muscle contraction, release of Ca^{2+} from the SR through the RyRs is initiated by depolarization of the exterior membranes of the cell: the surface membrane and its invaginations and the t tubules. A specific functional link exists between events in the exterior membranes and the response of the internal membrane system. The geometry of the SR in relation to the exterior membranes and the action of the exterior membrane Ca^{2+} channels (DHPRs) play a major role in defining this interaction. This section briefly covers structure and geometry of the jSR and then focuses on the interactions between RyRs and DHPRs (see Refs. 42, 88, 94, 270 for reviews).

A. Architecture of Junctional Sarcoplasmic Reticulum and Its Relationship to Exterior Membranes

The junctional face membrane of the jSR bears the RyRs, whose hydrophilic domains (feet) face the cytoplasm, and is associated with calsequestrin on its luminal side. Other proteins of the junctional complex (triadin, junctin) are also associated with this membrane. The jSR has two possible relationships with exterior membranes (Fig. 15). In most cases, the cytoplasmic side of the jSR is closely apposed either with the t tubules or with the surface membrane, forming junctions called triads, dyads, and peripheral couplings. Feet cross the narrow space between the two membranes, called junctional gap, and they appear to directly connect SR to exterior membranes. In this position the feet, or cytoplasmic domains of RyRs, provide a link between the Ca^{2+} release channel located in the jSR membrane and components of the exterior membranes that initiate e-c coupling events. Alternatively, the jSR may be present in the interior of the muscle fibers without bearing any relationship to exterior membranes, so that its feet face the cell cytoplasm. This is the so-called corbular or extended jSR (see Ref. 294 for a review). In skeletal muscle, jSR obligatorily forms junctions, and exceptions, if any, are very rare. In cardiac muscle, part of the jSR forms junctions, but corbular and/or extended jSR is also present in amounts that vary from a small percentage to as much as 70% of the feet-bearing SR (see Ref. 294 for a review). Evidence for release of Ca^{2+} at the sites of SR-t tubule junctions in the intact muscle fibers has come indirectly from the local stimulation experiments in skeletal muscle (120) and, more directly, in recent times, by the use of Ca^{2+} indicators and confocal microscopy (52, 77, 154, 282). Junctions between domains of the endoplasmic reticulum and the plasmalemma, mediated by feet, have also been shown in the Purkinje cells of the cerebellum (115).

Feet are disposed in arrays (82, 174) so that in thin sections they appear as evenly spaced densities. Rows of feet are seen in skeletal and cardiac muscle junctions (92, 296; Fig. 15, *A–C*) and in SR-surface junctions of Purkinje cells in the cerebellum (115). In muscles containing α- and β-isoforms, the two probably coexist within the same array. Examples of feet in junctions of dyspedic skeletal muscle have been detected, indicating that RyR3 may also be able to assemble into arrays in the absence of RyR1 (308, 315). The crooked neck dwarf mutation in the chicken, which results in loss of RyR1 but sustained in vivo level of RyR3 expression (6), has not yet been fully examined by electron microscopy. Extensive arrays of feet are also found in muscles of invertebrates, but the relationship of invertebrate to vertebrate isoforms has not been established.

The arrangement of feet has been well defined in the arrays of skeletal muscle and body muscles of some invertebrates (see Ref. 94 for a review). The two dispositions are slightly different but are both based on a similar interaction between the corners of cytoplasmic domains, resulting in an orthogonal array with overlap between the subunits of alternate feet. Although the centers of the feet define an orthogonal pattern, the feet themselves have a slightly skewed position. Formation of the array is an intrinsic property of the RyR, since expression of RyR1 in CHO cells results in ordered aggregates of feet (310). Inositol 1,4,5-trisphosphate receptors in Purkinje cells are arranged in a disposition similar to that of feet arrays, but with smaller spacings due to their smaller size (146).

B. Dihydropyridine Receptors and Excitation-Contraction Coupling

The primary event in the activation of striated muscle contraction is depolarization of the surface membrane.

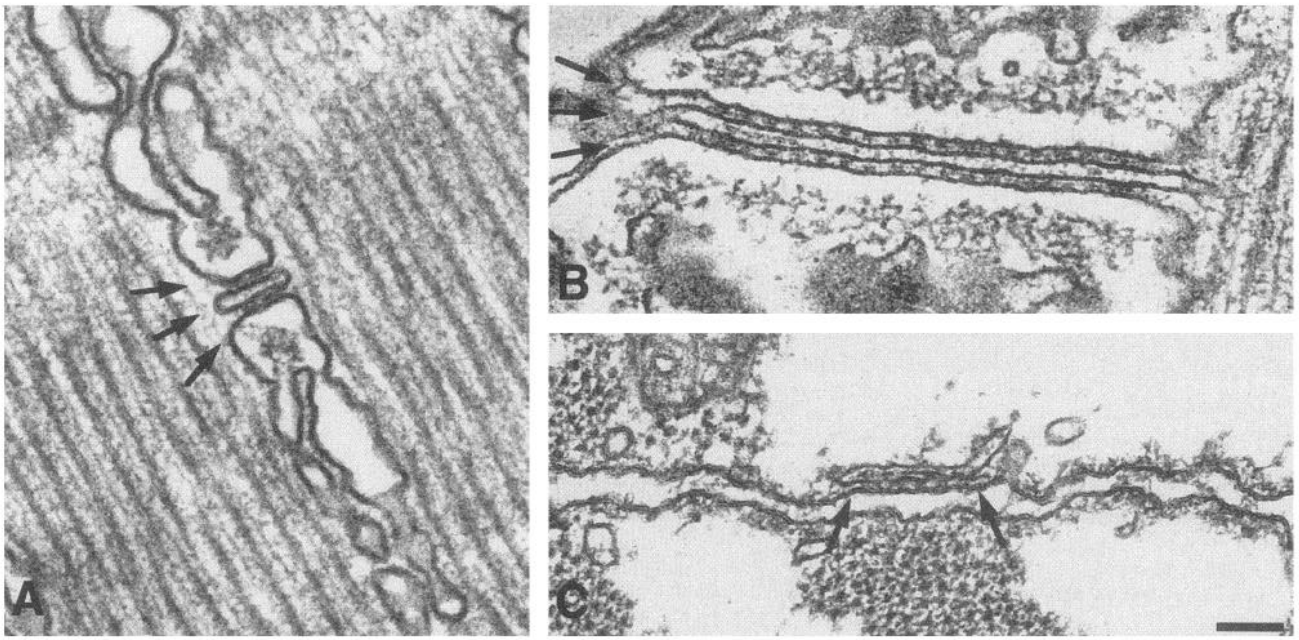

FIG. 15. Electron micrographs of triads in skeletal muscle (*A* and *B*, 3 arrows) and a peripheral coupling in cardiac muscle (*C*, between arrows). Evenly spaced densities or feet, representing junctional domains of RyRs, fill junctional gap. Bar, 0.1 μm. [From Protasi et al. (263).]

This in turn is sensed by voltage sensors, whose activity is detected in the form of a charge movement (278; see Ref. 277 for a review). The dihydropyridine-sensitive L-type Ca^{2+} channels of exterior membranes (DHPRs) are responsible for charge movement and the initiation of e-c coupling in skeletal muscle (1, 268, 318). The effects of charge movement are somehow felt by the RyRs, which become permeable to Ca^{2+}, thus initiating the next step, Ca^{2+} release. The DHPRs have five subunits (α_1, α_2, β, γ, and δ), and α_1 is the channel-forming, voltage-sensing, dihydropyridine-sensitive portion of the molecule (see Ref. 42 for a review). The α_1-DHPR is structurally similar to the voltage-sensitive Na^+channels, with four transmembrane domains (I–IV), each formed by six membrane-spanning segments (S1–S6) (215, 320). The cardiac α_1-DHPR is a related oligomeric complex, whose α_1-subunit has 66% homology with the skeletal counterpart and the same general transmembrane topology, but larger COOH- and NH_2-terminals (215). Motion of a positively charged portion of the DHPR (S4 in each domain) has been proposed, and later confirmed, to be responsible for charge movement providing the triggering signal that induces activation of RyRs in skeletal muscle (12, 320).

Three animal models bearing specific mutations of key e-c coupling components have played, and will continue to play, a key role in unraveling the interactions between DHPRs and RyRs in e-c coupling. One is the dysgenic mouse. In this spontaneously generated mutant, a single nucleotide deletion (nucleotide 4010) causes a shift in the translational reading frame for α_1, the channel-forming subunit of DHPR (44). This leads to loss of charge movement, currents, and e-c coupling (13, 14). The three functions are restored by transfection with cDNA for α_1-DHPR, indicating that they reside in this molecule (1, 318).

A second model is the transgenic mouse, resulting from a targeted mutation of the gene for RyR1 (311). Mutated, dyspedic, fibers lack e-c coupling and feet, but junctions between SR and exterior membranes are formed, although in a limited number (308).

A spontaneous mutation of RyR1 in chicken results in the crooked neck dwarf phenotype (3). The RyR3 isoform is expressed at later stages of development, but the fibers do not fully recover structure and function (6).

Basic differences in e-c coupling events in skeletal and cardiac muscles are due to differences in the interaction between DHPRs and RyRs, which in turn are based on different properties of both DHPRs and RyRs. In skeletal muscle, despite a well detectable charge movement, the Ca^{2+} current is slow and of small magnitude (Fig. 16*A*). The current peaks after tension development, and e-c coupling can occur normally in the absence of extracellular Ca^{2+}. Calcium transients, due to Ca^{2+} release from the SR, are fast and display a sigmoidal dependence of membrane voltage. Activation of RyR may be due to a direct action of the voltage sensor on the Ca^{2+} release channel. In cardiac muscle, on the other hand, the kinetics of DHPR activation are fast (Fig. 16*B*), Ca^{2+} currents precede contraction and are of large amplitude, Ca^{2+} permeation through the channels is a prerequisite of contraction, and Ca^{2+} transients are slow and have a bell-shaped dependence on voltage parallel to that of Ca^{2+} currents. Activation of cardiac RyRs may be by the indirect means of CICR (see Refs. 340, 341 for reviews).

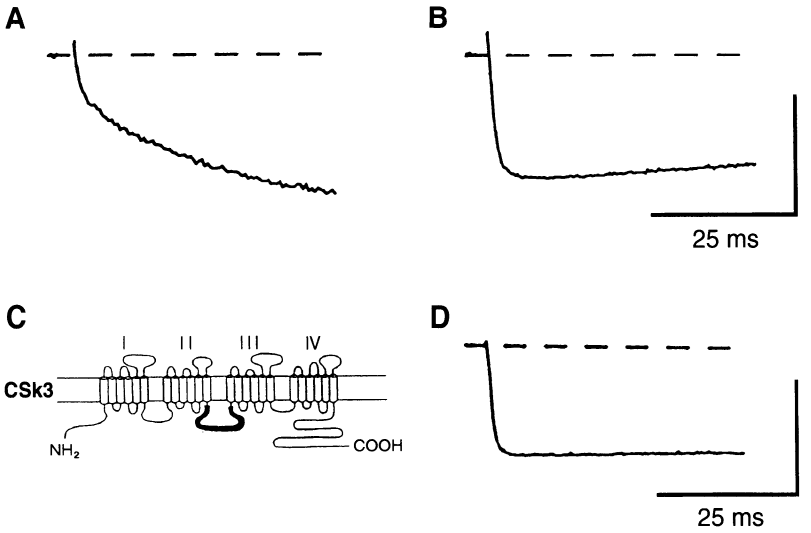

FIG. 16. Ca^{2+} currents of skeletal (*A*) and cardiac (*B*) DHPRs differ significantly in kinetic properties. *C*: skeletal-cardiac α_1-DHPR chimera (CSk3) constructed by substituting skeletal II-II loop (thick line) into a cardiac DHPR. Channel expressed in dysgenic myotubes is capable of cardiac-type Ca^{2+} currents (*right*) but results in skeletal-type excitation-contraction coupling, independent of extracellular Ca^{2+}. Vertical calibration corresponds to 1.5 nA (*A*), 15 nA (*B*), and 10 nA (*C*). [From Tanabe et al. (317).]

One possible explanation for the small Ca^{2+} currents of skeletal muscle DHPRs was sought in the existence of two isoforms of the α_1-subunit (64). It was proposed that the most abundant form, missing the COOH-terminal, acts only as a voltage sensor, whereas the full-length, longer transcript codes for a DHPR capable of opening its activation channel (see Ref. 42 for a review). However, the truncated version expressed in the dysgenic mouse can act both as voltage sensor and Ca^{2+} channel (12), and the conclusion is that skeletal DHPRs are excellent voltage sensors but sluggish Ca^{2+} channels. Indeed, DHPRs reconstituted in lipid bilayers have very low average probability, when stimulated by a voltage change (181).

Cardiac-skeletal chimeras of the α_1-subunit, expressed in dysgenic myotubes, have kinetic characteristics of either one or the other type, depending on the type of repeat I present in the chimera (316). More specifically, the S3 segment and the linker connecting S3 and S4 in the repeat I (226) are determinant of DHPR activation kinetics.

C. Spatial Relationship Between Ryanodine Receptors and Dihydropyridine Receptors: Clues to Excitation-Contraction Coupling

Dihydropyridine receptors are located in the surface/t-tubule membranes at sites of surface-SR junctions and face RyRs in skeletal and cardiac muscle and in cell lines derived from them (38, 86, 89, 263, 303, 348; Fig. 17). In this position, DHPRs are appropriately located to initiate the coupling between electrical signal and release of Ca^{2+} from the jSR. Indeed, development of e-c coupling mechanism and the appearance of DHPR-RyR associations are simultaneous in skeletal muscle myotubes (86). The α_1-subunit is responsible for anchoring DHPRs to the junctional regions, since in its absence (in dysgenic muscle) the α_2-subunit remains diffuse in the exterior membrane/t tubule (90). Biochemical evidence for a complex involving DHPRs and RyRs has been obtained (191), but it has not been confirmed structurally.

The specific location of DHPRs relative to the neighboring RyRs differs in skeletal and cardiac muscle, in a manner that is highly correlated with functional variations in e-c coupling mechanisms between the two muscles. In skeletal muscle, DHPRs are clustered in groups of four, or tetrads (Fig. 18, *A* and *C*). The tetrads are positioned in exact correspondence of the feet so that each DHPR is located immediately above one of the RyR subunits and thus available for a direct interaction (21, 96). However, unexpectedly, tetrads are associated with alternate feet. This disposition is found in several different types of fibers, and it is not dependent on the presence of two different RyR isoforms, so unattached feet can be RyR1s, as well as, probably, RyR3 (see Ref. 304 for further discussion). Identification of tetrads as groups of four DHPRs is based on the absence of tetrads in developing dysgenic muscle in vivo and in vitro (97, 307) and the restoration of tetrads after transfection with the cDNA for skeletal DHPR (307). One problem remains and that is an inconsistency between the ratio of DHPRs to RyRs predicted from structural considerations and the measured ratios of high-affinity [^{3}H]PN200–100 and [^{3}H]ryanodine binding. The alternate position of tetrads and feet results in an overall ratio of 2 DHPRs/RyR. Measured ratios vary in muscles from different sources and are mostly smaller than 2:1 (8, 18, 185). The observation that tetrads, although precisely located in correspondence of alternate feet, are often incomplete, i.e., they miss one or more particles, reduces but does not eliminate this discrepancy. A small subpopulation of "strong" triads resistance to dissociation and containing a higher density of DHPRs exists in rabbit skeletal muscle, indicating that DHPR levels may vary locally. However, it is not clear where these are located in the muscle fiber (27, 151), and structural evidence for local

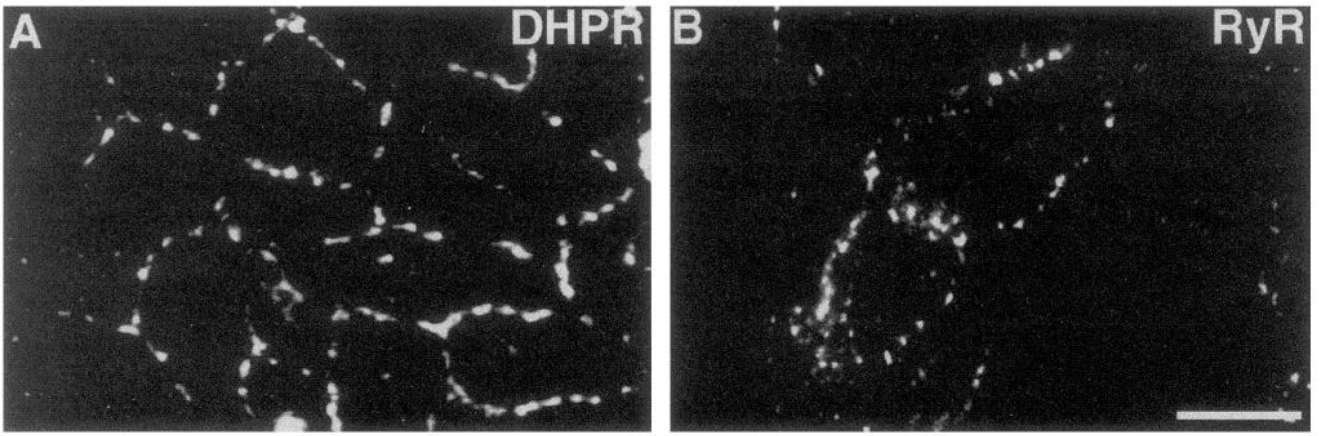

FIG. 17. Confocal images of cryosections from left ventricle of adult chicken myocardium immunolabeled for DHPRs (*A*) and RyRs (*B*). Images show discrete foci of 2 molecules marking location of peripheral couplings at cell periphery. Faint labeling by RyR antibody in interior of cell marks position of extended jSR. Bar, 10 μm. [From Protasi et al. (263).]

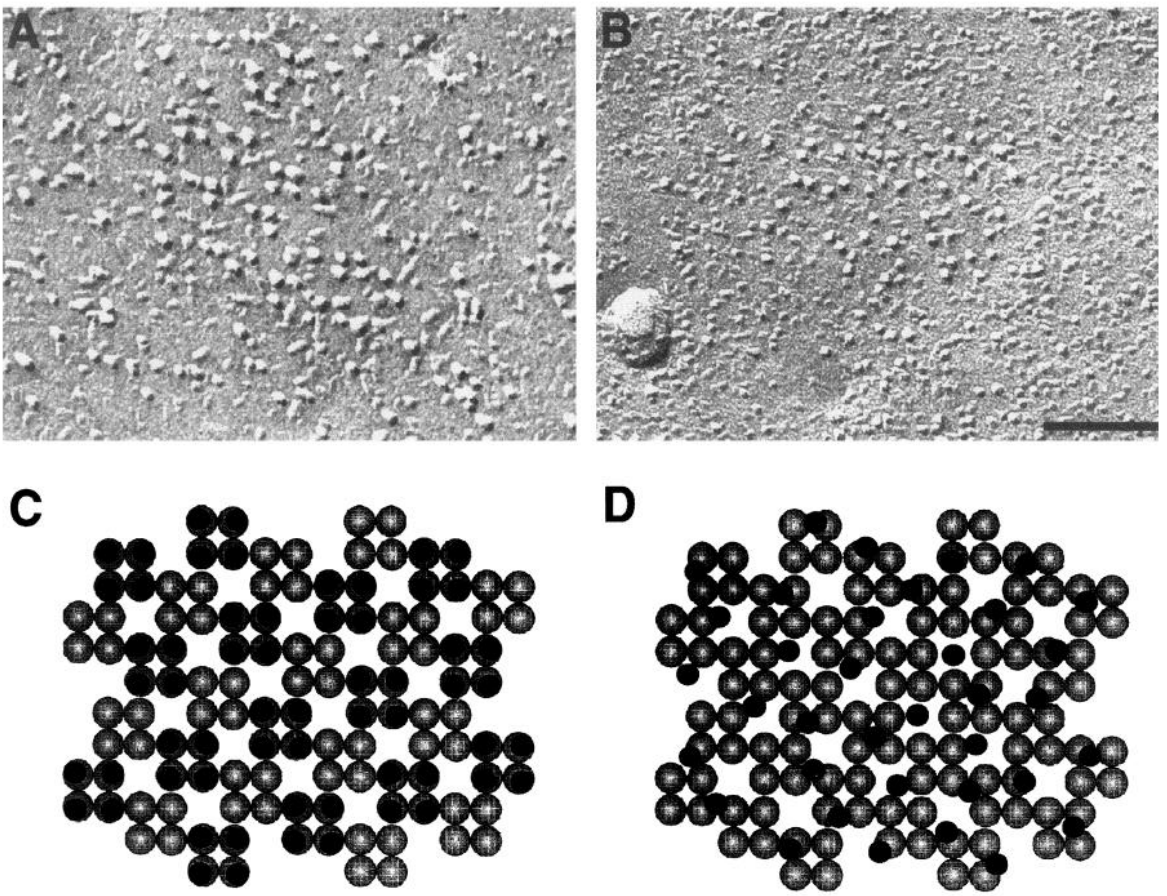

FIG. 18. *A* and *B*: freeze fractures of surface membrane from developing skeletal and adult cardiac muscles, showing DHPR clusters at sites of peripheral couplings. In skeletal muscle (*A*), DHPRs form groups of 4 (tetrads). Spacing between tetrads indicates a 1:2 correspondence of tetrads to feet. In cardiac muscle (*B*), DHPRs are randomly disposed within cluster. *C* and *D*: modeling of disposition of DHPRs (black circles) relative to underlying arrays of feet (represented by 4 gray circles). In skeletal muscle (*C*), best fit between arrays of tetrads and feet is obtained by exact superimposition of DHPR tetrads (4 black circles) over alternate feet. In cardiac muscle (*D*), DHPRs have a random dispositon and thus are not consistently located relative to feet. Note, however, that each DHPR is in very close proximity to one or more feet. Bar, 0.1 mm. [*A* from Franzini-Armstrong et al. (97); *B* from Protasi et al. (263).]

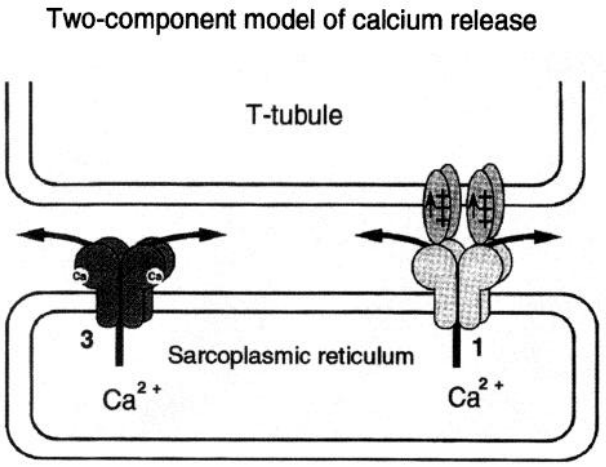

FIG. 19. Two-component model for Ca^{2+} release from SR. Alternate disposition of tetrads over feet implies that alternate feet are differently activated. One possibility, shown here, is that one RyR is directly activated by a tetrad of DHPRs, whereas the other is activated indirectly by CICR (see Ref. 271). In muscles with 2 isoforms, RyR1 would be directly coupled to foot, whereas RyR3 would be indirectly activated. However, in mammalian muscle, it must be assumed that coupled and uncoupled feet are both represented by RyR1s. [From Block et al. (22).]

variations in DHPR has not been directly obtained, for technical reasons.

An allosteric model for the control of one RyR by four DHPRs fits reasonably well the e-c coupling properties of skeletal muscle (269). However, as a result of the association of tetrads with alternate feet (Fig. 18*C*), some RyRs have the possibility of interacting directly with four DHPR, while the remaining feet are denied the interaction (21, 96, 262) (Fig. 19). Schemes in which the activity of alternate feet is directly initiated by interaction with DHPRs, whereas other are activated by CICR, have been proposed (see Refs. 22, 271 for reviews) (Fig. 19). Evidence both for and against Ca^{2+}-dependent mechanisms in addition to voltage-dependent effects and for voltage-dependent influence on Ca^{2+}-dependent release have been presented (8, 10, 67, 248; see Ref. 277 for a review).

Ryanodine receptors dictate the positioning of DHPRs into tetrads but are not necessary either for the formation of junctions between SR and exterior membranes, nor for the clustering of DHPRs in proximity of the junction. Developing myotubes from mice with a targeted mutation for RyR1 (311) form feetless (dyspedic) junctions in which calsequestrin-containing SR is closely apposed to surface membrane and t tubules (308). In addition, in a newly developed cell line carrying a similar targeted mutation of the RyR1 gene, DHPRs cluster at sites of the dyspedic junctions but remain randomly disposed, and do not form tetrads (F. Protasi, C. Franzini-Armstrong, and P. D. Allen, unpublished data).

In cardiac muscle, like in skeletal muscle, DHPRs are clustered at the SR-surface junctions (38, 263, 303). Indeed, the areas of surface membrane occupied by DHPRs are equal in size to the areas of SR associated with arrays of feet (263). However, there is no evidence for a direct association of DHPRs into tetrads (Fig. 18, *B* and *D*) and for a specific positioning of DHPRs relative to the feet in cardiac muscle. The apparent lack of specific association between DHPRs and RyRs in peripheral couplings of cardiac muscle is in keeping with the lack of a direct functional interaction of DHPRs with RyR2. However, clustering of RyRs and DHPRs into closely apposed patches of membrane increases the chance that a RyR can be affected by the Ca^{2+} current flowing through a nearby DHPR, thus allowing privileged DHPR-RyR communication (283).

Cardiac muscle presents an additional e-c coupling puzzle, due to the presence of extended jSR or corbular SR. These are SR sites containing high densities of calsequestrin and RyR2 (144, 140, 141), which do not face DHPRs (Fig. 20). In some avian myocardium, extended jSR constitutes a large proportion of the jSR, and thus it is expected that its RyRs participate in Ca^{2+} release (see Ref. 294 for a review). Clearly, this must be activated by some indirect means, such as a "saltatory conduction," that permits the spread of CICR from one site to the next (294). This hypothesis is supported by the observation that the distance between extended jSR sites is very small in avian muscle, thus favoring interactions from one site to the next (unpublished data).

Because the RyR3 isoform is normally combined with the RyR1 in skeletal muscle, it is not possible to know how it is activated under normal conditions. However, in cultured myotubes from *cn/cn* chicks, RyR1 is absent, whereas RyR3 is well expressed. *Cn/cn* myotubes show Ca^{2+} transients that are dependent on the presence of outside Ca^{2+}, remain localized, and rise slowly (133). It is not entirely clear whether these transients arise from the interaction of the cardiac-type DHPRs present in these myotubes with RyR3.

D. Functional Dihydropyridine Receptor-Ryanodine Receptor Interactions Are Isoform Dependent

The type of e-c coupling, skeletal (independent of extracellular Ca^{2+}) versus cardiac (dependent on intracellular Ca^{2+}), is determined by the DHPR isoform present in the cell. Expression of the skeletal-type α_1-DHPR in dysgenic myotubes, either after nuclear injection of the appropriate cDNA (1, 13, 318; see Ref. 14 for a review) or after fusion with wild-type fibroblasts (45, 61), restores skeletal-type e-c coupling. Transfection with cDNA for cardiac DHPR (pCARD1) results in cardiac-type e-c coupling, despite the presence of RyR1 in these cells (319). The expressed DHPRs maintain their native relationships between Ca^{2+} transients and Ca^{2+} currents (102). Activity of other Ca^{2+} channels fails to elicit e-c coupling in dysgenic myotubes (1, 327).

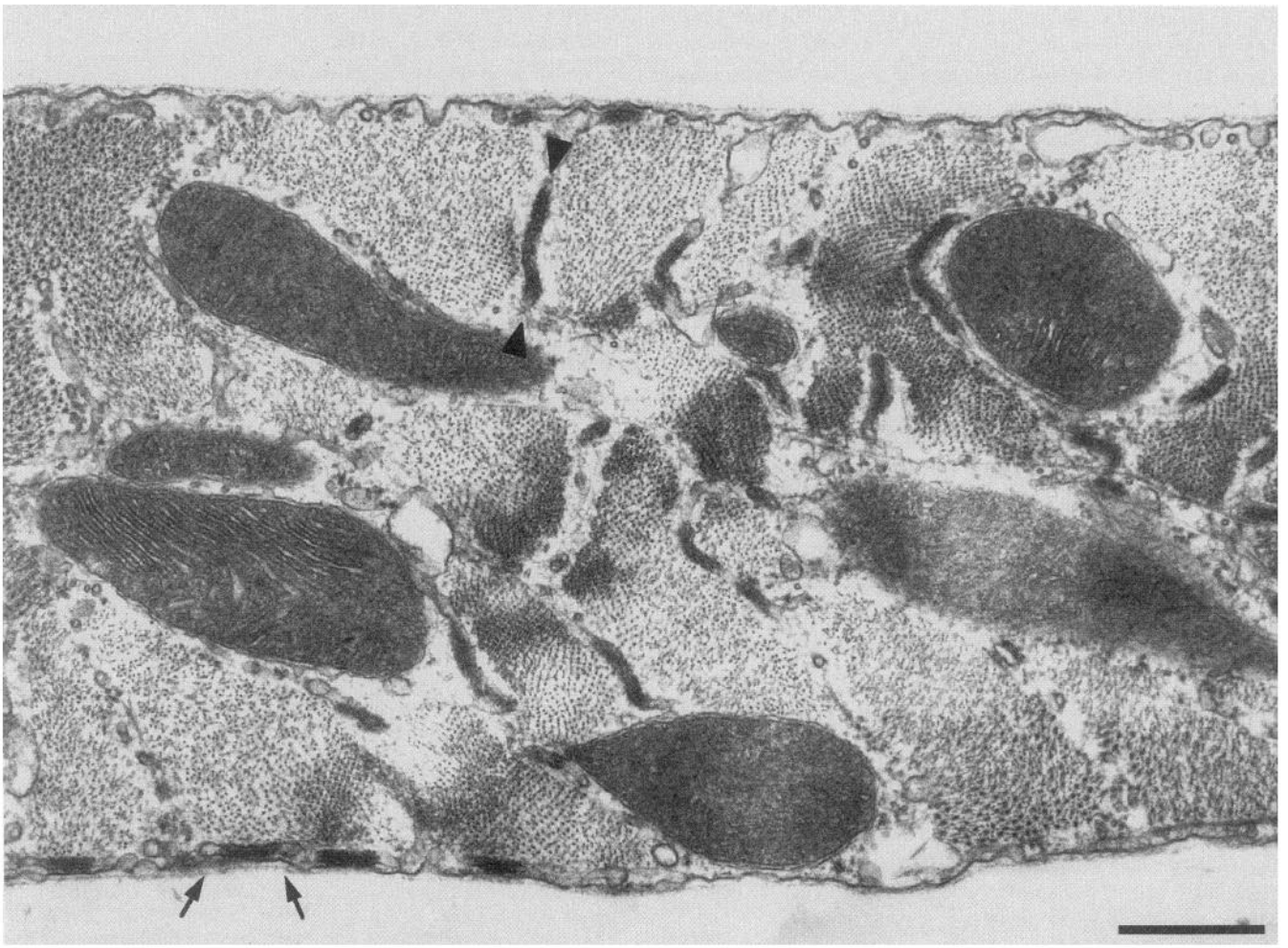

FIG. 20. Cross section through a cardiac myocyte from ventricle of finch. This muscle has no t tubules. jSR is either located at surface membrane, to form peripheral couplings (between arrows), or within interior of fiber, where it forms an extensive *Z*-line level network of extended jSR (see arrowheads). One extended jSR segment is indicated between arrowheads. Bar, 0.5 μm. (Courtesy of J. R. Sommer.)

With the use of skeletal-cardiac chimeras, in which the cytoplasmic loops of the cardiac α_1-DHPR are gradually substituted with their skeletal counterparts, the putative cytoplasmic loop between the II and III intramembrane segments (residues 666–791) was shown to be the major determinant of e-c coupling type (317; Fig. 16, *C* and *D*). In vitro experiments have not only confirmed the importance of the II-III loop but have also shown directly that this portion of the DHPR affects behavior of RyRs. However, several apparent inconsistencies between the results of different laboratories, and also between in vivo and in vitro results, exist. Peptides containing amino acids 666–791 do activate RyR channels in vitro, increasing open probability and ryanodine binding (176). However, contrary to what happens in vivo, peptides from skeletal and cardiac II-III loops are equally active on RyR1. A shorter peptide, comprising amino acids 666–726, and a phosphorylatable site at Ser-687, was found to have good activity, under the control of phosphorylation (177). However, this peptide does not overlap with the critical 17-amino acid segment, identified by in vivo experiments, which is located at amino acids 727–743 (229). Finally, two adjacent peptides within the loop have been identified. One, at amino acids 671–690, has an activating effect, and the other, at amino acids 724–760, inhibits the effect of the former (73, 74). Clearly, the II-III loop of DHPRs controls the state of activity of RyR1, but the interaction may be quite complex, very likely including various activities within the same segment as well as contributions by other loops. In addition, the RyR also has its voice in the RyR-DHPR interactions, since the complete skeletal II-III loop has no effect on RyR2 (177).

Although the above experiments strongly support the hypothesis of a molecular interaction between DHPRs and RyRs in skeletal muscle, retrograde interaction from RyR to DHPR gives further backing to this theory of e-c coupling. Dyspedic myotubes lacking RyR1 have greatly reduced L-type Ca^{2+} currents, but the maximum immobiliza-

tion-resistant charge movement related to surface area is comparable to that of control myotubes (227). Transfection with cDNA for RyR1 brings the ratio of Ca^{2+} current to charge movement back to the normal value (229). The inference is that dyspedic myotubes express DHPRs, and a retrograde action of RyR controls their gating. Thus the "mechanical" hypothesis of skeletal muscle e-c coupling, requiring direct interaction between surface membrane and SR components (278), has come of age at approximately the right time (~21 years after its proposal).

V. CONCLUSIONS

Paradigms for the identification of RyRs have been defined in striated muscles. Ryanodine binding and its effect, sequence, immunoreactivity, structure, channel conductivity, and pharmacology can be used to identify a member of this family of Ca^{2+} release channels of the internal membranes. With the use of any of these clues, it is clear that RyRs are present in all cells, together with $InsP_3$ receptors. However, striated muscle cells have considerably more RyRs than $InsP_3$ receptors, whereas nonmuscle cells have the opposite ratio. Interestingly, smooth muscle and Purkinje cells of the cerebellum are rich in both types of Ca^{2+} release channels. In smooth muscles, the two types of channels are involved in two distinct pathways for the activation of contraction (see Refs. 292, 293 for reviews). The rationale for the high concentration of skeletal-type RyRs and of $InsP_3$ receptors in Purkinje cells is not known.

Activity of RyRs is controlled by a variety of interactions with soluble and structural proteins. The interaction between two Ca^{2+} channels, DHPRs of the exterior membranes and RyRs of the SR, dominates e-c coupling. Variations in the properties and relative positions of the two channels explain differences in e-c coupling mechanisms in skeletal and cardiac muscle. Explorations of the molecular, functional, and structural basis for these difference have greatly strengthened the mechanical hypothesis for e-c coupling in skeletal muscle. This requires a direct functional interaction between DHPRs and RyRs, probably mediated by a physical contact between one or more cytoplasmic loops of DHPRs and the large cytoplasmic domain of RyRs. Other proteins of the SR, an accessory component of the RyR, and various solutes modulate, control, and stabilize the level of activity of the channel and its responsiveness to DHPRs. The molecular basis for these interactions and their functional role are quite complex, and their unraveling has just begun. A promising venue of research in the near future is the study of small spontaneous and activated release events by optical techniques (52).

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