Similar Ca^{2+} dependences of [^{3}H]ryanodine binding to α- and β-ryanodine receptors purified from bullfrog skeletal muscle in an isotonic medium

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Received 20 December 1995; revised version received 12 January 1996

Abstract To understand the functions of the two ryanodine receptor isoforms (α- and β-RyRs) in nonmammalian skeletal muscles, we determined [^{3}H]ryanodine binding to these isoforms purified from bullfrog skeletal muscle. In 0.17 M-NaCl medium, both isoforms demonstrated similar Ca^{2+} dependent ryanodine binding activities, while the Ca^{2+} sensitivity for activation of β-RyR was increased in 1 M-NaCl medium. This enhancement in Ca^{2+} sensitivity depended on the kind of salts used. These results imply that α- and β-RyRs may have similar properties as Ca^{2+}-induced Ca^{2+} release channels in bullfrog skeletal muscle.

Key words: Calcium ion dependence; Calcium ion release; Excitation–contraction coupling; Ryanodine receptor; Sarcoplasmic reticulum; Skeletal muscle (bullfrog)

1. Introduction

In vertebrate skeletal muscles, ryanodine receptor (RyR) functions as a Ca^{2+} release channel of sarcoplasmic reticulum (SR) [1–3]. RyR is believed to perform two distinct modes of Ca^{2+} release: 'depolarization-induced Ca^{2+} release (DICR)' that is triggered by depolarization of the T-tubule membrane irrespective of Ca^{2+} influx, and 'Ca^{2+}-induced Ca^{2+} release (CICR)' which requires Ca^{2+} for activation [4–7].

Frog and other non-mammalian skeletal muscles contain two isoforms of RyR, which are referred to as α-RyR and β-RyR on the basis of their mobilities on SDS-PAGE [3]. Their cDNA sequences for frog isoforms revealed that α- and β-RyR are most homologous to mammalian Ryr1 and Ryr3 which are derived from *ryr1* and *ryr3* genes, respectively [8]. It has been found that the *Crooked Neck Dwarf* mutant of chicken which lacks normal α-RyR failed to develop DICR on electrical or neuronal stimulation [9,10]. This suggests that α-RyR may function as the DICR channel in non-mammalian skeletal muscles. Little is known, however, about the role of β-RyR. We have reported that purified β-RyR of bullfrog was about 20 times as sensitive to Ca^{2+} as α-RyR in the Ca^{2+} dependent [^{3}H]ryanodine binding activity [11]. This leads to speculation that β-RyR could work in the manner of CICR as an amplifier of DICR through α-RyR in the SR. The experiments mentioned above, however, were carried out in a 1 M NaCl medium to stimulate the ryanodine binding [11]. To know the functions of α- and β-RyRs in vivo, determinations are desirable under an isotonic condition, e.g. in 0.17 M NaCl or KCl medium, because some differences in ryanodine binding to SR vesicles with or without CHAPS/phospholipids have been reported between 1 M and 0.17 M NaCl media [12,13].

2. Materials and methods

α- and β-RyRs were purified from SR vesicles from leg muscles of bullfrog (*Rana catesbeiana*), and determinations of [^{3}H]ryanodine binding to these isoforms were carried out as described [11]. Details are given in the legends to figures. Non-specific binding was determined in the presence of 50 μM unlabeled ryanodine.

[^{3}H]Ryanodine (60–90 Ci/mmol) was purchased from Du Pont New England Nuclear. Pure ryanodine was a generous gift from Wako Pure Chemical Industries. Egg lecithin (egg total phosphatide extract) was purchased from Avanti Polar-Lipids. Ruthenium red (45% purity) and procaine were from Sigma. All other reagents were of analytical grade. Free Ca^{2+} was calculated using values of 8.79×10^5 M^{-1} and 1.82×10^3 M^{-1} as the apparent binding constants for Ca^{2+} of EGTA [14] and of AMPOPCP [15], respectively, regardless of salt concentrations.

3. Results

Fig. 1 shows Ca^{2+} dependent [^{3}H]ryanodine binding activities of α- (panel A) and β-RyR (panel B) in 1 M NaCl medium. Both isoforms showed monophasically Ca^{2+} dependent [^{3}H]ryanodine binding in the absence of AMPOPCP or caffeine. Under the conditions used, β-RyR showed higher sensitivity for Ca^{2+} than α-RyR as reported previously [11]. The same situation was observed in the presence of AMPOPCP and/or caffeine. The results of similar determinations are summarized in Table 1 where the Ca^{2+} sensitivity is expressed as $(pCa)_{1/2}$, a pCa which would give half the maximal ryanodine binding. The differences in $(pCa)_{1/2}$ between α- and β-RyR were 0.9–1.3, indicating that β-RyR was 8–20 times as sensitive to Ca^{2+} as α-RyR.

In 0.17 M-NaCl medium (Fig. 2), in contrast, the Ca^{2+} dependences of α- and β-RyRs were very similar to each other under all conditions examined. Their Ca^{2+} sensitivities for activation were well consistent with those of α-RyR in 1 M NaCl medium and those of isolated SR vesicles under the corresponding conditions [12,16] (Table 1). This clearly indicates that the Ca^{2+} sensitivity of β-RyR was enhanced in 1 M NaCl medium, while that of α-RyR remained unchanged.

Figs. 1 and 2 reveal additional marked differences in ryanodine binding activities between 0.17 M and 1 M NaCl media. The maximal [^{3}H]ryanodine binding in the presence

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Abbreviations: AMPOPCP, β,γ-methylene adenosine triphosphate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CICR, Ca^{2+}-induced Ca^{2+} release; DICR, depolarization-induced Ca^{2+} release; EGTA, ethylene glycol bis (β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; MOPSO, 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid; RyR, ryanodine receptor; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SR, sarcoplasmic reticulum

SSDI S0014-5793(96)00053-1

Table 1
Summary of the apparent affinities for Ca^{2+} of α- and β-RyRs in the [^{3}H]ryanodine binding in 0.17 M and 1 M NaCl media

	$(pCa)_{1/2}$			
	α-RyR		β-RyR	
	0.17 M NaCl	1 M NaCl	0.17 M NaCl	1 M NaCl
None	5.05 ± 0.23 (3)*	4.56 ± 0.08 (3)	5.09 ± 0.16 (3)*	5.49 ± 0.03 (3)
AMPOPCP	5.04 ± 0.01 (4)	4.79 ± 0.03 (5)	5.06 ± 0.02 (4)	5.66 ± 0.06 (5)
Caffeine	5.25 ± 0.01 (2)*	5.56 ± 0.03 (2)	5.27 ± 0.06 (2)*	6.70 ± 0.01 (2)
AMPOPCP + caffeine	5.96 ± 0.00 (2)	5.81 ± 0.05 (2)	5.85 ± 0.08 (2)	7.12 ± 0.09 (2)

The results including those of Figs. 1 and 2 are summarized here. The apparent affinity for Ca^{2+} was indicated as $(pCa)_{1/2}$, i.e. negative logarithm of a Ca^{2+} concentration which would give the half-maximal ryanodine binding. The value was expressed as mean with S.E. (number of determinations) or with half range of the deviation in duplicate determinations. *Because of the small amount of ryanodine binding, these results are less reliable than the others.

of Ca^{2+} alone was no more than 10 pmol/mg protein in 0.17 M NaCl medium, while the counterpart in 1 M NaCl medium was about 200 pmol/mg protein which is near the saturation number of the sites. This small binding in 0.17 M NaCl medium can explain the lack of significant ryanodine binding to

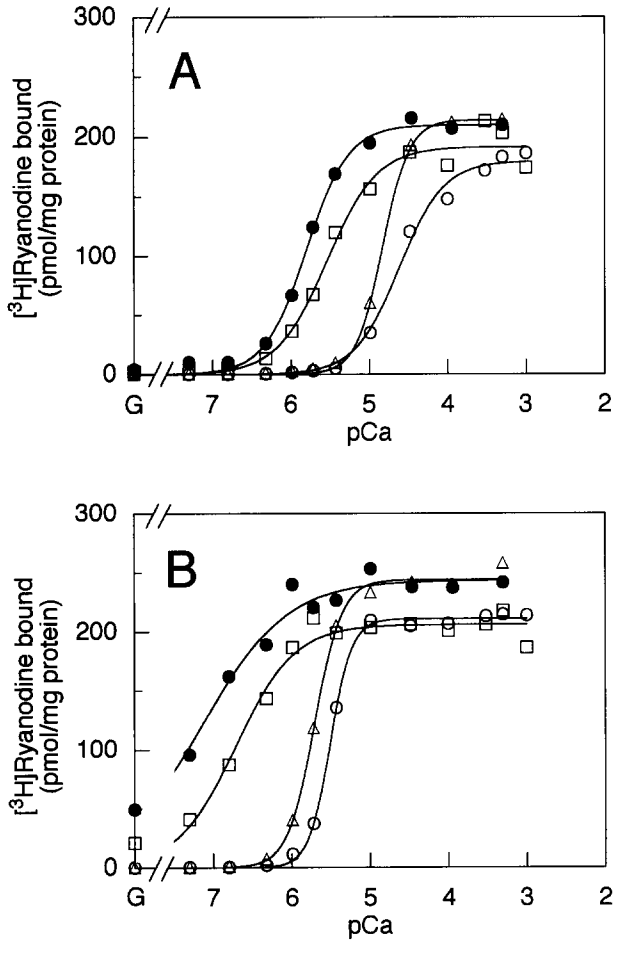

Fig. 1. Effect of Ca^{2+}, AMPOPCP, and caffeine on [^{3}H]ryanodine binding to the purified α- and β-RyRs in 1 M NaCl medium. α- (A) and β-RyR (B) were incubated with 8.5 nM [^{3}H]ryanodine for 3 h at 25°C in a buffer containing 1 M NaCl, 10 mM MOPSO/NaOH, pH 6.80, 1% CHAPS, 0.5% egg lecithin, and 2 mM dithiothreitol. Free Ca^{2+} lower than 20 μM was obtained using 1 mM $CaCl_2$ and a specified concentration of EGTA, while $CaCl_2$ was added to make the Ca^{2+} higher than 20 μM. Ligands other than Ca^{2+} were supplemented as follows; none (open circles), 1 mM AMPOPCP (open triangles), 10 mM caffeine (open squares), and 1 mM AMPOPCP plus 10 mM caffeine (closed circles). The results were means of duplicate or triplicate determinations.

SR vesicles under similar conditions [16]. One mM AMPOPCP markedly enhanced the binding (by a factor of more than 10 at pCa 4.5 for α- or β-RyR) with minor leftward shift of the pCa-binding curve in 0.17 M NaCl medium, while the stimulation was less marked in 1 M NaCl medium. Caffeine sensitized [^{3}H]ryanodine binding to Ca^{2+} activation with only slight increase in the maximal binding, in contrast to the effect of AMPOPCP. The effect of caffeine was weaker in 0.17 M NaCl-medium than that in 1 M NaCl-medium or that with SR vesicles in 0.17 M and 1 M NaCl medium [12,16]. The combined effect of AMPOPCP and caffeine appeared potentiating in 0.17 M NaCl medium, while it was additive in 1 M NaCl medium. Another marked difference is disappearance of the inhibitory action by high concentrations of Ca^{2+} in 1 M NaCl medium, while the inhibition is great in 0.17 M NaCl medium. Ca^{2+} dependence in 0.17 M NaCl medium was biphasic: Ca^{2+} lower than 0.1 mM was stimulatory but it was inhibitory at above 0.1 mM Ca^{2+}. In 1 M NaCl medium, in contrast, no inhibition was observed up to 1 mM Ca^{2+}.

We have reported that the Ca^{2+} sensitivity of isolated SR vesicles was enhanced when the vesicles were solubilized with CHAPS/phospholipids in 1 M NaCl medium [13]. This enhancement of Ca^{2+} sensitivity is consistent with the increased Ca^{2+} sensitivity for β-RyR and the unchanged Ca^{2+} sensitivity for α-RyR in 1 M NaCl medium. As shown in Table 1 and Fig. 2, the Ca^{2+} sensitivity was not increased in 0.17 M-NaCl medium; thus both 1 M NaCl and CHAPS/phospholipids were required for its enhancement. The Ca^{2+} sensitivity increased with the increase in NaCl concentration up to 1 M (data not shown). To know whether this Ca^{2+} sensitizing effect of NaCl is due to high salt concentrations or to specific ions, we examined the [^{3}H]ryanodine binding to β-RyR in media containing various kinds of salts at 1 M (Fig. 3). In a medium containing 1 M NaCl or KCl, β-RyR showed increased Ca^{2+} sensitivities ($(pCa)_{1/2}$s were 5.53 and 5.72 in NaCl and KCl media, respectively) (Fig. 3A). In a 1 M sodium propionate (NaPro) medium, in contrast, Ca^{2+} sensitivity for β-RyR was lower, i.e. $(pCa)_{1/2}$ of 4.78, which was similar to that for α-RyR in 1 M NaCl medium. The amount of the binding at pCa 5.4 was more than 50% of the maximum in NaCl or KCl medium, while significant binding was not observed in NaPro medium at pCa 5.4. We therefore determined [^{3}H]ryanodine binding at pCa 5.4 and at the optimal Ca^{2+} concentration (pCa 3.9) in the presence of various kinds of salts at 1 M (Fig. 3B). The increased Ca^{2+} sensitivity is neither due to ionic strength nor to the pharmacological action of Cl^-, because Ca^{2+} sensitivity remained unchanged in

Table 2
Effect of Mg^{2+}, ruthenium red, and procaine, on the [^{3}H]ryanodine binding to α- and β-RyRs in 0.17 M and 1 M NaCl media

	% Ryanodine bound			
	α-RyR		β-RyR	
	0.17 M NaCl	1 M NaCl	0.17 M NaCl	1 M NaCl
None	100	100	100	100
$MgCl_2$, 1 mM	47.8	82.0	54.1	87.2
$MgCl_2$, 10 mM	10.5	73.7	14.1	74.5
Ruthenium red, 1 μM	2.3	85.3	1.9	93.3
Ruthenium red, 10 μM	5.7	19.1	3.9	64.9
Ruthenium red, 100 μM	5.1	20.0	0.9	35.0
Procaine, 10 mM	7.0	35.7	5.1	78.3

[^{3}H]Ryanodine binding was performed as in Figs. 1 and 2 except for 5.5 nM [^{3}H]ryanodine, 1 mM AMPOPCP and 0.3 mM $CaCl_2$ (pCa 3.9) in the presence of indicated CICR inhibitors. The absolute values in the absence of inhibitors for α- and β-RyR which were referred to as 100 were 91.4 and 108.4 pmol/mg protein in 0.17 M-NaCl medium, and 203.6 and 212.0 pmol/mg protein in 1 M-NaCl medium, respectively. The results were means of duplicate determinations.

LiCl and TrisCl media, although it increased in NaCl, KCl, CsCl and CholineCl. It should be mentioned that the effect of CsCl was dependent on AMPOPCP: ryanodine binding in 1 M CsCl medium was greatly reduced (to no more than 10% of the maximum value) in the absence of AMPOPCP, unlike cases shown in Fig. 3B which were determined in its presence (data not shown).

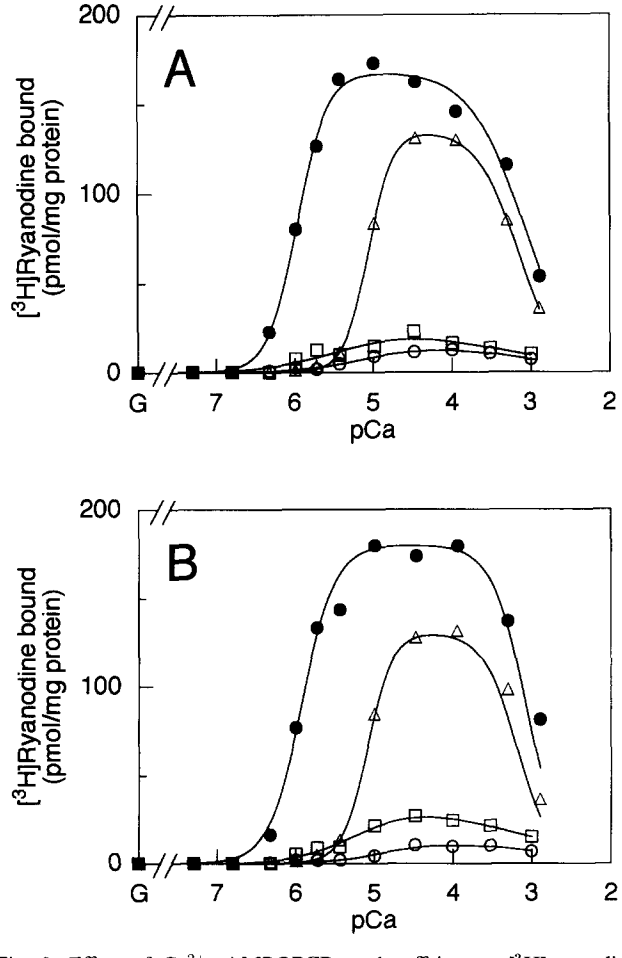

Fig. 2. Effect of Ca^{2+}, AMPOPCP, and caffeine on [^{3}H]ryanodine binding to the purified α- and β-RyRs in 0.17 M-NaCl medium. The determinations were performed as in Fig. 1 except that 0.17 M NaCl was used instead of 1 M NaCl. Symbols are the same as in Fig. 1. The results were means of duplicate determinations. A, α-RyR; B, β-RyR.

In 1 M NaCl medium, disinhibition of [^{3}H]ryanodine binding at higher Ca^{2+} concentrations was noted (Fig. 1). We examined the effect of other CICR inhibitors to learn if this were also the case in 1 M NaCl medium. As summarized in Table 2, reductions of [^{3}H]ryanodine binding by CICR inhibitors were more marked in 0.17 M NaCl medium than in 1 M NaCl medium. While 1 mM and 10 mM Mg^{2+} decreased ryanodine binding to about half and 10–15% of the control values in the 0.17 M NaCl medium, respectively, about three quarters of the activity were still detected even in the presence of 10 mM Mg^{2+} in 1 M NaCl medium. In the presence of 1 μM ruthenium red which completely inhibited the binding in 0.17 M NaCl medium, the binding decreased only slightly in 1 M NaCl medium, and more than 20% of the control was still retained even at 100 μM. The inhibition by procaine was also weak in 1 M NaCl medium. A similar conclusion that the inhibitory action of these reagents was weaker in the 1 M NaCl medium was also obtained with RyR from rabbit skeletal muscle [17] and brain microsomes [18]. It should be noted that β-RyR was more resistant to ruthenium red and procaine in 1 M NaCl medium, consistent with the results with tetracaine by O'Brien et al. [19].

4. Discussion

Ryanodine is considered to be a good probe for investigating the functional state of RyRs [20,21]. It is reasonable to think that the [^{3}H]ryanodine binding activities of RyRs well reflect their CICR channel activities [3,20,21]. In this study, α- and β-RyRs showed almost identical [^{3}H]ryanodine binding activities including their sensitivities to Ca^{2+} activation under an isotonic salt concentration of 0.17 M NaCl. This indicates that the two isoforms may have similar CICR channel properties in vivo. This conclusion is strongly supported by the finding that our results of Fig. 2 are consistent with the rates of CICR from SR in skinned frog skeletal muscle fibers under corresponding conditions, i.e. in the absence and presence of AMPOPCP and/or caffeine [3,22]. Our conclusion that the two isoforms are equally potent as CICR channels is at variance with the speculation that β-RyR may serve as a CICR channel that amplifies Ca^{2+} release [19,23]. However, the possibility that the dihydropyridine receptor or some other component(s) in vivo may modulate the channel activities of RyR remains to be confirmed.

Several groups have recently reported the properties of two

RyR isoforms in non-mammalian skeletal muscles [19,23,24]. Bull and Marengo [24] reported two types of Ca^{2+}-release channels with distinct Ca^{2+} dependences in SR vesicles isolated from frog skeletal muscle, one of biphasic dependence with marked inhibition at high Ca^{2+} concentrations and the other of monophasic dependence with little inhibition. Similar results were obtained from SR vesicles of toadfish white swimming muscles by O'Brien et al. [19]. Percival et al. [23] investigated the ion channel properties of α- and β-RyRs partially purified from SR of chicken skeletal muscle and reported that the two isoforms embodied Ca^{2+} channels with similar conductances but different gating properties on activation by Ca^{2+} and ATP, and on inactivation by Ca^{2+}. Thus these investigators have concluded that the two RyR isoforms in non-mammalian skeletal muscles have distinct CICR properties, in contrast to our conclusion. It is possible that this is due to differences of the species examined, in view of the limited immunologic cross-reactivities among α-RyRs from different species [25].

The activity of CICR with skinned muscle fibers or with isolated SR vesicles which contain the two RyR isoforms is considered to be summation of the contribution from each isoform. In frog, [^{3}H]ryanodine binding to isolated SR vesicles [16] and CICR activity of SR in skinned skeletal muscle fibers [3,22] are consistent with [^{3}H]ryanodine binding to α-and β-RyR in 0.17 M NaCl medium where they are equally potent (Fig. 2). In contrast, if the two isoforms have different Ca^{2+} dependences, SR where they coexist would show an intermediate Ca^{2+} dependence, as Murayama and Ogawa demonstrated with solubilized SR in 1 M NaCl medium [13]. It is therefore interesting to know the Ca^{2+} dependence of CICR in muscles from species which are claimed to show different Ca^{2+} dependences of α-and β-RyRs [19,23,24].

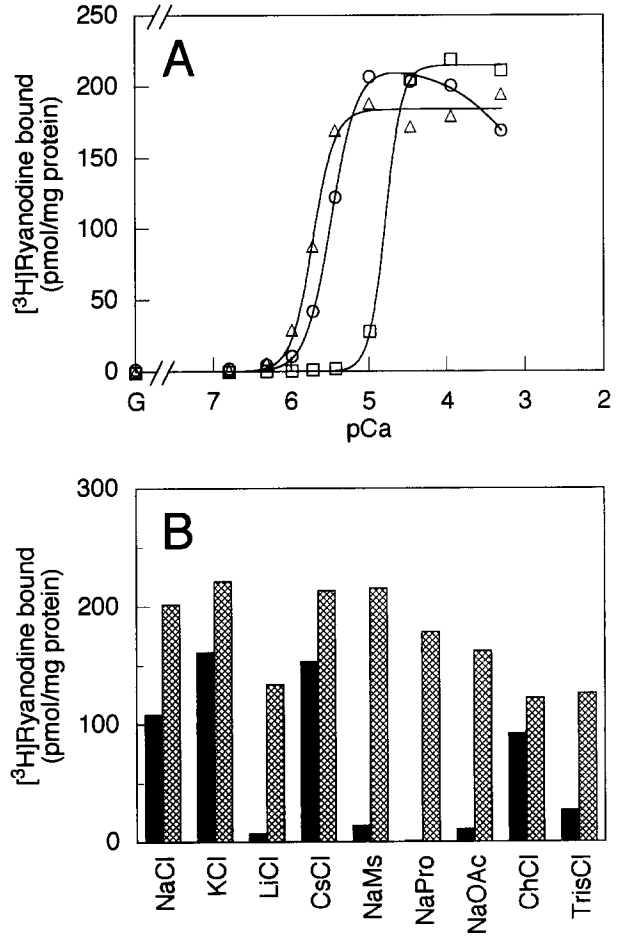

Fig. 3. Differential effects of various kinds of salts on Ca^{2+}-dependent [^{3}H]ryanodine binding to β-RyR. Determinations were carried out as in Fig. 1 in the presence of 1 mM AMPOPCP. A, Ca^{2+} dependences of [^{3}H]ryanodine binding to β-RyR in the medium containing 1 M of NaCl (circles), KCl (triangles), or sodium propionate (squares). B, [^{3}H]Ryanodine binding in the presence of various salts at the concentration of 1 M. Free Ca^{2+} concentrations of the medium were pCa 5.4 (filled column) and 3.9 (hatched column). The results were means of 2–4 determinations. NaMs, sodium methanesulfonate; NaPro, sodium propionate; NaOAc, sodium acetate; ChCl, choline chloride.

In mammalian skeletal muscles, Ryr1 functions as the DICR channel, because skeletal muscles of *ryr1*-targeted mice (dispedic mice) fail to show DICR from SR [26]. Recently, mRNA for Ryr3 has been detected in mammalian skeletal muscles [26,27]. However, the amount of the isoform is considered to be very minute, because only a single band corresponding to Ryr1 is visible on SDS-PAGE of SR vesicles from these muscles in contrast to two bands which are observed in SR vesicles from bullfrog skeletal muscle. Takeshima et al. [26] reported that the CICR activity in the dispedic mice in which mRNA for Ryr3 was detected was about 10 times less sensitive to Ca^{2+} than that in the control mice. One possible reason for the different result might be the difference in species. A minute amount of Ryr3 in mammalian skeletal muscles might be another possible explanation. The modulation of CICR by component(s) other than RyR could also be considered as a reasonable explanation. In this connection, it is noted that CICR in the dispedic mice was also less sensitive to adenine nucleotide or caffeine [26].

Properties of [^{3}H]ryanodine binding in 1 M NaCl medium may represent other characteristics of Ca^{2+} release channels which RyRs could show. RyR from mammalian cardiac muscle (Ryr2) is reported to have higher Ca^{2+} sensitivity than mammalian Ryr1 and to be much less sensitive to stimulation by ATP and inhibition by high Ca^{2+} concentrations [1–3]. These seem similar to the results of [^{3}H]ryanodine binding in 1 M NaCl medium which were described here. Thus Ryr2 might easily take a conformation which would correspond to the one assumed here. The slight effect of inhibitors of CICR in 1 M NaCl medium is also interesting because DICR is reported to be insensitive to local anesthetics such as procaine and tetracaine [4,22].

Acknowledgements: This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan and by the Uehara Memorial Foundation. We thank Dr. Nagomi Kurebayashi for her helpful discussion and Miss Naomi Ariji for her expert secretarial assistance.

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