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RYANODINE RECEPTOR ISOFORMS OF NON-MAMMALIAN SKELETAL MUSCLE

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1. ABSTRACT

Whereas mammalian skeletal muscles express primarily a single isoform of ryanodine receptor (RyR) as the Ca2+ releasing channel, many non-mammalian vertebrate skeletal muscles express two isoforms in almost similar amount, alpha- and beta-RyR which are homologues of mammalian isoforms RyR1 and 3, respectively. alpha-RyR is believed to be directly involved in excitation-contraction coupling in skeletal muscles and is variable in its properties among animals and fibers, while beta-RyR shows similar properties and is variable in its content. alpha- and beta-RyR purified from frog skeletal muscle, a favorite material for physiological and morphological experiments, are very similar in Ca2+ dependent [3H]ryanodine binding. On the SR membrane, however, alpha-RyR is selectively suppressed in the ligand binding, indicating that the Ca2+-induced Ca2+ release (CICR) activity in skeletal muscle is conducted primarily by beta-RyR. We also stressed here that Ca2+ binding to the activating site is a necessary but not a sufficient condition for CICR. The maximum activity attainable under a specified condition is also a critical parameter to be determined. Taking these findings into consideration, we conclude that CICR is too slow to explain the physiological Ca2+ release on depolarization.

2. INTRODUCTION

How the action potential in the sarcolemma (an electrical event) is transformed to the contraction of myofibrils in the myoplasm (a mechanical event) is the central theme in excitation-contraction coupling (ECC). Since establishment of the fact that myofibrillar contraction mechanism underlying Ca^{2+} release from the sarcoplasmic is regulated by Ca^{2+} (1), there is new focus on the reticulum (SR) on depolarization of the T-tubule. Identification of relaxing factor with Ca^{2+}-ATPase on the SR led to the finding of a Ca^{2+} release mechanism, Ca^{2+}-induced Ca^{2+} release (CICR) (2). The well-known experiment of local stimulation (3, 4) preceded identification of the T-tubule, invagination of the sarcolemma, which served as conduit for inward spreading of excitation, and of the foot structure, electron dense material spanning between T-tubule and the junctional face of SR in triad, which was thought to play the critical role in Ca^{2+} release in ECC (5, 6). Demonstration of charge movements suggested a hypothesis that the conformation change of the voltage sensor was transmitted to the Ca^{2+} release channel, resulting in Ca^{2+} release from the store (depolarization-induced Ca^{2+} release, DICR) (7). The finding that ryanodine strongly binds to CICR channels led to isolation of the ryanodine receptor (RyR) (8). Electron microscopic observation of RyR molecules revealed that the foot structure was the cytoplasmic part of RyR tetramers (8). Understanding of the molecular mechanism of ECC has been deepened by molecular biological studies using dysgenic and dyspedic (RyR-knockout) mice (9-11). Interaction between dihydropyridine receptor (DHPR), the voltage sensor, and RyR, the Ca^{2+} release channel, is critical in ECC. It is now clear that RyR could operate two modes of Ca^{2+}release, CICR and DICR. Although Ca^{2+} influx is unnecessary for skeletal muscle contraction, the role of CICR in ECC remains a heated argument (12, 13).

Mammalian RyR has three genetically distinct isoforms: RyR1 is the primary isoform in skeletal muscle, RyR2 is the isoform in the cardiac muscle, and RyR3 is the

isoform usually found in a miniscule amount in various kinds of cells, tissues and organs. In skeletal muscles, protein staining usually showed a single band of RyR1, although Western blot may detect a faint band of RyR3 in addition to the main band of RyR1 (14, 15). It should be mentioned that the primary isoform in the brain was RyR2, but RyR1 was detected in the Purkinje neuron in the cerebellum, and RyR3, faintly but characteristically, in the corpus striatum, hippocampus and thalamus (14, 15). Many non-mammalian skeletal muscles, in contrast, showed two bands of RyR, alpha- and beta-RyR, in similar density on protein staining after SDS-PAGE (14, 16). cDNA sequences showed that alpha- and beta-RyR were homologues of RyR1 and RyR3, respectively, although their genetic loci were not yet identified. In this article, the terms alpha- and beta-RyR refer to non-mammalian origins, whereas RyR1 and 3 to mammalian origins.

In this review we will summarize findings on alpha- and beta-RyR of non-mammalian vertebrate skeletal muscle compared with the mammalian counterparts (RyR1 and 3). Discussion will concentrate on their CICR which has been well studied to learn the biological roles of the two isoforms. For want of space, we must defer many aspects to the authors of other chapters of this book. There are also many excellent reviews which can be consulted for general matter on RyR (17-23).

3.COEXPRESSION OF TWO DISTINCT ISOFORMS, ALPHA- AND BETA-RyR

Sutko and his colleagues first detected two distinct isoforms, alpha- and beta-RyR, in the SR vesicles from skeletal muscles of chicken, frog and fish (24, 25). The densities of the two bands on the SDS-PAGE pattern were similar. beta-RyR moves slightly faster than alpha-RyR on SDS-PAGE; its mobility was similar to that of RyR2, whereas alpha-RyR was similar to RyR1. beta-RyR cannot be a fragment of alpha-RyR, because they showed distinct peptide-map patterns on limited proteolysis, and also because they showed different immunologic reactivities (24). beta-RyR cannot be the cardiac isoform, because they detected an additional separate isoform (probably corresponding to RyR2) in chicken heart (26, 27). On the basis of immunological coprecipitation, they concluded that alpha- and beta-RyR formed homotetramers (24). The cultured embryonic cells from a crooked neck dwarf chick (28, 29) where alpha-RyR was lacking failed to show Ca^{2+} release on electrical stimulation in a Ca^{2+}-free medium, but they caused Ca^{2+} release in response to caffeine, indicating that alpha-RyR is directly involved in ECC (see later) (30).

Block and her colleagues (31) extensively examined RyR isoforms expressed in non-mammalian vertebrate skeletal muscles. They found that coexpression of two isoforms was not a general rule in non-mammalian vertebrates: among reptiles, turtle and alligator coexpressed the two isoforms, whereas lizard and snake expressed alpha-isoform alone. Extraocular muscles of fishes (toadfish, striped bass, tuna and blue marlin) and avians, and toadfish swim bladder muscles expressed alpha-isoform alone, whereas these body muscles expressed the two isoforms. Murayama recently detected beta-RyR in extraocular muscles of bullfrog (unpublished results). The content of beta-RyR in frog extraocular muscles may be variable among fibers.

Murayama and Ogawa (32) purified alpha- and beta-RyR from bullfrog skeletal muscle by Mono-Q column chromatography, confirming homotetramers of each of the two isoforms. They are very similar in Ca^{2+}-dependent [^{3}H]ryanodine binding (33). Using polyclonal antibodies specific to alpha- and beta-RyR, they examined cross-reactivity of the two isoforms from various species of animals (carp, 5 species of frog, two species of toad, chicken and 4 species of mammals) (34). Western blot analysis of the SDS-PAGE pattern of SR vesicles showed that anti-alpha-RyR antibody positively reacted only to the frogs examined, *Rana catesbeiana, R. nigromaculata, R.* japonica, and R. temporaria. It was negative or very weakly positive to the other animals including toads and a frog Rhacophorus. In contrast, anti beta-RyR antibody showed positive reactions to all non-mammalian vertebrates examined, although more weakly in those with carp muscle. Mammals were negative to anti alpha-RyR or anti beta-RyR antibodies. Mobilities of alpha-RyRs on SDS-PAGE were variable among animals, whereas those of beta-RyR were consistent among non-mammalian vertebrates.

Oyamada *et al.* (35) cloned and sequenced the cDNAs for alpha- and beta- RyRs in bullfrog skeletal muscles, and they concluded that alpha-RyR and beta-RyR were homologous to RyR1 and RyR3, respectively. Particularly, RyR3 and beta-RyR lacked the D2 region. It was also confirmed that chicken beta-RyR was homologous to RyR3 (36). Franck *et al.* (37) reported that alpha-RyR in skeletal muscles of fish was fiber-type specific: distinct in its cDNA sequence between slow-twitch (red) and fast-twitch (white) muscles. They also showed that identities of the amino acid sequences of RyR1 and alpha-RyR among rabbit, frog and fish were 73-78%, whereas those of RyR3 and beta-RyR were 85-86% among rabbit, chicken and frog. These results suggest that alpha-RyRs may be more variable among animals than beta-RyRs which are similar to each other (34).

RyR3 isoform was at first detected as a novel isoform by its mRNA or cDNA in specified regions of the brain (38) and lung epithelial cells (39). Later it was found that RyR3 was ubiquitously distributed, but in a miniscule amount (40, 41). In the rabbit brain, RyR3 was about 2% of RyR2, the main isoform in the brain (the RyR amount in the brain was about 3% of that in the skeletal muscle) (42, 43). In adult skeletal muscles, diaphragm is the richest source of RyR3 (41) (less than 1% of RyR1 in rabbit diaphragm (44), and about 5% of RyR1 in bovine diaphragm (45)). Whereas RyR1 appeared at the early stage of the development, RyR3 appeared just before hatching, increased transiently and decreased with age (in mouse, the content reached maximum around 2 weeks after birth and then decreased to the adult level or disappeared) (46, 47). In chicken, similar time courses for alpha- and beta-RyR during development were reported (48).

We can conclude that RyR3 and beta-RyR are similar in their properties, but their contents in skeletal muscles are greatly variable, depending on age, fiber type and animal species. Block *et al.* (49) clearly showed that alpha-RyR in toadfish swim bladder aligned characteristically in two rows on the junctional face of the SR in the triad, and dihydropyridine receptors (DHPR) taking the form of the tetrad on the T-tubule are in precise register to every alternate foot. This characteristic configuration of voltage sensor (tetrad/DHPR) and foot (RyR1 or alpha-RyR) is now accepted as the basic structure for ECC in skeletal muscle (6, 50). Along this line, RyR1 and RyR3 isoforms were found to coexist in the same triads of the mammalian skeletal muscles (47). Recently, Felder and Franzini-Armstrong (51) reported that there were additional feet-like structures in 1-2 rows in either side of the junctional position on the SR, parajunctional feet, and that their occurrence is in parallel with the content of RyR3 or beta-RyR in fibers. They also reported that the packing arrangement was different between the two kinds of feet.

4. CA^{2+} DEPENDENCES OF ALPHA- AND BETA-RyR

[^{3}H]ryanodine binding to alpha- and beta-RyR from bullfrog skeletal muscles showed very similar biphasic Ca^{2+} dependence: Ca^{2+} less than 0.1 mM stimulates [^{3}H]ryanodine binding (EC_{50} ~ 0.01 mM) and Ca^{2+} higher than this concentration decreases it (IC_{50} ~ 3 mM) (33, see also Figure 2B). [^{3}H]ryanodine binding to RyR3 from rabbit brain and diaphragm showed similar Ca^{2+} dependence (42, 44). [^{3}H]ryanodine binding to RyR1 from rabbit skeletal muscle was about 10 times as sensitive to Ca^{2+} as RyR3 (42, 44). Takeshima *et al.* (52) had observed similar results of CICR in neonatal skeletal muscle cells from wild type (mainly RyR1) and dyspedic mutant (RyR3) mice. Franck *et al.* (37) reported that alpha-RyRs from slow-twitch red muscles of fishes was about 10 times more sensitive to Ca^{2+} than those from fast-twitch white muscles and toadfish swim bladder. They also concluded that the Ca^{2+} sensitivity of alpha-RyR was not affected by coexisting beta-RyR. Therefore we can conclude that RyR3 and beta-RyR, irrespective of their origins, showed similar Ca^{2+} sensitivity, whereas RyR1 and alpha-RyR can show distinct Ca^{2+} sensitivity among the sources. In other words, the difference of Ca^{2+} sensitivity should be ascribed to the species specificity, rather than to the isoform specificity (43, 44).

The conclusions mentioned above were drawn from the results of CICR and of [3H]ryanodine binding in an isotonic medium. Because hypertonic media gave different Ca2+ dependences (32, 33, 43, 53-55), attention to the composition of the medium is required. Ca2+ dependence determined by lipid bilayer experiments may also give different conclusions (56-60). Bull and Marengo (56) first reported that the SR vesicles from frog skeletal muscle showed two types of channel activity: higher sensitivity to Ca2+ and monophasic Ca2+ dependence without inhibition at high Ca2+ concentrations, which was similar to RyR2, and a lower sensitivity to Ca2+ and biphasic Ca2+ dependence with a marked inhibition at mM Ca2+ concentrations, which was similar to RyR1. Later, these authors and Hidalgo (57) reported that these types of channel activity were transformable and due to oxidation of channels, concluding that the two types of Ca^{2+} dependence could not be ascribed to the difference of the isoforms. Many investigators including us observed that beta-RyR (from fish, chicken, and frog skeletal muscles) (58, 59) and RyR3 (23, 44, 45, 60) showed very steep Ca^{2+} dependence with very weak or no inhibition at high Ca^{2+} concentrations, and that the peak Po (open probability) can often reach as high as almost 1 in an isotonic medium without an adenine nucleotide or caffeine. Murayama *et al.* (44) showed that RyR3 which showed peak Po~1 had very low [^{3}H]ryanodine binding under the corresponding conditions. The reason for the discrepancy between the two methodologies is not yet fully understood. It should be pointed out that lipid bilayer experiments report the activity of only the active channels, but not of silent ones, whereas the other methods give information on the averaged activity of all molecules. Po shows a time-averaged value of an (or several) active channel(s), but not an averaged number of open channels during a specified period. Because [^{3}H]ryanodine binding was consistent with CICR, we believe that the Ca^{2+} dependence based on [^{3}H]ryanodine binding is more suitable.

5. QUANTITATIVE ANALYSIS OF CICR

5.1. Ca^{2+} and Mg^{2+}

The biphasic Ca^{2+} dependence can be explained by Ca^{2+} binding to high-affinity activating Ca^{2+} sites (A-sites) and low-affinity inactivating Ca^{2+} sites (I-sites) of RyR. Mg^{2+} serves as a competitive antagonist in the A-site and an agonist in the I-site (Figure 1A). These findings can be expressed by the following equation (61):

$$A = A_{max} * f_A * (1-f_I) \quad \text{----------} \quad (1)$$

where A_{max} is the maximum activity attainable under the specified conditions, and f_A and f_I were fractions of A- and I-sites, respectively, which were occupied by Ca^{2+} and/or Mg^{2+}.

$$f_A = Ca\text{^}n1 / \{Ca\text{^}n1 + K1\text{^}n1 * (1 + Mg\text{^}n2 / K2\text{^}n2)\}$$

$$1 - f_I = 1 / (1 + Ca\text{^}n3 / K3\text{^}n3 + Mg\text{^}n4 / K4\text{^}n4)$$

where K1 and n1; and K3 and n3 represent dissociation constants and Hill coefficients for Ca2+ of A- and I- sites, respectively, and K2 and n2; and K4 and n4, parameters for Mg2+ of A- and I-sites, respectively. Here, Ca and Mg stand for [Ca2+] and [Mg2+], respectively. The results obtained with purified alpha- and beta-RyR and with CICR in skinned frog skeletal muscle fibers are summarized in Table 1 and drawn in Figure 1A (61). The A-site favored Ca2+ 20- to 30-fold over Mg2+, whereas the I-site was nonselective between the two cations. It is interesting that the Hill coefficient for Ca2+ in the A-site was 2, whereas that for Mg2+ was 1, probably because of difference in the favorable configurations of the two cation complexes. Purified alpha- and beta-RyR are very similar within the accuracy of determinations. Intact SR showed higher sensitivity to Ca2+ and Mg2+ than purified isoforms (61). This difference can be reasonably accounted for by the following considerations: the different state of channels, RyR

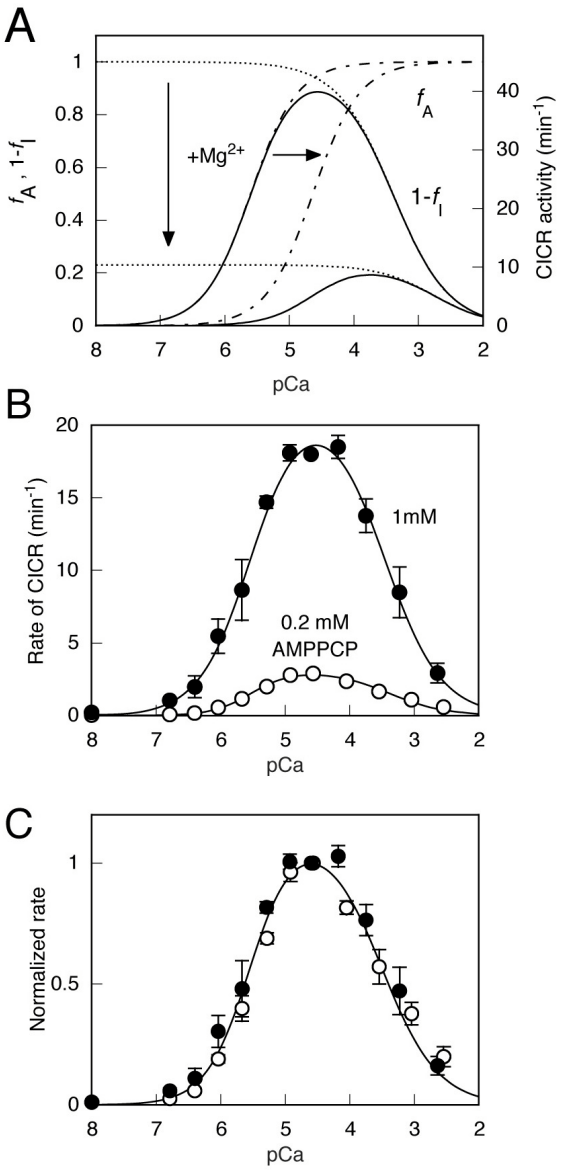


Figure 1. Quantitative analysis of CICR. A. The pCa-CICR activity curves in an isotonic medium containing 4 mM AMPPCP with and without 1 mM Mg^{2+} were drawn using parameters in Table 1 and the effect of Mg^{2+} was analyzed according to equation 1. Mg^{2+} shifted curve f_A (interrupted line) to a higher Ca^{2+} concentration range and reduced the maximum value for (1 - f_I) (dotted line), resulting in a rightward shifted pCa-CICR activity curve with reduced peak activity. B. Dose-dependent effects of AMPPCP on CICR. C. Each curve shown in panel B was normalized with its own peak activity. The Ca^{2+} dependence was unchanged by AMPPCP. Open circles, 0.2 mM AMPPCP; closed circles, 1 mM AMPPCP. For details, see ref. 61 (courtesy of the *Biophysical Journal*).

complexed with accessory proteins and purified RyR, presence and absence of the detergent, and [^{3}H]ryanodine binding and Ca^{2+} release. Effect of Mg^{2+} (1 mM) on CICR of frog skinned fibers is shown in Figure 1A.

5.2. Effects of an adenine nucleotide

It is well known that adenine nucleotides including ATP, AMPPCP, ADP and AMP stimulate CICR channel/RyR through the effects on the same site(s). However, it is unknown whether MgATP exerts the same magnitude of effect as free ATP. This is critical in considering the in situ effect because MgATP is the major form in the sarcoplasm. Murayama *et al.* (61) addressed this problem comparing the effect of AMP which has very weak affinity for Mg^{2+} with that of AMPPCP which has an affinity for Mg^{2+} comparable to that of ATP. The conclusion is that the effect of an adenine nucleotide is independent of the form and that it is the total concentration which is important.

As shown in Figure 1B, the rate of CICR in skinned fibers increased with the increase in concentration of AMPPCP. The Ca^{2+} dependence, however, remained unchanged, because normalized Ca^{2+} dependence was coincident (Figure 1C). Namely, the main effect of an adenine nucleotide is the increase in A_{max} alone. Another change is the decrease in n1 to around 1, probably because of conformation change in RyR. These findings indicate that not only affinities for Ca^{2+} and Mg^{2+} of A- and I-sites but also A_{max} value in equation 1 is a parameter variable with experimental conditions. The conclusion that A_{max} is not fixed but variable is also supported by the finding that the inhibition of procaine was independent of Ca^{2+} concentrations (62). In other words, the occupation of the A-site with Ca^{2+} is a necessary but not a sufficient condition for CICR. Murayama *et al.* (61) also reported that AMPPCP definitely stimulates Ca^{2+} release in a dose-dependent manner in the virtual absence of Ca^{2+}. This indicates that an adenine nucleotide may also stimulate and amplify DICR which may be triggered by a mode different from that in CICR.

5.3. Effects of caffeine

Murayama *et al.* (61) analyzed the effect of caffeine according to the procedure proposed. They reported that the effect of caffeine was two-fold: an increase in A_{max} and a decrease in K1. No effect on K2, K3 or K4 was found. It is notable that caffeine exerts differential effects on Ca^{2+} affinity than on Mg^{2+} affinity in the A-sites. The I-sites, in contrast, are not affected by caffeine, excluding the possibility that the decrease of Mg^{2+} inhibition might be the underlying mechanism. Although the increase in the affinity for Ca^{2+} in the A-site is well known, attention has not been paid to the increase in A_{max} except by Endo (2, 63) and the authors (14, 22, 61, 64). Decrease in K1 was saturated at 10 mM caffeine, but the increase in A_{max} was not yet saturated at this concentration (64). There was no difference in effect between alpha- and beta-RyR from frog skeletal muscle. In comparison with mammalian RyR1, the extent of the decrease in K1 was similar between rabbit and frog. But increase in A_{max} in frog is greater than that in rabbit (64). Caffeine contracture was difficult to observe in mammalian skeletal muscle, and was sometimes abortive. In the presence of a SERCA inhibitor, cyclopiazonic acid or thapsigargin, however, caffeine contracture can be unfailingly observed (65). With skinned fibers, caffeine-induced Ca^{2+} release

Table 1. Properties of A-sites and I-sites of CICR channels/RyR and alpha- and beta-RyR purified from bullfrog skeletal muscles

	A-site				I-site			
	Ca^{2+}		Mg^{2+}		Ca^{2+}		Mg^{2+}	
	K1(mM)	n1	K2(mM)	n2	K3(mM)	n3	K4(mM)	n4
alpha-RyR	0.010	2	0.3	1	2.4	1	2.8	1
beta-RyR	0.018	2	0.3	1	2.3	1	3.1	1
"CICR"	0.0025	~1.2	0.075	1	0.4	1	0.3	1

"CICR" means CICR channels/RyR in the SR of skinned frog skeletal muscle fibers.

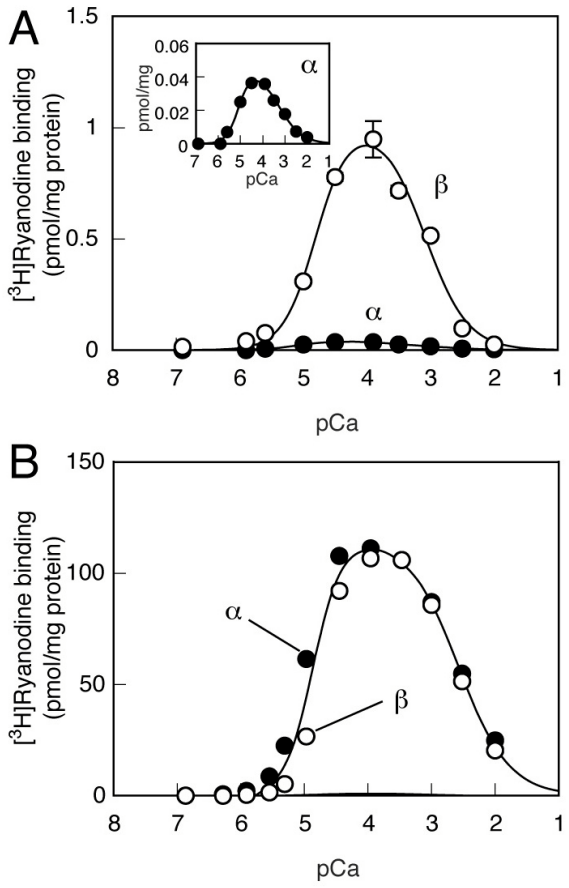


Figure 2. Selective suppression of alpha-RyR in the SR. A. [^{3}H]Ryanodine binding to alpha- and beta-RyR in the SR. B. [^{3}H]Ryanodine binding to purified alpha- and beta-RyR. [^{3}H]Ryanodine binding to beta-RyR in SR was equivalent to that of purified beta-RyR, considering the content of beta-RyR in SR and effect of the detergent used for purification. Note that Ca^{2+} dependence of alpha-RyR in SR (inset) was very similar to that of beta-RyR in SR, but that the peak value of the former was as low as 4% that of beta-RyR. alpha- and beta-RyR in SR were the same in the sensitivity to caffeine and AMPPCP as well as to Ca^{2+}. alpha-RyR in SR showed a lower affinity for [^{3}H]ryanodine without change in the maximum number of binding sites. For details, see ref. 66 (courtesy of the *Journal of Biological Chemistry*).

can be constantly observed in the absence of ATP. For the net Ca^{2+} release to be observed, the rate of this release must be in excess of the Ca^{2+}-pump activity. These findings indicate that the increase in A_{max} is a prevailing factor for caffeine-induced Ca^{2+} release.

6. SELECTIVE SUPPRESSION OF alpha-RYR IN SR IN CICR ACTIVITY

Because CHAPS and exogenous phospholipids which were used for purification of RyR was not inert to [^{3}H]ryanodine binding to RyR (54, 64), Murayama and Ogawa (66) examined whether RyR in SR and purified RyR were identical in this binding. The procedure for determination was as follows. The SR vesicles were subjected to [^{3}H]ryanodine binding in an isotonic medium containing Ca^{2+} and AMPPCP at room temperature, supplemented with an excess amount of non-radiolabeled ryanodine to prevent extra [^{3}H]ryanodine binding after the reaction had reached the steady state, and then were cooled down to about 4°C. Because the bound ligand would not dissociate at 4°C (67, 68), the following solubilization and separation of alpha- and beta-RyR were conducted at low temperature. After the SR vesicles were solubilized by CHAPS and phospholipids, alpha-RyR was immunoprecipitated with a monoclonal antibody against the alpha-RyR, beta-RyR remaining in the supernatant. The contents of alpha- and beta-RyR in SR vesicles were 45 and 55% respectively. Surprisingly, [^{3}H]ryanodine binding to alpha-RyR in the SR was as low as ~4% that of beta-RyR in the SR vesicles, which was comparable to that of purified beta-RyR (Figure 2). The two isoforms, however, showed no difference in sensitivity to Ca^{2+}, adenine nucleotide or caffeine. This reduced binding of alpha-RyR was ascribed to the low affinity for [^{3}H]ryanodine, with no change in the maximal number of binding sites. This means, in other words, that A_{max} for alpha-RyR in equation 1 will be markedly reduced in the SR vesicles. These results indicate that the CICR activity in the SR of frog skeletal muscle is carried out largely by beta-RyR. Murayama recently found that this was also the case with mammalian skeletal muscles (unpublished results). This is consistent with the finding by Shirokova et al. (69) that Ca^{2+} sparks were easily observable in frog skeletal muscles, but scarcely in mammalian skeletal muscles because of the scanty RyR3 in the latter.

Coexistence of beta-RyR cannot be the cause for the suppression of alpha-RyR in SR membrane, because a mixture of purified alpha- and beta-RyR shows the result of their simple addition. Solubilization of SR with CHAPS partly remedied this non-equivalence, whereas 1 M NaCl was ineffective. Accessory proteins which form the Ca^{2+} release channel complex might be responsible. 12 kDa

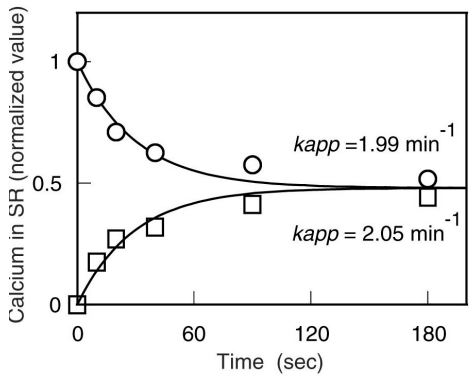


Figure 3. Ca^{2+} release from loaded SR (circles) and Ca^{2+} influx into the empty SR (squares). The medium Ca^{2+} concentration was 2 mM. The unit for "Calcium in SR" stands for the loading level after 2 min incubation with 0.0003 mM Ca^{2+} in the presence of 4 mM MgATP at room temperature. For details, see ref. 62 (courtesy of the *Biophysical Journal*).

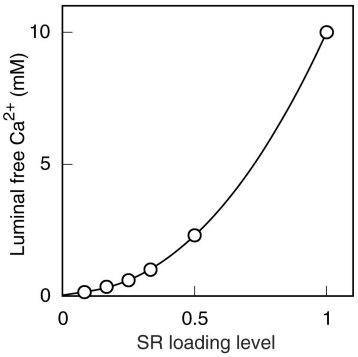


Figure 4. Non-linear relationship between the SR loading level and luminal free Ca^{2+} concentration. The luminal free Ca^{2+} concentration at the unit SR loading level (see Figure 3) was determined to be 10 mM. This level corresponds to 90% of the maximum loading and also to two- to three-fold of the physiological loading. The luminal Ca^{2+} buffer sites were assumed to be 14 mM with K_D of 1 mM. For details, see ref. 62.

FKBP (12 or 12.6), however, could not be responsible for the suppression, because FK506 treatment did not eliminate the suppression in contrast to marked removal of FKBP12 or 12.6 from alpha-RyR. Nor can calmodulin be the factor, because supplementation of calmodulin did not selectively affect it. At the present time, the underlying mechanism including the possibility of change in the intermolecular interaction among alpha-RyR monomers remains to be elucidated.

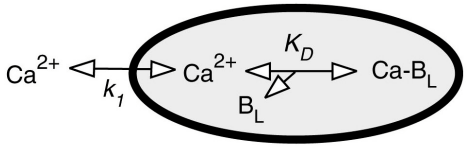
7. EFFECTS OF LUMINAL CA^{2+}

Calsequestrin and triadin are localized in the lumen of the terminal cisternae and their molecular interactions with RyR have been considered (17, 18, 22). Ca^{2+} release might be regulated by these proteins and/or luminal Ca^{2+} (70-73). Kurebayashi and Ogawa (62) addressed this problem using skinned fibers, determining the size and properties of the luminal Ca^{2+} binding sites. They also showed how Ca^{2+} release kinetics was apparently modulated by the Ca^{2+}-buffer sites and suggested the effect of luminal Ca^{2+} on the Ca^{2+} release channels. Because voltage-sensitive and caffeine-sensitive Ca^{2+} stores in skeletal muscles can be depleted simultaneously, the Ca^{2+} store in skeletal muscles can be regarded as a single compartment (65). Kurebayashi and Ogawa (62) observed in frog skinned fiber that Ca^{2+} influx into the empty SR occurred in a single exponential time course and that the rate constants were affected by Ca^{2+}, Mg^{2+}, adenine nucleotide, and procaine in the same manner as those in the CICR; they concluded that the Ca^{2+} influx occurred through the CICR channels, but in the direction opposite to the Ca^{2+} release. They also showed that the Ca^{2+} release from the loaded SR and the Ca^{2+} influx into the empty SR at a specified medium Ca^{2+} concentration reached the same final steady state level following single exponential time courses with almost the same rate constants (Figure 3). The steady state level was determined only by the Ca^{2+} concentration of the incubating medium, indicating that the medium and luminal free Ca^{2+} concentrations must be equilibrated at this level. The relationship of the steady state levels at various Ca^{2+} concentrations allowed them to determine the luminal Ca^{2+} binding sites: B_{max} = 38 mM, K_D = 5 mM, n_H (Hill coefficient) = 0.65; or B_{max} = 14 mM, K_D = 1 mM, n_H = 1.0. The former corresponds to the case of heterogeneous classes of binding sites, and the latter to the case of the homogeneous binding sites composed of calsequestrin and the luminal low affinity sites of Ca^{2+}-ATPase. In either case, the same conclusions can be reached in the following discussion where we will deal with the latter case alone, just for simplicity.

Because the SR membrane is permeable to K^+, the membrane potential difference can be easily compensated. Therefore, the driving force for Ca^{2+} release is primarily the Ca^{2+} concentration gradient between the medium and the lumen. A model simulation using parameters obtained here allows us the following predictions. First, the luminal free Ca^{2+} concentration changes in a non-linear relationship to SR loading level (Figure 4). Second, the calculated time courses for Ca^{2+} release or for Ca^{2+} influx did not follow simple exponential time courses. They could be approximated, however, by single exponential curves (Figure 5A). The apparent rate constant for Ca^{2+} release thus obtained was definitely greater than that for Ca^{2+} influx (Figure 5A), being at variance with the experimental results shown in Figure 3. This difference can be accommodated by the assumption that the luminal Ca^{2+} exerts an inhibitory effect on RyR1 which is expressed in the following equation:

$$k / k_1 = 1 - Ca_L / (K_i + Ca_L)$$

where k_1 stands for intrinsic rate constant for Ca^{2+} release, and Ca_L and K_i are the luminal Ca^{2+} concentration and the dissociation constant for inhibition. When K_i is assumed to be 2 mM, the apparent rate constants for Ca^{2+} release and Ca^{2+} influx in the medium containing 2 mM Ca^{2+} became similar to the observed value (~2 min^{-1}) (Figure 5B). The inhibition of the luminal Ca^{2+} also accounts for



$$k = k_1 * K_i / (K_i + Ca_L)$$

$$-\frac{dX}{dt} = k\,(Ca_L - Ca_C)$$

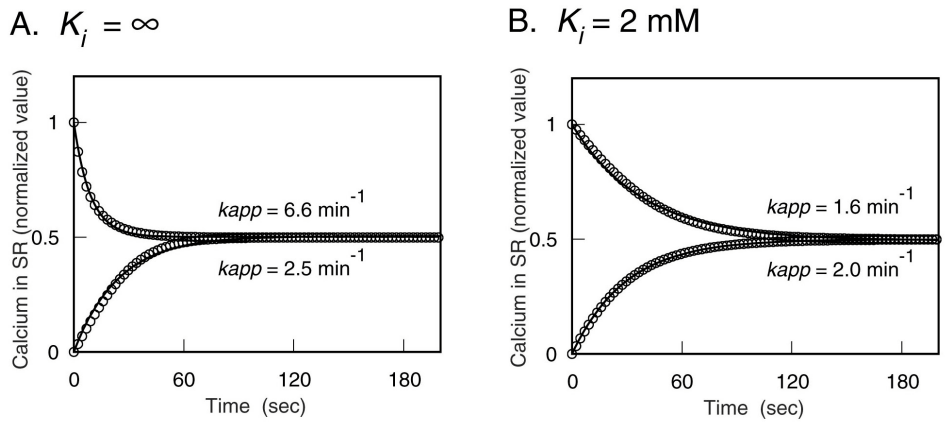


Figure 5. Calculated time courses of Ca^{2+} release from the loaded SR and of Ca^{2+} influx into the empty SR. The luminal Ca^{2+}-buffer sites (B_L) and their dissociation constant (K_D) were assumed to be 14 mM and 1 mM, respectively. Ca^{2+} association to and dissociation from B_L are assumed to be instantaneous. Ca^{2+} fluxes are driven by the free Ca^{2+} concentration gradient between the medium (Ca_C) and luminal sides (Ca_L) with a rate constant (k) as shown in the following equation: - dX / dt = k * (Ca_L – Ca_C) where X stands for total calcium in SR. k was a function of luminal free Ca^{2+} concentrations as expressed by $k = k_1 * K_i / (K_i + Ca_L)$ where k_1 (an intrinsic rate constant) was arbitrarily determined to be 10.5 min^{-1} here. In this case, Ca_C = 2 mM as is the case in Figure 3. A. Calculated time courses under the assumption of no inhibition of the luminal Ca^{2+} (open circles, total Ca^{2+}). The lines are best-fitted exponential curves with apparent rate constants (k_{app}) indicated in the Figure. Note that the calculated fluxes were approximated by single exponential curves with distinct k_{app} values (k_{app} for efflux > k_{app} for influx). For unit calcium in SR, refer to Figure 3. B. Assuming inhibition by luminal Ca^{2+}. In the equation, $k = k_1 * K_i / (K_i + Ca_L)$, K_i = 2 mM was assumed. Note that, then, k_{app} for efflux ≈ k_{app} for influx.

the observation that the apparent rate constants for Ca^{2+} release were almost independent of the initial loading level (62, see also ref. 75). It also suggests that the apparent rate constant may be dependent on the endpoint of the Ca^{2+} release.

8. BIOLOGICAL ROLES OF CICR

Table 2 shows our estimates of CICR in frog skinned skeletal muscle fibers under various conditions in a medium simulating the myoplasm and compares those with the rate constant for depolarization-induced Ca^{2+} release (61). Under the assumption of 4 and 1 mM for myoplasmic ATP and free Mg^{2+} concentrations, respectively, the rate constant for CICR at an activating Ca^{2+} (0.01 – 0.1 mM Ca^{2+}) would be as low as no more than 10 min^{-1} (see also Figure 1A), whereas the physiological depolarization-induced Ca^{2+} release is estimated to be as high as 1200 – 3000 min^{-1} (74, 75). If myoplasmic ATP concentration should be 8 mM (76), the rate constant for CICR would be at most twice that. Even if either A-site or I-site were freed of Mg^{2+} inhibition (76, 77), the CICR would be much less than 100 min^{-1} at an activating Ca^{2+} or 10 min^{-1} at a resting Ca^{2+} (cf. Figure 1A). These results indicate that the CICR is too slow to make a measurable contribution to physiological depolarization-induced Ca^{2+} release. This conclusion is supported by other lines of evidence in spite of the irrelevant criticism by Lamb *et al.* (76).

In frog skeletal muscle, alpha-RyR in situ is selectively suppressed in the CICR activity. Therefore, CICR must be conducted exclusively by beta-RyR. As shown previously, Ca^{2+} sensitivity of beta-RyR (EC_{50} of about 0.03 mM in the presence of 1mM Mg^{2+} (Figure 1A)) was much lower than that of the contractile system (EC_{50} of about 0.002 mM in the presence of 1 mM Mg^{2+} (Kurebayashi & Ogawa (78))). In mammalian skeletal muscle where RyR3 is miniscule, RyR1 in situ is also suppressed in CICR. In vertebrate skeletal muscle, irrespective of whether mammalian or non-mammalian, CICR contributes in only a minor way to the physiological Ca^{2+} release on depolarization.

Table 2. Comparison of the release rate by CICR with that of physiological depolarization-induced Ca^{2+} release

Conditions	Rate constant (min^{-1})
Physiological release rate	1200 - 3000
CICR in the presence of 4 mM ATP	
0 mM Mg^{2+}, at an activating Ca^{2+}	40
1 mM Mg^{2+}, at an activating Ca^{2+}	~10
+ 5 mM caffeine	~50
0 mM Mg^{2+}, at a resting Ca^{2+}	~5
1 mM Mg^{2+}, at a resting Ca^{2+}	0.03
with the A-site free of Mg^{2+} inhibition	~1.1

However, CICR has a critical contribution to such pathological conditions as malignant hyperthermia ((79,80), also refer to other chapters of this book). The mutated sites of RyR1 in malignant hyperthermia are classified largely into three regions: region 1 (residues 35-614), region 2 (residues 2162-2458) and region 3 (residues 4637-4898) (79, 80). When RyR1 mutated in regions 1 and 2 was expressed in dyspedic myotube, EC50 of the membrane potential for Ca2+ transient was shifted to the hyperpolarized side (81, 82). It would be interesting if this finding might have some relation with a de-suppression mechanism for reduced CICR activity of RyR1 in situ. In any event, we must keep in mind that the seizure is evident only when it is triggered by psychic stress and drugs such as halothane, but otherwise ECC appears to be normal. Further investigation is required.

9. PERSPECTIVES

There are two distinct modes for triggering Ca^{2+} release from the SR: depolarization-induced Ca^{2+} release (DICR) and Ca^{2+}-induced Ca^{2+} release (CICR). The direct interaction between DHPR and RyR is the trigger for the former, whereas the increased cytoplasmic Ca^{2+} is the trigger for the latter. It is noteworthy, however, that Ca^{2+} is a necessary though not a sufficient condition for CICR. alpha-RyR and RyR1 are responsible both for DICR and for CICR, but their CICR activity is selectively suppressed in situ; the underlying mechanism for this remains to be elucidated. beta-RyR and RyR3 appear not to be responsible for DICR, but their CICR activities were not suppressed. Therefore, CICR activity in frog skeletal muscles is largely conducted by beta-RyR. CICR activity in frog skeletal muscle is thought to be too low to make a significant contribution to Ca^{2+} release during depolarization.

Because the experimental system for CICR has been well established, understanding of CICR has deepened. Although some experimental models have been proposed for DICR, they are not satisfactory, and establishment of such a model is desired as early as possible. Despite differences in their triggering modes, it is quite probable that some modulators of CICR might also affect DICR. They share the following two characteristics: the driving force for Ca^{2+} release is primarily the luminal free Ca^{2+} concentration and the luminal free Ca^{2+} may exert an inhibitory effect on RyR.

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