Role of Mg^{2+} in Ca^{2+}-Induced Ca^{2+} Release through Ryanodine Receptors of Frog Skeletal Muscle: Modulations by Adenine Nucleotides and Caffeine

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ABSTRACT Mg^{2+} serves as a competitive antagonist against Ca^{2+} in the high-affinity Ca^{2+} activation site (A-site) and as an agonist of Ca^{2+} in the low-affinity Ca^{2+} inactivation site (I-site) of the ryanodine receptor (RyR), which mediates Ca^{2+}-induced Ca^{2+} release (CICR). This paper presents the quantitative determination of the affinities for Ca^{2+} and Mg^{2+} of A- and I-sites of RyR in frog skeletal muscles by measuring [^{3}H]ryanodine binding to purified α- and β-RyRs and CICR activity in skinned fibers. There was only a minor difference in affinity at most between α- and β-RyRs. The A-site favored Ca^{2+} 20- to 30-fold over Mg^{2+}, whereas the I-site was nonselective between the two cations. The RyR in situ showed fivefold higher affinities for Ca^{2+} and Mg^{2+} of both sites than the purified α- and β-RyRs with unchanged cation selectivity. Adenine nucleotides, whose stimulating effect was found to be indistinguishable between free and complexed forms, did not alter the affinities for cations in either site, except for the increased maximum activity of RyR. Caffeine increased not only the affinity of the A-site for Ca^{2+} alone, but also the maximum activity of RyR with otherwise minor changes. The results presented here suggest that the rate of CICR in frog skeletal muscles appears to be too low to explain the physiological Ca^{2+} release, even though Mg^{2+} inhibition disappears.

INTRODUCTION

The ryanodine receptor (RyR) is a Ca^{2+} release channel on the sarcoplasmic reticulum (SR) (Coronado et al., 1994; Meissner, 1994; Ogawa, 1994; Sutko and Airey, 1996). The RyR is a large (~2.3 MDa) homotetrameric protein complex and forms the "foot" structure in situ, which spans the gap between transverse tubule and terminal cisternae of SR (Franzini-Armstrong and Protasi, 1997; Wagenknecht and Radermacher, 1997). To date, three genetically distinct isoforms of RyR (RyR1–3) have been identified in mammalian tissues. RyR1 is a primary isoform in mammalian skeletal muscles and plays an important role in excitation-contraction (E-C) coupling. In many skeletal muscles of nonmammalian vertebrates, in contrast, two isoforms of RyR, α- and β-RyRs, which are homologs of mammalian RyR1 and RyR3, respectively, coexist in nearly equal amounts (Ogawa, 1994; Sutko and Airey, 1996).

The RyR shows properties of Ca^{2+}-induced Ca^{2+} release (CICR), which is modulated by several endogenous and exogenous ligands (Coronado et al., 1994; Meissner, 1994; Ogawa, 1994). Among them, Mg^{2+}, adenine nucleotides, and caffeine have attracted much interest because of their physiological and pharmacological relevance (Endo, 1977, 1981). The myoplasmic ATP concentration of the skeletal muscle is reported to be 3–9 mM, of which the major form is MgATP (more than 90%) (Godt and Maughan, 1988), and the free Mg^{2+} concentration is estimated to be ~1 mM (Westerblad and Allen, 1992; Konishi et al., 1993).

CICR activity of RyR is biphasically regulated by Ca^{2+}: micromolar Ca^{2+} activates the channel, whereas millimolar Ca^{2+} inhibits it. The biphasic effect of Ca^{2+} suggests the existence of two classes of Ca^{2+} sites: a high-affinity Ca^{2+} activation site (A-site) and a low-affinity Ca^{2+} inactivation site (I-site) (Meissner, 1994; Ogawa, 1994). Mg^{2+} decreases the peak value of CICR activity with a reduction in the Ca^{2+} sensitivity for activation (Endo, 1977, 1981). The extent of inhibition by Mg^{2+} depends on the Ca^{2+} concentration, in marked contrast to the case of procaine, which shows Ca^{2+}-independent inhibition (Kurebayashi and Ogawa, 1998). This is accounted for by the dual effects of Mg^{2+} on the two sites as follows: a competitive antagonist against Ca^{2+} in the former site and an agonist in the latter site (Endo, 1981; Laver et al., 1997; Meissner et al., 1997). It should be noted, however, that the effect of Mg^{2+} was assumed to be due primarily to competitive antagonism on the A-site in many experiments with skinned or cut fibers (Lamb and Stephenson, 1991; Jacquemond and Schneider, 1992; Lacampagne et al., 1998).

It is well known that ATP increases CICR activity without changing its Ca^{2+} dependence (Endo, 1981). However, it remains unknown whether ATP affects Mg^{2+} sensitivity. This is due to difficulty in analyzing the effect of Mg^{2+} in the presence of ATP, because it is unclear whether MgATP is as potent as free ATP in stimulating CICR. Caffeine is known to stimulate CICR activity by increasing the Ca^{2+} sensitivity for activation (Endo, 1981). However, its effect on Ca^{2+} inactivation is unclear, and whether the affinity for Mg^{2+} of either the A-site or the I-site is affected remains to be determined. To understand the physiological and pharmacological roles of the CICR activity of RyR in situ,

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knowledge of the affinities for Mg^{2+} of these A- and I-sites and their modulations by endogenous ligands or drugs is essential. In frog muscle, where two isoforms are coexpressed, the role of each isoform in E-C coupling cannot be discussed without knowing the CICR activity of each one in the presence of ~1 mM Mg^{2+}.

In this study, we examined the affinity for Mg^{2+} as well as for Ca^{2+} of the A- and I-sites of α- and β-RyRs on the basis of the model for the actions of Ca^{2+} and Mg^{2+}. We used a [^{3}H]ryanodine binding assay with the two isoforms purified from bullfrog skeletal muscle, which enabled us to analyze the effect of Mg^{2+} on the individual isoforms (Murayama and Ogawa, 1992, 1996). Because it has been reported that affinities for Ca^{2+} and Mg^{2+} are affected by many factors, including ionic or nonionic solutes, detergents, and phospholipids (Ogawa et al., 1999), their affinity must be assessed under conditions that are as close as possible to the physiological environment. Taking advantage of the finding that there was only a minor difference at most in the [^{3}H]ryanodine binding activity between the two purified isoforms, we extended the same analysis procedure to the CICR experiments to determine affinities for Ca^{2+} and Mg^{2+} of both A- and I-sites of the Ca^{2+} release channel, using frog skinned skeletal muscle fibers; in these the molecular organization of biological components involved in Ca^{2+} release from SR is well maintained (Kurebayashi and Ogawa, 1986, 1998). Based on the results of these experiments, we will discuss the role of Mg^{2+} in the CICR activity of RyRs in situ, with particular reference to the effects of adenine nucleotides and caffeine. A preliminary report appeared earlier (Murayama et al., 1998a).

MATERIALS AND METHODS

Materials

[^{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. All other reagents were of analytical grade. Concentrations of AMP and β-, γ-methyleneadenosine triphosphate (AMPPCP) were determined by spectrometry, using molar extinction coefficients at 259 nm of 15.4×10^3 and 14.2×10^3 M^{-1} cm^{-1}, respectively. Free Ca^{2+} and Mg^{2+} concentrations were calculated using dissociation constants as follows: EGTA for Ca^{2+} (pK_{app} = 5.94 for [^{3}H]ryanodine binding, 6.02 and 6.43 for CICR experiments at pH 6.8 and 7.0, respectively) was taken from Harafuji and Ogawa (1980), EGTA for Mg^{2+} (pK_{app} = 0.49 at pH 6.8) was from Martell and Smith (1974), AMPPCP for Ca^{2+} and Mg^{2+} (pK_a = 7.7; pK_{Ca} = 4.16; pK_{Mg} = 4.68) was by Ogawa et al. (1986), and AMP for Ca^{2+} and Mg^{2+} (pK_a = 6.26; pK_{Ca} = 1.86; pK_{Mg} = 1.92) was by Khan and Martell (1967).

[^{3}H]Ryanodine binding

α- and β-RyRs were purified from the heavy fraction of SR vesicles of bullfrog skeletal muscle (Murayama and Ogawa, 1992). Assays of [^{3}H]ryanodine binding to each of the two isoforms were carried out as described in Murayama and Ogawa (1996), with some modifications. Purified RyR (1–2 μg) was incubated with 8.5 nM [^{3}H]ryanodine for 5 h at 25°C in 200 μl of a reaction medium containing 0.17 M NaCl, 20 mM 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid (MOPSO)/NaOH (pH 6.8), 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (CHAPS), 0.5% phospholipids, 2 mM dithiothreitol, and 4 mM AMP. A free Ca^{2+} concentration was set by mixing 10 mM EGTA with a specified amount of $CaCl_2$. In experiments with Mg^{2+}, $MgCl_2$ was added to the medium and NaCl was reduced to keep the ionic strength constant. This care is critical, particularly in the presence of high Ca^{2+} and Mg^{2+} concentrations, because the increased ionic strength may weaken the inhibition (Murayama and Ogawa, 1996; Murayama et al., 1998b). Samples were then filtered through Whatman GF/B glass filters that had been soaked with 2% polyethylenimine. Filters were rinsed twice with ice-cold water and dried. Radioactivity retained on the filters was counted in a liquid scintillation counter. Nonspecific binding was determined in the presence of 50 μM unlabeled ryanodine.

CICR experiments

A single skinned muscle fiber was obtained from the iliofibularis muscle of *Rana japonica* and mounted in the experimental chamber for optical determination of Ca^{2+} concentrations as described (Kurebayashi and Ogawa, 1998; Murayama et al., 1998b). All experiments were performed at a temperature of 16°C.

Table 1 shows the composition of solutions used for the experiments. A relaxing solution (RS) and Ca^{2+} loading solution (LS) contained 4 mM MgATP and 1 mM free Mg^{2+}, and the other solutions, i.e., three kinds of washing solutions (W1-W3), test solutions (TS), and a discharging solution (DS), did not contain ATP, to avoid active transport of Ca^{2+} into SR by Ca^{2+} pump. W3 and DS contained 0.1 mM EGTA and 1 μM fura-2 for determination of Ca^{2+} released from SR. TS contained 10 mM EGTA and 0–10 mM total calcium to achieve a desired cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_C$) when $[Ca^{2+}]_C \leq 0.1$ mM. If $0.1 < [Ca^{2+}]_C < 10$ mM, it contained 10 mM $CaCl_2$ and EGTA calculated as $(10 - [Ca^{2+}]_C)$ mM. AMP, AMPPCP, Mg^{2+}, and/or caffeine were added to the TS if necessary.

The experimental protocol for determinations of the activity of CICR in skinned fibers was similar to that described (Kurebayashi and Ogawa, 1998). The rate of Ca^{2+} release was determined by the rate of decrease in the total amount of calcium remaining in the SR (Ca in SR) after a certain stimulus, as follows. A skinned fiber was initially treated with DS to empty SR of Ca^{2+}, and then SR was actively loaded to a constant level (prescriptive loading level) by incubation with LS (pCa 6.5) for 2 min. After

TABLE 1 Composition of experimental solutions

Solution	EGTA/K_2 (mM)	Ca/X_2* (mM)	Mg/X_2* (mM)	Fura 2/K_5 (μM)	ATP/Na_2 (mM)	Others (mM)
RS	2	—	5	—	4.3	—
LS	2	0.5	5	—	4.3	—
W1	10	—	1	—	—	—
W2	10	—	—	1.0	—	—
W3	0.1	—	—	1.0	—	—
TS	7–10†	0–10†	‡	—	—	‡
DS	0.1	—	—	1.0	—	25 caffeine 1 AMP

All solutions contained 20 mM 3(*N*-morpholino) propanesulfonic acid and 2 μg ml^{-1} leupeptin. Their pHs were adjusted to 6.8 with KOH and ionic strengths to 0.16 with KCl or K-methanesulfonate (Ms).

*X was Cl in experiments in Fig. 4 and Ms in Figs. 5–7.

†Free Ca^{2+} concentration was made by mixing EGTA/K_2 and Ca/X_2 as follows: $[Ca^{2+}] \leq 0.1$ mM, 10 mM EGTA plus calculated Ca/X_2; 0.1 mM $< [Ca^{2+}] <$ 10 mM, 10 mM Ca/X_2 plus calculated EGTA.

‡Mg^{2+}, AMP, AMPPCP, and caffeine were added if necessary.

removal of ATP by washing successively with W1 for 60 s and W2 for 30 s, the skinned fiber was treated with a TS for a specified period ($t \geq 3$ s). The fiber was then successively washed with W1, W2, and W3 and challenged with DS to discharge all releasable Ca^{2+} in SR. The amount of discharged Ca^{2+} (Ca in SR) was determined from the fluorescence ratio signal of fura-2 in DS. The protocol was repeated with the same fiber. The prescriptive loading level without TS treatment was determined in every three to five series of experiments for the standard in calibration, and Ca in SR remaining after incubation for a specified period (t) in TS was expressed as its relative value (Y). Decay of Ca in SR apparently follows a first-order kinetics in TS, and the final steady level, S, is dependent on cytoplasmic $[Ca^{2+}]$ ($[Ca^{2+}]_C$), as described previously (Kurebayashi and Ogawa, 1998). The time course of Ca^{2+} release can be best fitted by the equation $Y = (1 - S) \times \exp(-k_{app} \times t) + S$, where k_{app} stands for an apparent rate constant. k_{app} is a measure of the Ca^{2+} release channel activity. S was practically zero at 0.02 mM $[Ca^{2+}]_C$ (pCa 4.7) or less. At $[Ca^{2+}]_C$ higher than 0.02 mM, S was determined by incubating the fiber for 300 s in the TS solution.

Corrections for k_{app} at mM cytoplasmic Ca^{2+}

As described above, the obtained k_{app} is the value of the decrease in total calcium in SR (Ca in SR), i.e., the sum of free Ca^{2+} and bound calcium in SR, under the assumption that its time course may be approximated in a first-order kinetics. In principle, the Ca^{2+} flux is caused by the downhill movement of free Ca^{2+} through a channel showing an intrinsic permeability. Therefore, if all of the Ca^{2+} stored in the SR were free, the rate constant for the Ca^{2+} release would be equal to the intrinsic rate constant. The presence of the massive Ca^{2+}-binding sites in the lumen, such as calsequestrin and Ca^{2+}-ATPase, however, may lower the concentration of free Ca^{2+} with the same total amount of calcium in SR. In this case, the decrease in the luminal free Ca^{2+} concentration during Ca^{2+} release is buffered by the luminal bound Ca^{2+}. The apparent rate constant for this Ca^{2+} release, k_{app}, will be smaller than the intrinsic rate constant, even if Ca^{2+} instantaneously dissociates from the binding sites. In the Ca^{2+}-loading conditions mentioned here, the luminal free Ca^{2+} concentration is estimated to be 10 mM, because Ca in SR was unchanged after the incubation with a solution of 10 mM Ca^{2+} in the absence of Ca^{2+} pump activity by removal of ATP, whereas it was increased and decreased after the incubation with a higher and lower Ca^{2+} concentration, respectively (Kurebayashi and Ogawa, 1998). We also found that the luminal Ca^{2+}-binding sites might be homogeneous and independent, and that the total sites would be 14 mM with $K_D = 1$ mM. Therefore, Ca in SR at the initial loading level is calculated to consist of 13 mM bound calcium and 10 mM free Ca^{2+}. The apparent rate constant for the Ca^{2+} release from the SR at the loading level to a solution of a high Ca^{2+} concentration, say 3 mM, is greater than that to a low Ca^{2+} concentration solution, say 10 μM, for the reason mentioned below. The final luminal free Ca^{2+} and bound calcium are calculated to be 3 and 10 mM in the former case, and 0.01 and 0.14 mM in the latter case, respectively. In the former, Ca^{2+} release was largely driven by change in free Ca^{2+}, whereas there was a more marked contribution of the bound calcium in the latter. This difference in the apparent rate constant that depends on the Ca^{2+} concentration of the incubation medium must be corrected for. Furthermore, we have suggested that the luminal Ca^{2+} exerts an inhibitory effect on the intrinsic rate constant of the Ca^{2+} release channel with $K_i \approx 2$ mM (Kurebayashi and Ogawa, 1998). Taking these findings into consideration, the correction factors at various Ca^{2+} concentrations were obtained as follows: 1.00 at pCa > 4.9, 1.02 at pCa 4.6, 1.05 at pCa 4.0, 1.15 at pCa 3.5, 1.44 at pCa 3.0, and 1.75 at pCa 2.5. For the analysis of CICR activity at a high $[Ca^{2+}]_C$, k_{app} was divided by this factor, and the corrected value was denoted as k'_{app}. This correction, however, actually affects only the dissociation constant for Ca^{2+} in the I-site. The other parameters for the A-site or I-site were not significantly affected.

Data analysis

The results of [^{3}H]ryanodine binding and CICR experiments were fitted to equations obtained according to a model for the actions of Ca^{2+} and Mg^{2+} on the Ca^{2+} release channel to yield parameters for these divalent cations of the two Ca^{2+} sites (see Results). The curve fit was performed using nonlinear regression by Sigma Plot, version 5 for Macintosh (Jandel Scientific). Data are expressed as means ± SE, except as otherwise stated.

RESULTS

[^{3}H]Ryanodine binding to the purified α- and β-RyRs

Effect of Mg^{2+}

[^{3}H]Ryanodine binding to α- and β-RyRs that were purified from bullfrog skeletal muscle was carried out in an isotonic medium containing 0.17 M NaCl. In such a medium frog RyRs show only a small amount of [^{3}H]ryanodine binding (~10 pmol/mg protein) without any added ligand other than Ca^{2+}, even in the presence of its optimal concentrations (Ogawa and Harafuji, 1990; Murayama and Ogawa, 1996). Therefore, 4 mM AMP, which showed very weak affinity for divalent cations, was added to the medium to stimulate the activity of RyR without significant change in its Ca^{2+} sensitivity. The binding at the optimal Ca^{2+} concentration in the absence of Mg^{2+} amounted to 100–120 pmol/mg protein for both isoforms.

Fig. 1 shows the effects of Mg^{2+} on the Ca^{2+}-dependent [^{3}H]ryanodine binding to α-RyR. The Ca^{2+}-dependent [^{3}H]ryanodine binding was biphasic in the absence of Mg^{2+}: Ca^{2+} was stimulatory at a concentration lower than 0.1 mM and inhibitory at a higher concentration (Fig. 1 *A*, *open circles*). Mg^{2+} (5.9 mM) depressed the peak value to about one-third of the control value and shifted the stimulatory Ca^{2+} to a higher concentration range: the EC_{50} value of Ca^{2+} was increased from 10 μM in control to 32 μM in the presence of 5.9 mM Mg^{2+} (Fig. 1 *A*, *filled circles*).

The widely accepted explanation for the inhibitory effects of Mg^{2+} is as follows. The RyR or Ca^{2+} release channel has both the high-affinity Ca^{2+} activation site (A-site) and the low-affinity Ca^{2+} inactivation site (I-site). Mg^{2+} serves as a competitive antagonist on the A-site and as an agonist on the I-site (Laver et al., 1997; Meissner et al., 1997). Therefore, the following equations are predicted according to the model corresponding to the above explanation. Channels with the A-site occupied by Ca^{2+} (the probability of f_A) and the I-site free of Ca^{2+} or Mg^{2+} (the probability of $(1 - f_I)$) are in the activated state. f_A and $1 - f_I$ are expressed as follows:

$$f_A = [Ca^{2+}]^{n_{A,Ca}}/\{[Ca^{2+}]^{n_{A,Ca}} + K_{A,Ca}^{n_{A,Ca}}(1 + [Mg^{2+}]^{n_{A,Mg}}/K_{A,Mg}^{n_{A,Mg}})\}$$

$$1 - f_I = 1/(1 + [Ca^{2+}]^{n_{I,Ca}}/K_{I,Ca}^{n_{I,Ca}} + [Mg^{2+}]^{n_{I,Mg}}/K_{I,Mg}^{n_{I,Mg}})$$

where $K_{A,Ca}$, $K_{A,Mg}$, $K_{I,Ca}$, and $K_{I,Mg}$ represent the dissociation constants for Ca^{2+} and Mg^{2+} of the A- and I-sites,

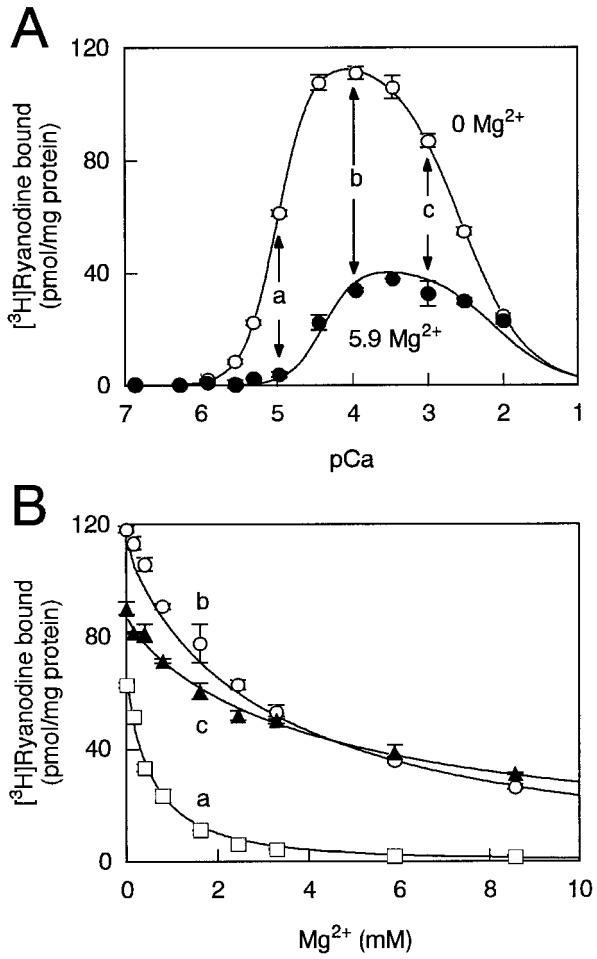

FIGURE 1 Scheme illustrating the procedure for determinations of the affinities for Ca^{2+} and Mg^{2+} of the A- and I-sites of frog RyRs. [^{3}H]Ryanodine binding to the purified α-RyR was carried out as described in Materials and Methods in a reaction medium containing 8.5 nM [^{3}H]ryanodine, 0.17 M NaCl, 20 mM MOPSO/NaOH (pH 6.8), 1% CHAPS, 0.5% phospholipids, 2 mM dithiothreitol, 4 mM AMP, and various free Ca^{2+} and Mg^{2+}. (*A*) Ca^{2+} dependence of the ryanodine binding in the presence (●) and absence (○) of 5.9 mM Mg^{2+}. Data are means ± SE (n = 4). (*B*) Dose-dependent inhibition by Mg^{2+} of [^{3}H]ryanodine binding at three different Ca^{2+} concentrations (*arrows a–c* are as indicated in *A*): 10.7 μM (*a*), 110 μM (*b*), and 1.0 mM (*c*). Data are means ± SE (n = 3). Parameters for Ca^{2+} and Mg^{2+} of the A- and I-sites were determined in three consecutive steps. First, the data in the absence of Mg^{2+} (○ in *A*) were fitted to Eq. 2 to yield the following parameters: B_{max} = 117.4 pmol/mg protein; $K_{A,Ca}$ = 10.1 μM; $n_{A,Ca}$ = 2.0; $K_{I,Ca}$ = 2.73 mM; $n_{I,Ca}$ = 1.0. Second, the dose-dependent inhibition by Mg^{2+} (▲ in *B*) in the presence of 1 mM Ca^{2+} (*arrow c* in *A*), where competition of Mg^{2+} for the A-site is negligible, was fitted to Eq. 3 to obtain $K_{I,Mg}$ (3.16 mM) and $n_{I,Mg}$ (0.9). Third, the data (□ in *B*) at 10.7 μM Ca^{2+} (*arrow a* in *A*), where competitive inhibition by Mg^{2+} is prominent, were fitted to Eq. 1 to estimate $K_{A,Mg}$ (396 μM) and $n_{A,Mg}$ (1.1). The validity of the obtained parameters was confirmed by comparing the curve computed according to Eq. 1 with the data (*curve b* in *B*) at 110 μM Ca^{2+} (*arrow b* in *A*). These parameter values were also verified by the curve fit to the Ca^{2+}-dependent [^{3}H]ryanodine binding in the presence of 5.9 mM Mg^{2+} as shown in *A*.

respectively. $n_{A,Ca}$, $n_{A,Mg}$, $n_{I,Ca}$, and $n_{I,Mg}$ represent the Hill coefficients of the relevant sites. Because [^{3}H]ryanodine is considered to bind only to the open channel (Coronado et al., 1994; Meissner, 1994; Ogawa, 1994), the [^{3}H]ryanodine binding (B) in the presence of a specified concentration of [^{3}H]ryanodine as determined in Fig. 1 *A* can be expressed by Eq. 1:

$$B = B_{max} \times f_A \times (1 - f_I)$$
$$= B_{max} \times \frac{[Ca^{2+}]^{n_{A,Ca}}}{[Ca^{2+}]^{n_{A,Ca}} + K_{A,Ca}^{n_{A,Ca}}(1 + [Mg^{2+}]^{n_{A,Mg}}/K_{A,Mg}^{n_{A,Mg}})} \quad (1)$$
$$\times \{1/(1 + [Ca^{2+}]^{n_{I,Ca}}/K_{I,Ca}^{n_{I,Ca}} + [Mg^{2+}]^{n_{I,Mg}}/K_{I,Mg}^{n_{I,Mg}})\}$$

where B_{max} is the maximum amount of [^{3}H]ryanodine binding to be expected in the presence of a specified concentration of the ligand. It should be noted that B_{max} is different from the conventional value for maximum binding, which refers to the value in the presence of an infinite amount of [^{3}H]ryanodine. B_{max} is also affected by modulators such as adenine nucleotides and caffeine. A preliminary attempt to determine all nine parameters, including B_{max} in Eq. 1 by curve fit of the data of Ca^{2+} dependence, as shown in Fig. 1 *A*, was unsuccessful, because there were too many parameters to fix for the data points currently available. To determine these parameters accurately with a relatively small number of data points, we designed an analysis procedure comprising three consecutive steps and confirmed of the validity of the results. This procedure was found to work well, as shown in Fig. 2. First, in the absence of Mg^{2+} ($[Mg^{2+}] = 0$), Eq. 1 is simplified into Eq. 2:

$$B = B_{max} \times f_A \times (1 - f_I)$$
$$= B_{max} \times \{[Ca^{2+}]^{n_{A,Ca}}/([Ca^{2+}]^{n_{A,Ca}} + K_{A,Ca}^{n_{A,Ca}})\}$$
$$\times \{1 - [Ca^{2+}]^{n_{I,Ca}}/([Ca^{2+}]^{n_{I,Ca}} + K_{I,Ca}^{n_{I,Ca}})\} \quad (2)$$

The results of Ca^{2+}-dependent [^{3}H]ryanodine binding in the absence of Mg^{2+} (Fig. 1 *A*, *open symbols*) were fitted to Eq. 2 to yield B_{max} and the parameters for Ca^{2+} of the A-site ($K_{A,Ca}$, $n_{A,Ca}$) and the I-site ($K_{I,Ca}$, and $n_{I,Ca}$). Second, at a $[Ca^{2+}]$ much higher than $K_{A,Ca}$ (e.g., Fig. 1 *A*, *arrow c*), where competitive inhibition by Mg^{2+} on the A-site is negligible, the inhibition by Mg^{2+} can be explained by the action on the I-site alone. Under the circumstances, Eq. 1 is simplified into Eq. 3:

$$B = B_{max} \times (1 - f_I)$$
$$= B_{max} \times \{1/(1 + [Ca^{2+}]^{n_{I,Ca}}/K_{I,Ca}^{n_{I,Ca}} + [Mg^{2+}]^{n_{I,Mg}}/K_{I,Mg}^{n_{I,Mg}})\} \quad (3)$$

The results of dose-dependent inhibition by Mg^{2+} in the presence of 1 mM Ca^{2+} (Fig. 1 *B*, *triangles*, *curve c*) were fitted to Eq. 3 to obtain the parameters for Mg^{2+} of the I-site

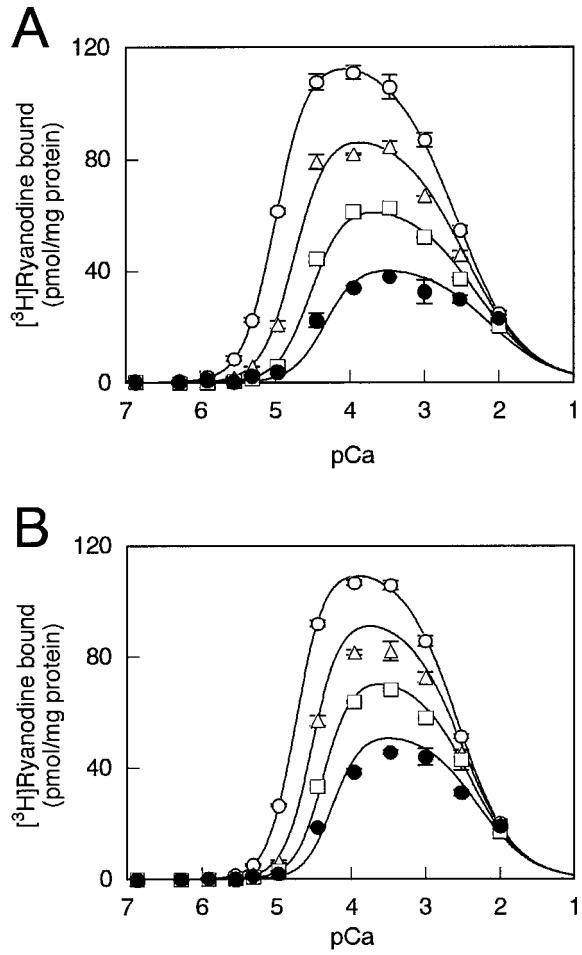

FIGURE 2 Effect of Mg^{2+} on the Ca^{2+}-dependent [^{3}H]ryanodine binding to frog RyRs. [^{3}H]Ryanodine binding to the purified α-RyR (*A*) and β-RyR (*B*) was carried out as in Fig. 1 at various free Ca^{2+} concentrations in the presence of 0 (○), 0.8 (△), 2.5 (□), and 5.9 (●) mM free Mg^{2+}. Data are means ± half-range of deviations of duplicate determinations. Curves in *A* and *B* were drawn according to Eq. 1, using the following parameters: B_{max} = 117.4 and 113.1 pmol/mg protein, $K_{A,Ca}$ = 10.1 and 18.1 μM, $n_{A,Ca}$ = 2.0 and 2.3, $K_{I,Ca}$ = 2.73 and 2.72 mM, $n_{I,Ca}$ = 1.0 and 1.2, $K_{A,Mg}$ = 396 and 350 μM, $n_{A,Mg}$ = 1.1 and 0.9, $K_{I,Mg}$ = 3.16 and 5.23 mM, and $n_{I,Mg}$ = 0.9 and 0.9 for α-RyR and β-RyR, respectively.

($K_{I,Mg}$ and $n_{I,Mg}$), using the other parameters already fixed. Third, the results of Mg^{2+} dependence (Fig. 1 *B, squares, curve a*) at a [Ca^{2+}] near $K_{A,Ca}$ (Fig. 1 *A, arrow a*), where competitive inhibition by Mg^{2+} is prominent, would give the parameters for Mg^{2+} of the A-site ($K_{A,Mg}$ and $n_{A,Mg}$) according to Eq. 1, because the other parameters used in the equation are already known. The validity of the obtained parameters was confirmed by comparing the results at a mid-range of free Ca^{2+} concentration (e.g., 0.1 mM Ca^{2+}) (Fig. 1 *B, circles, curve b*; see also Fig. 1 *A, arrow b*). These parameters also can predict well the Ca^{2+}-dependent [^{3}H]ryanodine binding in the presence of 5.9 mM Mg^{2+}, as shown in Fig. 1 *A*.

We would like to point out that the inhibition by Mg^{2+} depended on the Ca^{2+} concentration where the dose-effect curve for Mg^{2+} was obtained. Fig. 1 *B* shows that IC_{50} values for Mg^{2+} were 0.48, 2.7, and 4.1 mM at 10.7 (*open squares*), 110 (*open circles*), and 1000 (*filled triangles*) μM Ca^{2+}, respectively. The three-step procedure of analysis is indispensable for the coherent understanding of these effects of Mg^{2+}, which are of apparently variable grade. These systematic determinations and the analysis procedure are the fundamental principle throughout these experiments.

Fig. 2 demonstrates Ca^{2+}-dependent [^{3}H]ryanodine binding to α-RyR (Fig. 2 *A*) and β-RyR (Fig. 2 *B*) in the presence of 0–5.9 mM free Mg^{2+}. Computed curves using parameters determined as described above corresponded well to the experimental data points for both RyR isoforms at every Mg^{2+} concentration. A similar set of parameters was also obtained all at once by fitting all of the data points for each isoform (Fig. 2) in the presence of various Ca^{2+} and Mg^{2+} concentrations to Eq. 1, using a 3D curve fitter (Sigma Plot, version 5, for Macintosh) (data not shown). These results suggest that the model and the parameters determined by the three-step procedure well explain the effects of Mg^{2+} on the [^{3}H]ryanodine binding activity of both α- and β-RyRs.

Table 2 shows a summary of six to eight similar determinations. Although $K_{A,Ca}$ for β-RyR (18.1 μM) appears to be slightly larger than that for α-RyR (11.0 μM), the difference was not great, and we may conclude that they were similar to each other. There was no difference between α- and β-RyRs in $K_{I,Ca}$ (2.38 versus 2.34 mM), $K_{A,Mg}$ (324 versus 325 μM), or $K_{I,Mg}$ (2.79 versus 3.06 mM). The A-site showed 20–30-fold higher affinity for Ca^{2+} than for Mg^{2+}. It should be noted that the Hill coefficient for Mg^{2+} ($n_{A,Mg}$ = 0.9–1.0) was significantly smaller than that for Ca^{2+} ($n_{A,Ca}$ = 2.1–2.3). On the other hand, the I-site showed similar affinity (K_D of 2–3 mM) for Ca^{2+} and Mg^{2+}, with a Hill coefficient of ~1. In addition to these parameters, the B_{max} values of the two isoforms were also similar (107 versus 118 pmol/mg protein).

Effect of caffeine

Caffeine is a well-known activator of RyRs and is thought to enhance the Ca^{2+} sensitivity for activation. However, it is still unclear how the effect of the drug is modulated in the presence of Mg^{2+}. We examined in detail the effect of caffeine on [^{3}H]ryanodine binding in the presence of various concentrations of Ca^{2+} and Mg^{2+} and analyzed the effect of the drug on the A- and I-sites. Fig. 3 *A* demonstrates the effects of various concentrations of caffeine on Ca^{2+}-dependent [^{3}H]ryanodine binding to α-RyR. Caffeine dose-dependently enhanced the apparent Ca^{2+} sensitivity for activation: EC_{50} for Ca^{2+} was reduced from 9.9 μM (control) to 3.6 and 1.1 μM with 2 and 10 mM caffeine, respectively. No further Ca^{2+}-sensitizing effect was ob-

TABLE 2 Summary of parameters for Ca^{2+} and Mg^{2+} of the A- and I-sites and B_{max}, which were determined from [^{3}H]ryanodine binding to the purified RyRs

	A-site				I-site			
	Ca^{2+}		Mg^{2+}		Ca^{2+}		Mg^{2+}	
B_{max} (pmol/mg)	$K_{A,Ca}$ (μM)	$n_{A,Ca}$	$K_{A,Mg}$ (μM)	$n_{A,Mg}$	$K_{I,Ca}$ (mM)	$n_{I,Ca}$	$K_{I,Mg}$ (mM)	$n_{I,Mg}$
α-RyR								
Control								
107.3 ± 4.3 (8)	11.0 ± 0.4 (8)	2.1 ± 0.1 (8)	324 ± 34 (6)	1.0 ± 0.1 (6)	2.38 ± 0.13 (8)	1.1 ± 0.1 (8)	2.79 ± 0.25 (6)	0.9 ± 0.1 (6)
+10 mM caffeine								
138.2 ± 1.6 (4)	1.12 ± 0.04 (4)	1.5 ± 0.1 (4)	394 ± 19 (3)	1.3 ± 0.1 (3)	3.99 ± 0.20 (4)	1.2 ± 0.1 (4)	5.22 ± 0.19 (3)	1.2 ± 0.1 (3)
β-RyR								
Control								
118.1 ± 3.1 (7)	18.1 ± 0.6 (7)	2.3 ± 0.1 (7)	325 ± 51 (6)	0.9 ± 0.1 (6)	2.34 ± 0.11 (7)	1.1 ± 0.1 (7)	3.06 ± 0.57 (6)	0.9 ± 0.2 (6)
+10 mM caffeine								
138.8 ± 2.4 (3)	3.12 ± 0.15 (3)	1.7 ± 0.1 (3)	351 ± 23 (3)	1.3 ± 0.1 (3)	5.09 ± 0.35 (3)	1.3 ± 0.1 (3)	7.77 ± 0.30 (3)	1.4 ± 0.1 (3)

Data are presented as means ± SE of determinations numbered in parentheses.

served at 15 mM caffeine (data not shown). Furthermore, it slightly reduced the inactivation in the presence of high Ca^{2+} concentrations. In addition, 2 mM or more caffeine increased the binding at optimal Ca^{2+} by ~20%. Similar results were obtained with the purified β-RyR (see Table 2). These findings were consistent with previous studies of [^{3}H]ryanodine binding to the purified RyRs (Murayama and Ogawa, 1996) and isolated SR vesicles (Ogawa and Harafuji, 1990).

Fig. 3 *B* shows the effects of Mg^{2+} on the Ca^{2+}-dependent [^{3}H]ryanodine binding to α-RyR in the presence of 10 mM caffeine. Mg^{2+} decreased both the binding at the optimal Ca^{2+} and apparent Ca^{2+} sensitivity for activation in a dose-dependent manner, as is the case without caffeine (see Fig. 2). EC_{50} for Ca^{2+} was increased from 1.1 μM (control) to 2.4, 4.2, and 6.6 μM by 0.8, 2.5, and 5.9 mM Mg^{2+}, respectively. The analysis was carried out to obtain parameters as described above in the three-step procedure. Table 2 shows the summary of parameters thus determined, including those for β-RyR. The experimental results obtained in the presence of varying amounts of Mg^{2+} in Fig. 3 *B* coincided well with computed curves, when we used the parameters shown in Table 2. Caffeine markedly reduced $K_{A,Ca}$ for both isoforms (from 11 to 1 μM, 10-fold, with α-RyR and from 18 to 3 μM, sixfold, with β-RyR) with a slight decrease in $n_{A,Ca}$ (from 2.1 to 1.5 with α-RyR and from 2.3 to 1.7 with β-RyR). In contrast, $K_{A,Mg}$ and $n_{A,Mg}$ were not changed substantially by the reagent. These results suggest that caffeine increases the affinity of the A-site for Ca^{2+} but not for Mg^{2+}. In addition to the effects on the A-site, caffeine slightly increased $K_{I,Ca}$ (from 2.4 to 4.0 mM for α-RyR and from 2.3 to 5.1 mM for β-RyR) and $K_{I,Mg}$ (from 2.8 to 5.2 mM for α-RyR and from 3.1 to 7.8 mM for β-RyR). This may be consistent with the decreased inactivation in the presence of high Ca^{2+} concentrations. The B_{max} was increased by 20–30%. This increase in B_{max} may partly contribute to the enhanced peak value of the binding. The enhancement appeared to be saturated at 2 mM caffeine in these experiments (see Fig. 3 *A*). With SR vesicles, however, the peak values of [^{3}H]ryanodine binding increased up to threefold or more as caffeine was increased to 15 mM (Ogawa and Harafuji, 1990; Ogawa et al., 1999). This difference may be explained by the effect of CHAPS, which potentiates the effect of adenine nucleotides (Ogawa et al., 1999).

CICR activity in skinned fibers

Effect of Mg^{2+}

Fig. 4 shows the Ca^{2+} dependence of the rates of CICR that were determined in a chloride salt medium (Cl medium) containing 4 mM AMP and 0–1.6 mM Mg^{2+}. In the absence of Mg^{2+}, CICR shows a bell-shaped Ca^{2+} dependence similar to that of [^{3}H]ryanodine binding (Fig. 2). The peak value of the rate constant was ~7 min^{-1}, and EC_{50} and IC_{50} for Ca^{2+} were 3 μM and 1 mM, respectively. Mg^{2+} had two distinct effects on the pCa-CICR activity relationship: it dose-dependently decreased the peak rate of Ca^{2+} release with an IC_{50} of 0.3 mM and lowered Ca^{2+} sensitivity for activation. The EC_{50} for Ca^{2+} was increased to 5, 10, and 20 μM by 0.4, 0.8, and 1.6 mM Mg^{2+}, respectively.

We also followed the procedure of analysis in the case of [^{3}H]ryanodine binding. k_{max} and k'_{app}, however, replace B_{max} and B in Eqs. 1–3, respectively. k_{max} is the maximum value of k'_{app}. The curve for circles in the absence of Mg^{2+} (Fig. 4) was drawn by using $K_{A,Ca}$ = 3.2 μM, $n_{A,Ca}$ = 1.6, $K_{I,Ca}$ = 1.1 mM, and k_{max} = 7.0 min^{-1} as the best fit parameters. In this calculation, we assumed $n_{I,Ca}$ to be unity in all of the skinned fiber experiments, because determinations with 21 fibers gave an average of 0.92 ± 0.15 (mean ± SD) for the parameter. This conclusion is consistent with the results of [^{3}H]ryanodine binding (Table 2). The Hill coefficient for the A-site, $n_{A,Ca}$, was calculated as a

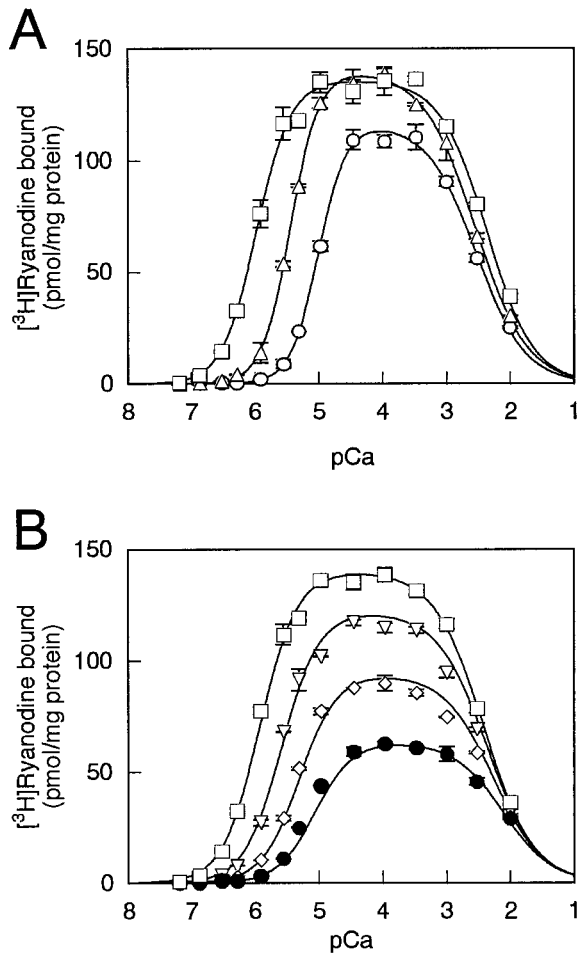

FIGURE 3 (*A*) Effect of caffeine on the Ca^{2+}-dependent [^{3}H]ryanodine binding to frog RyRs. [^{3}H]Ryanodine binding to α-RyR was carried out as in Fig. 1 in the presence of 0 (○), 2 (△), and 10 (□) mM caffeine. Data are means ± half-range of deviations of duplicate determinations. (*B*) Effect of Mg^{2+} on the Ca^{2+}-dependent [^{3}H]ryanodine binding in the presence of 10 mM caffeine. [^{3}H]Ryanodine binding to α-RyR was determined with 0 (□), 0.8 (▽), 2.5 (◇), and 5.9 (●) mM Mg^{2+}. Computed curves were drawn using Eq. 1 and the following parameters: B_{max} = 140.2 pmol/mg protein; $K_{A,Ca}$ = 1.19 μM; $n_{A,Ca}$ = 1.5; $K_{I,Ca}$ = 3.85 mM; $n_{I,Ca}$ = 1.1; $K_{A,Mg}$ = 379 μM; $n_{A,Mg}$ = 1.1; $K_{I,Mg}$ = 4.92 mM; $n_{I,Mg}$ = 1.1.

parameter to be determined, because it varied between 1.0 and 2.0, depending on species and concentrations of adenine nucleotides. Using the parameters for Ca^{2+} described above ($K_{A,Ca}$, $n_{A,Ca}$, $K_{I,Ca}$), the relation between k'_{app} and Mg^{2+} concentration in the presence of 63 μM Ca^{2+} (pCa 4.2) was analyzed according to Eq. 3. The best fit was obtained when the dissociation constant of the I-site for Mg^{2+} ($K_{I,Mg}$) was 0.42 mM. Furthermore, a $K_{A,Mg}$ of 150 μM was obtained according to Eq. 1 on the basis of Mg^{2+}-dependent inhibition of k'_{app} at pCa 5.2. Hill coefficients, $n_{A,Mg}$ and $n_{I,Mg}$, were also fixed at 1.0, based on the results of [^{3}H]ryanodine binding (Table 2). To verify whether the parameters obtained here explain all of the data at various concentrations of Ca^{2+} and Mg^{2+}, CICR activity was recomputed using

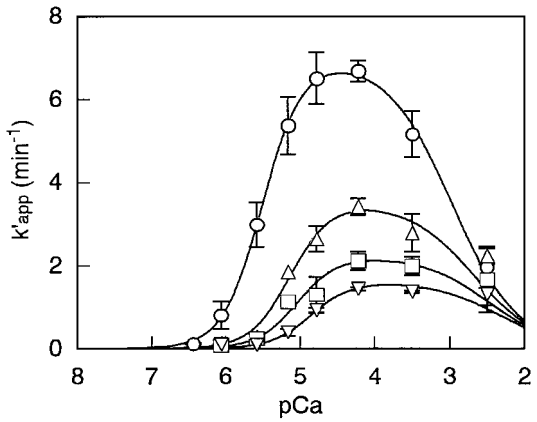

FIGURE 4 Effect of Mg^{2+} on the Ca^{2+} dependence of CICR in skinned muscle fibers. Rate constants of the Ca^{2+} release from SR using six skinned fibers were determined in the Cl medium containing 4 mM AMP and various Ca^{2+} concentrations. Effects of various Mg^{2+} concentrations were examined: 0 (○), 0.4 (△), 0.8 (□), or 1.6 mM (▽) Mg^{2+}. The results, corrected for as described in Materials and Methods, are plotted. pH was adjusted to 7.0 for these experiments. Parameters determined according to the procedure described in Fig. 1 were k_{max} = 7.0 min^{-1}, $K_{A,Ca}$ = 3.2 μM, $n_{A,Ca}$ = 1.6, $K_{I,Ca}$ = 1.1 mM, $K_{I,Mg}$ = 0.42 mM, $K_{A,Mg}$ = 0.15 mM. The drawn lines are curves calculated for indicated Mg^{2+} concentrations, using these parameters. $n_{I,Ca}$, $n_{A,Mg}$, and $n_{I,Mg}$ were fixed at 1.0 in these simulations.

these parameters, and curves for 0.4, 0.8, and 1.6 mM Mg^{2+} were drawn in Fig. 4. All of the CICR activity determined in the presence of varied Ca^{2+} and Mg^{2+} is consistent with the values anticipated by the model. A similar set of parameters was also obtained all at once, using the 3D curve fitter (Sigma Plot, version 5, for Macintosh) as mentioned above (data not shown), supporting the assumption that the appropriate values for the parameters were obtained by the three-step procedure. We would like to point out that the corrected k_{app} (k'_{app}) was used here as described in Materials and Methods. This correction was significant at pCa 2.5 and 3.0. Without this correction, $K_{I,Ca}$ and $n_{I,Ca}$ would be 80% larger and 16% smaller, respectively. Other parameters ($K_{A,Ca}$, $n_{A,Ca}$, $K_{A,Mg}$, $n_{A,Mg}$, $K_{I,Mg}$, and $n_{I,Mg}$) were not significantly affected by the correction. Table 3 (the first group) summarizes similar experiments at pH 6.8 with varied AMP concentrations.

The stimulatory principle of an adenine nucleotide: free or bound form

Adenine nucleotides such as ATP, AMPPCP, ADP, and AMP are believed to stimulate CICR through the common responsible site(s) (Endo, 1981). To assess the effect of Mg^{2+} on Ca^{2+} release in situ, CICR activity should be determined in the presence of ATP. Its use, however, prevents us from analyzing clearly the Ca^{2+} release itself because it also drives Ca^{2+} uptake by activating the Ca^{2+} pump. Therefore, we performed experiments in the presence of AMPPCP, a nonhydrolyzable ATP analog, which is very

TABLE 3 Summary of parameters for Ca^{2+} and Mg^{2+} of the A- and I-sites and k_{max}, which were determined from CICR experiments with skinned fibers

		A-site				I-site			
		Ca^{2+}		Mg^{2+}		Ca^{2+}		Mg^{2+}	
	k_{max} (min^{-1})	$K_{A,Ca}$ (μM)	$n_{A,Ca}$	$K_{A,Mg}$ (μM)	$n_{A,Mg}$*	$K_{I,Ca}$ (mM)	$n_{I,Ca}$*	$K_{I,Mg}$ (mM)	$n_{I,Mg}$*
1 mM AMP	2.6 ± 0.1 (3)	4.3 ± 0.2 (3)	2.0 ± 0.2 (3)	37 ± 16 (2)	1.0	0.75 ± 0.07 (3)	1.0	0.31 ± 0.04 (2)	1.0
4 mM AMP	5.6 ± 0.5 (4)	3.5 ± 0.7 (4)	1.6 ± 0.4 (4)	95 ± 28 (2)	1.0	0.40 ± 0.12 (4)	1.0	0.36 ± 0.04 (2)	1.0
0.2 mM AMPPCP	2.9 ± 0.5 (5)	3.0 ± 0.3 (5)	1.4 ± 0.2 (5)	97 ± 16 (3)	1.0	0.40 ± 0.06 (5)	1.0	0.36 ± 0.03 (6)	1.0
1 mM AMPPCP	18 ± 2.4 (3)	2.5 ± 0.4 (3)	1.2 ± 0.1 (3)	49 ± 5 (3)	1.0	0.39 ± 0.07 (3)	1.0	0.18 ± 0.02 (3)	1.0
1 mM AMP, 5 mM caffeine	15 ± 0.6 (3)	0.7 ± 0.10 (3)	1.7 ± 0.4 (3)	48 ± 18 (2)	1.0	0.86 ± 0.18 (3)	1.0	0.29 ± 0.03 (2)	1.0
0.2 mM AMPPCP, 5 mM caffeine	11 ± 1.0 (3)	0.4 ± 0.07 (3)	1.0 ± 0.1 (3)	74 ± 12 (3)	1.0	1.2 ± 0.21 (3)	1.0	0.31 ± 0.03 (3)	1.0

Data are means ± SE (number of determinations) or means ± half-range of the deviation in duplicate determinations. Experiments with AMP were performed in Cl medium, whereas those with AMPPCP were performed in Ms medium. *Hill coefficients were fixed at 1.0.

similar to ATP in stimulating Ca^{2+} release but does not support Ca^{2+} uptake (Kakuta, 1984). For these experiments, we used methanesulfonate salt medium (Ms medium) instead of Cl medium because the latter has been reported to have some stimulating effects on RyR (Meissner et al., 1997). In our experiments with skinned fibers, however, there were no significant differences in the Ca^{2+} sensitivities of activation and inactivation sites between the two media, while k_{max} in the Ms medium was slightly smaller than that in the Cl medium (averaged k_{max} values in the presence of 4 mM AMP were 5.6 ± 0.5 min^{-1} ($n = 4$) and 3.6 ± 1.0 min^{-1} ($n = 3$) in Cl and Ms media, respectively).

An obstacle in analyzing the effect of Mg^{2+} on CICR activity in the presence of AMPPCP is that a substantial fraction of AMPPCP binds Mg^{2+} and Ca^{2+} with an apparent K_D of ~0.2 mM. Therefore, the question arises whether the nucleotide complexed with a divalent cation is equivalent to free nucleotide in stimulating CICR. To clarify this point, we compared CICR activities in the presence of AMPPCP with those in the presence of AMP, which has a much lower affinity to divalent cations. The top of Fig. 5 *A* shows the Ca^{2+} dependence of the relative CICR activities normalized with the values at pCa 4.5 in the presence of 4 mM AMP (*filled symbols*) and 0.2 mM AMPPCP (*open symbols*). A suitable set of parameters in Eq. 2 can predict all of the data points, irrespective of AMP or AMPPCP, as shown by the curve in Fig. 5 *A*. Fractions of free to total nucleotide at various Ca^{2+} concentrations were calculated and are plotted in the lower panel. A substantial fraction of AMPPCP binds to Ca^{2+} at calcium concentrations higher than 0.1 mM, whereas AMP is almost free up to 3 mM Ca^{2+} (*bottom panel*). The Ca^{2+} dependences of k'_{app}, however, were not significantly different between the two nucleotides. The IC_{50} values of Ca^{2+} were 0.38 ± 0.12 mM and 0.43 ± 0.03 mM in the presence of AMP and AMPPCP, respectively. Similar IC_{50} values with AMP were also obtained in Cl medium. Fig. 5 *B* shows the Mg^{2+}-dependent inhibition of CICR activities in the presence of AMP or AMPPCP at 90 μM Ca^{2+}, where the activation site was expected to be fully saturated with Ca^{2+}. The Mg^{2+} dependence with AMPPCP was very similar to that with AMP. The IC_{50} values of Mg^{2+} were 0.52 ± 0.09 mM and 0.44 ± 0.03 mM in the presence of AMP and AMPPCP, respectively. The fraction of free AMPPCP, however, was much less than that of AMP in the range of $[Mg^{2+}] > 0.1$ mM (*bottom panel*). These results are consistent with the idea that free AMPPCP, MgAMPPCP, and CaAMPPCP are equally potent in stimulating CICR. Therefore, in the following experiments we calculated the affinities of the A- and I-sites for Ca^{2+} and Mg^{2+} under the assumption that the free and complexed forms of AMPPCP are equivalent to each other in stimulating CICR.

We examined the effect of AMPPCP on each of the parameters for CICR in situ by determining the Ca^{2+} release in the presence of various concentrations of AMPPCP. Initially, we determined the dependence of k'_{app} on AMPPCP concentration at pCa 4.05, which was around the optimum concentration (Fig. 6 *A*). In the absence of AMPPCP, the k'_{app} was very small (0.1–0.3 min^{-1}). The k'_{app} determined in a single fiber increased with increase in AMPPCP concentration up to 3 mM (Fig. 6 *A*, *open circles*). At a concentration higher than 3 mM, k'_{app} was too great to be reliable in our experimental system. Results in Fig. 6 *A* (*open circles*) can be satisfactorily predicted by the conventional law of the mass reaction with an apparent dissociation constant (5 mM) and the saturated value for k'_{app} (53 min^{-1}). Similar results were obtained in three different fibers. The averages for k'_{app} values in the presence of 0.2 and 1 mM AMPPCP were 2.1 ± 0.8 ($n = 9$) and 13.2 ± 2.0 ($n = 6$) min^{-1}, respectively (*filled circles*). In the presence of 1 mM Mg^{2+}, a similar dose-dependent increase in CICR was also observed at pCa 4.05 (Fig. 6 *A*, *open triangles*).

The Ca^{2+}-dependent k'_{app} values in the presence of 0.2 mM and 1 mM AMPPCP were normalized by each peak value in Fig. 6 *B*. They were homologous to each other in their Ca^{2+} dependences. The second group in Table 3

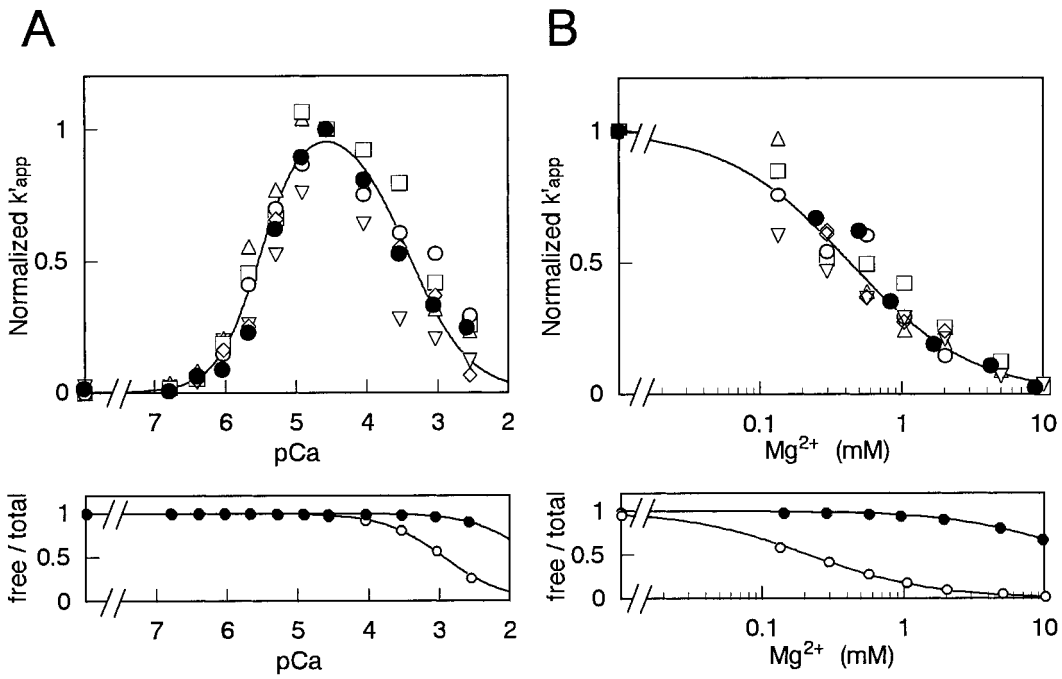

FIGURE 5 Comparison of effects of AMP and AMPPCP on Ca^{2+} and Mg^{2+} dependences of CICR in skinned fibers. (*A*) Ca^{2+} dependence in the absence of Mg^{2+}. The top panel shows Ca^{2+} dependence of the relative k'_{app}, which was normalized with the value at pCa 4.5. CICR was determined in the Ms medium containing 4 mM AMP (●) or 0.2 mM AMPPCP (○, □, △, ▽, ◇). Different symbols indicate different preparations. The bottom panel indicates the calculated fractions of the free form to total AMP (●) or AMPPCP (○). AMP is mostly in the free form, whereas the complexed form of AMPPCP makes up a considerable fraction of the total. The solid line in the top panel was drawn based on data with 0.2 mM AMPPCP according to Eq. 2, where the parameters were $K_{A,Ca}$ = 3.9 μM, $n_{A,Ca}$ = 1.4, $K_{I,Ca}$ = 0.43 mM, and $n_{I,Ca}$ = 1. (*B*) Mg^{2+}-dependent inhibition in the presence of 90 μM Ca^{2+} is shown in the top panel, and the calculated ratio of free to total nucleotide is shown in the bottom panel. The results were normalized with those in the absence of Mg^{2+}. The drawn line shows the curve of Eq. 3 best fit to data with 0.2 mM AMPPCP, where parameters were $K_{I,Mg}$ = 0.36 mM and $n_{I,Mg}$ = 1.0, in addition to those determined in *A*. Note that CICR activities in the presence of AMP and AMPPCP showed very similar Ca^{2+} (*A*) and Mg^{2+} dependences (*B*), although their ratio of the free form to total nucleotides was very different in the presence of high concentrations of Ca^{2+} and Mg^{2+}.

summarizes the results of analysis according to Eqs. 1–3 of determinations with 0.2 and 1 mM AMPPCP in the presence of various Ca^{2+} and Mg^{2+} concentrations. As AMPPCP was increased from 0.2 to 1 mM, k_{max} was enhanced as much as sixfold. Neither $K_{A,Ca}$ nor $K_{I,Ca}$, however, was significantly affected. This result was in accordance with previous reports of unchanged Ca^{2+} dependence with skinned fibers (Endo, 1981) and with [^{3}H]ryanodine binding to SR vesicles (Ogawa and Harafuji, 1990). The Hill coefficient of the A-site ($n_{A,Ca}$), however, appears to be slightly smaller at a higher concentration of AMPPCP: 1.4 and 1.2 at 0.2 mM and 1 mM AMPPCP, respectively. The average of $K_{I,Mg}$ at 1 mM AMPPCP was 0.18 ± 0.02 mM (ranging from 0.13 to 0.22 mM), while that at 0.2 mM was 0.36 ± 0.03 mM (ranging from 0.26 to 0.46 mM). Under the experimental conditions where Mg^{2+} and AMPPCP were used, some variations of the K_D values for Mg^{2+} of AMPPCP may lead to a different conclusion. For example, if pK_{Mg} is 4.38, which is two times larger than what we used (4.68), the range of $K_{I,Mg}$ would be 0.27–0.49 mM and 0.17–0.28 mM in the presence of 0.2 mM and 1.0 mM AMPPCP, respectively. We may conclude that the affinity of the I-site for Mg^{2+} was similar between 0.2 and 1 mM AMPPCP. This is also the case with the affinity of the A-site for Mg^{2+} (Table 3). It should also be noted that k'_{app} in the absence of Ca^{2+} (10 mM EGTA, pCa ~9) at 1.0 mM AMPPCP (0.17 ± 0.07 min^{-1}, n = 3) was significantly higher than that at 0.2 mM AMPPCP (0.03 ± 0.02 min^{-1}, n = 4). This activity was completely suppressed by less than 1 mM Mg^{2+}. As AMPPCP was increased, Ca^{2+} release in the virtual absence of Ca^{2+} was more prominent, but it would be only a minor fraction (no more than 1%) of the peak rate of CICR (data not shown). In summary, the stimulating effect of adenine nucleotides such as AMP and AMPPCP can be explained by enhanced k_{max}, but affinities for Ca^{2+} and Mg^{2+} of A- and I-sites remain unchanged, being independent of the concentrations of nucleotides. This conclusion was proved regardless of the medium used (Ms or Cl) (data not shown).

Effect of caffeine

We then examined the effect of caffeine on CICR in skinned fibers in the presence of varying amounts of Ca^{2+} and

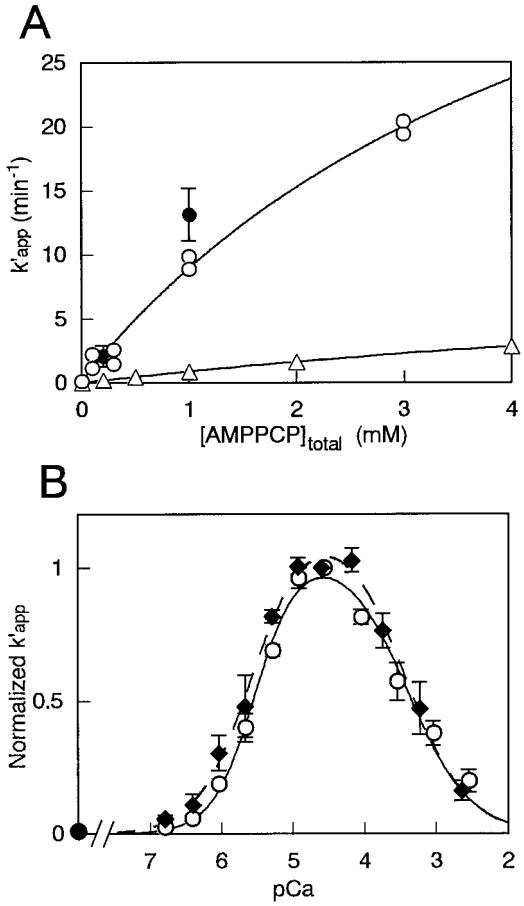

FIGURE 6 (*A*) Dose-dependent potentiation of CICR by AMPPCP. The k'_{app} values were determined in the Ms medium containing 90 μM Ca^{2+} and various levels of AMPPCP with (△) or without (○) 1 mM Mg^{2+}, using different single fibers. Solid lines correspond to simple equations of the law of mass reaction with a dissociation constant and a saturated value for k'_{app} of, respectively, 5 mM and 53 min^{-1} in the absence of Mg^{2+} and 10 mM and 11 min^{-1} in its presence. ●, Average with SE of all determinations (n = 9 and 6 for 0.2 mM and 1 mM AMPPCP, respectively) in the absence of Mg^{2+}, including the results of open circles. These results suggest that k'_{app} in the presence of 4 mM AMPPCP would be ~35–50 min^{-1} at pCa 4.05. (*B*) Normalized Ca^{2+}-dependent k'_{app} values in the presence of 0.2 mM (○) or 1 mM AMPPCP (◆). Parameters in Eq. 2 that would give a best fit are listed in Table 3. Note that the two fitted curves are very similar to each other. The averaged k_{max} values were 2.9 min^{-1} and 18 min^{-1} in the presence of 0.2 mM and 1 mM AMPPCP, respectively.

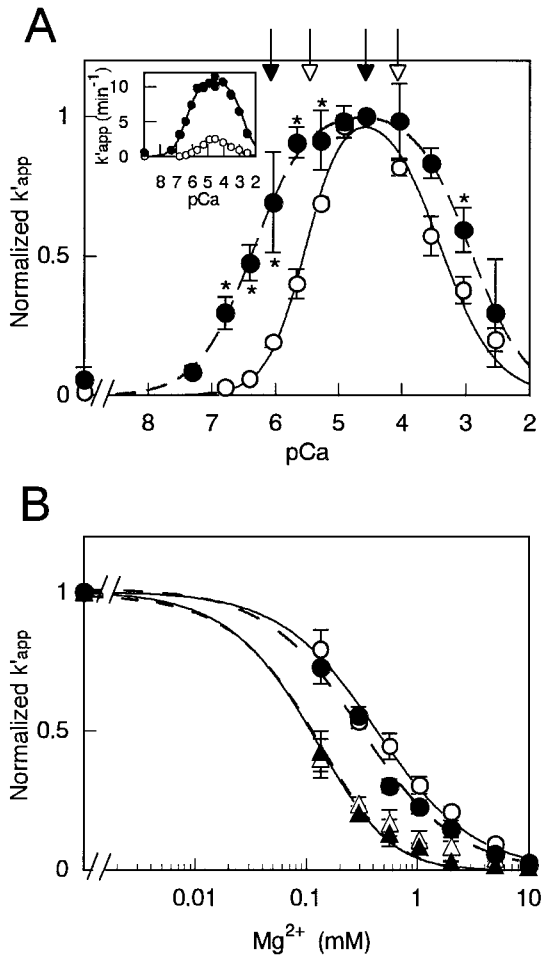

FIGURE 7 Effect of caffeine on the affinities of A- and I-sites for Ca^{2+} and Mg^{2+}. All determinations were carried out in the Ms medium containing 0.2 mM AMPPCP. (*A*) Normalized Ca^{2+}-dependent k'_{app} values in the presence (●) and absence (○) of 5 mM caffeine. *Significant difference between their normalized values ($p < 0.05$). Parameters that would give the best fits are listed in Table 3. Determined values of k'_{app} are plotted in the inset. Note that caffeine enhanced the Ca^{2+} sensitivity for activation by sevenfold and the k'_{app} at the optimal Ca^{2+} concentrations by fourfold. (*B*) Inhibition by Mg^{2+} in the presence and absence of 5 mM caffeine. Determinations were carried out at the pCas indicated by arrows in *A* (*open arrows*, no caffeine; *filled arrows*, 5 mM caffeine). Rate constants of CICR at various concentrations of Mg^{2+} in the absence of caffeine (——) were determined at pCa 4.05 (○) and 5.44 (△) whereas in the presence of caffeine (– – –) at pCa 4.56 (●) and 6.03 (▲). The results were normalized by the value in the absence of Mg^{2+} in each series of determinations. Best-fit parameters in Eqs. 1–3 are listed in the third section of Table 3.

Mg^{2+} (Fig. 7), and the results are summarized in the third group of Table 3. Fig. 7 *A* shows the potentiating effect of 5 mM caffeine on the Ca^{2+}-dependent CICR activity in the presence of 0.2 mM AMPPCP. Caffeine had two distinct effects: it increased the maximum rate constant of Ca^{2+} release by fourfold (k_{max} = 2.9 versus 11.2 min^{-1}) (Fig. 7 *A*, *inset*) and increased the sensitivity of the Ca^{2+} activation ($K_{A,Ca}$ = 3.0 versus 0.4 μM) by sevenfold (Fig. 7 *A*). The averaged affinity of the I-site for Ca^{2+} appeared to be lowered to one-third by caffeine ($K_{I,Ca}$ = 0.40 versus 1.2 mM) (Fig. 7 *A*). However, significant differences were not detected ($p > 0.05$, *t*-test) between normalized values in the presence and absence of caffeine at pCa < 4.5, except for pCa 3.0, because the experimental variation was large at higher Ca^{2+} concentrations (Fig. 7 *A*). The same conclusion was also obtained by ANOVA analysis ($p \approx 0.09$) with StatView 5.0 for Macintosh.

The inhibition by Mg^{2+} of the CICR activity determined at Ca^{2+} concentrations where the occupation with Ca^{2+} of the A-site was hardly affected by Mg^{2+} (pCa 4.56 and 4.05,

in the presence and absence of caffeine, respectively) is shown in Fig. 7 *B* (the *two right curves with circles*). The normalized Mg^{2+} dependence in the presence of caffeine (Fig. 7 *B, filled circles*) was very similar to that in its absence (Fig. 7 *B, open circles*), although the absolute k'_{app} values were increased severalfold by caffeine. $K_{I,Mg}$ values determined by Eq. 3 were not significantly changed by caffeine ($K_{I,Mg}$ = 0.31 versus 0.36 mM). The two left curves (*triangles*) in Fig. 7 *B* represent the Mg^{2+} dependences obtained at Ca^{2+} concentrations that gave ~60% of the maximum activity (pCa 6.03 and 5.44, with and without caffeine, respectively). The curve in the presence of caffeine (Fig. 7 *B, filled triangles*) was not significantly different from that in the absence of caffeine (Fig. 7, *open triangles*). Taken together, similar Mg^{2+} dependences under the two specified conditions indicate that caffeine does not change the affinity for Mg^{2+} of the A- or I-sites. Actually, calculated $K_{A,Mg}$ in the presence of caffeine (74 μM) was also indistinguishable from that without caffeine (97 μM). In contrast to the case for Ca^{2+}, the affinity for Mg^{2+} of the A-site was not affected by caffeine. In the presence of 1 mM AMP in the Cl medium, very similar results were also observed for the effect of caffeine: an approximately sixfold increase in k_{max} and an approximately sixfold reduction in $K_{A,Ca}$ without a significant change in other parameters (Table 3). The four- to sixfold enhancement of k_{app} by 5 mM caffeine at the optimal Ca^{2+} was consistently obtained in the presence of 0–10 mM AMP (data not shown). These observations indicate that caffeine and adenine nucleotide may act independently through the different underlying mechanisms. This is consistent with previous reports (Endo, 1981; Sitsapesan and Williams, 1990; Coronado et al., 1994; Meissner, 1994; Ogawa, 1994; Sutko and Airey, 1996).

DISCUSSION

It is well known that Mg^{2+} decreases the biphasically Ca^{2+}-dependent CICR activity of the Ca^{2+} release channel (RyR) with a shift to a higher Ca^{2+} concentration range. However, there was no quantitative consideration on this matter until recent investigations made by Laver et al. (1997) and Meissner et al. (1997). During the course of this investigation, we have learned that there are some possible reasons for the difficulty. One of these is a substantial overlap between the Ca^{2+} activation curve, f_A, and the Ca^{2+} inactivation curve, $(1 - f_I)$. As shown in Fig. 8 *A*, Mg^{2+} affects the f_A and $(1 - f_I)$ curves differently. These effects of Mg^{2+} in particular make determination of the parameters difficult without numerous systematic experiments such as those shown here. To be freed from this difficulty, Meissner et al. (1997) performed experiments under different conditions to obtain separately parameters for the A- or I-site: some determinations were carried out in a solution containing 0.5 M choline chloride, where the contribution of the I-site was claimed to be negligible. It is still unclear, however, whether these parameters can be valid in situ, because the sensitivities for Ca^{2+} and Mg^{2+} were largely affected by several factors, including ionic species and their concentrations (Murayama and Ogawa, 1996; Ogawa et al., 1999). Laver et al. (1997), on the other hand, determined these parameters with cardiac RyR, where the inactivation by Ca^{2+} and Mg^{2+} was claimed to be weak, and extended these findings to the skeletal muscle RyR to obtain a simplified relationship with a necessary approximation.

We performed systematic [^{3}H]ryanodine binding experiments with α- and β-RyRs purified from frog skeletal muscle in the presence of various concentrations of Ca^{2+} and Mg^{2+} in an isotonic medium that simulated the characteristics of the sarcoplasm as far as possible and determined the parameters according to the three-step procedure with the aid of computer simulation. The [^{3}H]ryanodine binding activity in an isotonic salt solution is too low to be precisely analyzed in the absence of any stimulator other than Ca^{2+} (Murayama and Ogawa, 1996). Here, AMP was used to increase the activity without a significant change in Ca^{2+} dependence. The adenine nucleotide has another advantage in that it shows very weak affinity for Ca^{2+} and Mg^{2+}. Another important precaution is to keep the ionic strength of the medium constant, because the activity in the presence of high concentrations of Ca^{2+} and Mg^{2+} may otherwise be changed. After these considerations, the parameters for Ca^{2+} and Mg^{2+} of the two Ca^{2+} sites on individual isoforms can be obtained under the same conditions. To know the parameters for the Ca^{2+} release channel in situ that maintain the organization of RyR and related proteins, furthermore, we measured the CICR activity, using frog skinned skeletal muscle fibers under conditions as close as possible to those of the physiological environment. In analyzing these results, we took advantage of the determinations of $n_{A,Mg} = n_{I,Ca} = n_{I,Mg} = 1$ in [^{3}H]ryanodine binding experiments, because more laborious maneuvers of CICR experiments impeded such numerous determinations, as in [^{3}H]ryanodine binding.

All of the results obtained are summarized in Table 2 for [^{3}H]ryanodine binding experiments and in Table 3 for CICR from SR in frog skinned skeletal muscle fibers.

Comparison between Ca^{2+} release channel in SR and purified RyR

Table 2 shows that α- and β-RyRs are very similar in the values for all parameters in the presence of 4 mM AMP. This means that there was only a minor difference at most in the Ca^{2+}-dependent [^{3}H]ryanodine binding and its modulation by Mg^{2+} between the two isoforms. Frog skeletal muscles express the two isoforms in almost equal amounts (Murayama and Ogawa, 1992; 1994). This indicates that both α- and β-RyRs contribute to the CICR activity in frog

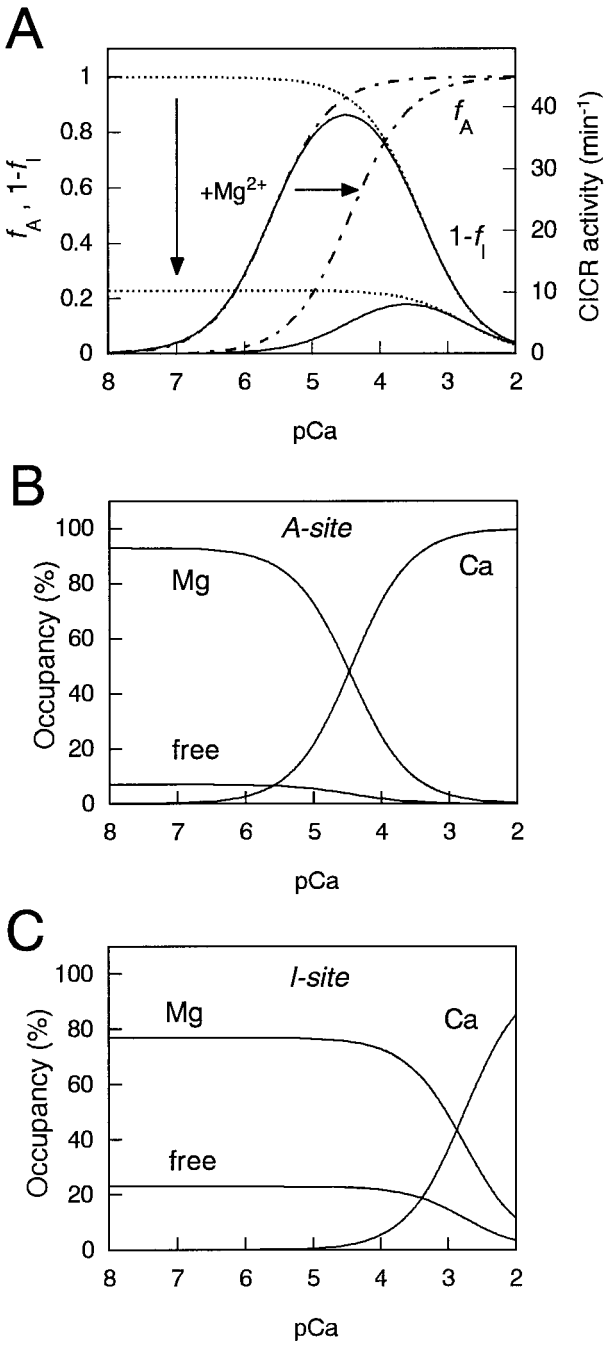

FIGURE 8 Simulations of effects of Mg^{2+} on the CICR activity in situ and analysis of the occupancy by divalent cations of the A- and I-sites. (*A*) The fractions of Ca^{2+}-occupied A-site (f_A) and vacant I-site (1-f_I) and the anticipated CICR activity ($k = k_{max} \times f_A \times (1 - f_I)$) were calculated in the presence or absence of 1 mM Mg^{2+}. f_A (– – –), $1 - f_I$ (·····), and k (——) were calculated as a function of free Ca^{2+}, using the following parameters, which are assumed to be those for the Ca^{2+} release channel of SR in situ: $k_{max} = 45\ min^{-1}$; $K_{A,Ca} = 2.5\ \mu M$; $n_{A,Ca} = 1$; $K_{I,Ca} = 0.4$ mM; $n_{I,Ca} = 1$; $K_{A,Mg} = 75\ \mu M$; $n_{A,Mg} = 1$; $K_{I,Mg} = 0.3$ mM; $n_{I,Mg} = 1$. We assumed $n_{A,Ca}$ to be 1 in the presence of 4 mM AMPPCP, based on the finding that $n_{A,Ca}$ values decreased with an increase in AMPPCP concentration (from 1.4 at 0.2 mM to 1.2 at 1 mM; see Table 3). Mg^{2+} (1 mM) shifts curve f_A rightward along the abscissa to decrease the apparent Ca^{2+} sensitivity and reduces $(1 - f_I)$ throughout all Ca^{2+} concentrations, resulting in the decreased peak value. (*B* and *C*) The probabilities of occupancy by divalent cations of A- (*B*) and I- (*C*) sites were calculated as a function of free Ca^{2+} in the presence of 1 mM Mg^{2+}. The same parameter values as those in *A* were used for calculation. See text for details.

skinned skeletal muscle fibers, although we cannot exclude the possibility that either isoform might be silent in situ.

Comparison of Tables 2 and 3 tells us that purified RyR shows lower affinities for Ca^{2+} and Mg^{2+} than does the Ca^{2+} release channel in SR: $K_{A,Ca}$ (~10 μM versus ~3 μM), $K_{A,Mg}$ (~300 μM versus ~70 μM), $K_{I,Ca}$ and $K_{I,Mg}$ (~2 mM versus ~0.4 mM). However, $K_{A,Mg}/K_{A,Ca}$ (= 10–30) and $K_{I,Mg}/K_{I,Ca}$ (= 1) remained constant. This means that the selectivity between Ca^{2+} and Mg^{2+} in each of the A- and I-sites was unchanged, although their affinities for divalent cations were varied. Addition of CHAPS to SR vesicles and the following process of purification altered the Ca^{2+} sensitivity of both A- and I-sites (Ogawa et al., 1999). Changes in the environment around RyRs (e.g., phospholipids, detergent, associating protein(s), and so on) may affect properties of the RyR molecule or Ca^{2+} release channels. We would like to point out that troponin C in the thin filament shows a 10-fold higher affinity for Ca^{2+} than purified troponin C (Ebashi and Ogawa, 1988).

Effect of adenine nucleotides on the activity of RyR

Although ATP is thought to be an endogenous activator of RyR in situ, it has not been known whether MgATP is as potent as free ATP in stimulating RyR. The comparison of divalent cation dependencies in the presence of AMP and AMPPCP (Fig. 5) suggests that free AMPPCP, CaAMPPCP, and MgAMPPCP are equipotent in stimulating CICR. Because AMPPCP and ATP are reported to be very similar in this stimulation (Kakuta, 1984), these results suggest that MgATP stimulates RyR as much as free ATP does in situ.

The CICR activity in the absence of any adenine nucleotide was as low as 0.1–0.3 min^{-1}, even at the optimum pCa, whereas that in the presence of 1 mM AMPPCP was around 13 min^{-1} (see Fig. 6 *A*). In the presence of 3–9 mM ATP, which corresponds to the myoplasmic concentration in frog skeletal muscle (Godt and Maughan, 1988), the enhancement of CICR activity by ATP would be as great as >200-fold. These findings suggest that ATP is critically important for activation of RyRs in frog skeletal muscle.

Whereas Meissner et al. (1986, 1997) reported that adenine nucleotides render RyR less sensitive to inhibition by Ca^{2+} and Mg^{2+}, we did not find any evidence of a decrease in Ca^{2+}/Mg^{2+} sensitivity with increase in adenine nucleotide concentration. However, we cannot exclude the possibility that the I-site of CICR activity might be more sensitive to Mg^{2+} in the absence of adenine nucleotide than in its presence. We did not determine the affinity for Mg^{2+} in the

absence of any adenine nucleotide where the RyR activity was too small to analyze the effect of Mg^{2+}.

It has long been known that adenine nucleotides enhance CICR without changing Ca^{2+} sensitivities (Endo, 1981). The results of the present study showed that the affinities for Ca^{2+} and Mg^{2+} of both A- and I-sites were not significantly affected by any species or concentrations of adenine nucleotides in skinned fibers (Fig. 6 and Table 3). Only k_{max}, the calculated maximum CICR activity, was largely enhanced by adenine nucleotides. These findings indicate that the occupation of the A-site by Ca^{2+} is not the sole determinant for the CICR activity of RyR. In other words, the occupation by Ca^{2+} of the A-site may be a necessary but not a sufficient condition for Ca^{2+} release. The stimulating effect of ATP was also observed in the absence of Ca^{2+} as well as in its presence. ATP may affect the transition between closed and open states of the Ca^{2+} release channel in favor of the open state (Smith et al., 1986; Coronado et al., 1994; Meissner, 1994). The increase in k_{max} and B_{max} may reflect this effect (Figs. 6 and 8).

Actions of caffeine on the function of RyR

Caffeine has been known as a potent stimulator of CICR. The main action of the drug was thought to be to enhance the Ca^{2+} sensitivity for the activation of CICR (Endo, 1981). We here showed that caffeine specifically increased the affinity for Ca^{2+} of the A-site but left that for Mg^{2+} unchanged (Tables 2 and 3). This is characteristic of caffeine. Furthermore, the drug decreases slightly the affinities for Ca^{2+} and Mg^{2+} of the I-sites of α- and β-RyR, to an almost equal extent. With SR in skinned fibers, the affinity for Ca^{2+} of the I-site appeared to be decreased in the presence of 0.2 mM AMPPCP but unchanged in the presence of 1 mM AMP (Table 3). The drug did not change the affinity for Mg^{2+} of the I-site in either case, suggesting minor effects on the I-site. These results suggest that caffeine may only minimally affect the interaction of Mg^{2+} with the A- or I-sites. The removal of inactivation by Ca^{2+} or Mg^{2+} cannot be the underlying mechanism for the Ca^{2+}-releasing action of caffeine, although this was claimed to be the case for several drugs, such as bastadins (Mack et al., 1994).

In addition to the effects on the A-sites, caffeine at 5 mM enhanced k_{max} by four- to sixfold in CICR activity. The increase by caffeine of the peak CICR activity at the optimum Ca^{2+} concentration has also been reported by Endo (1981). Such a great enhancement in maximum activity was also observed with [^{3}H]ryanodine binding with SR vesicles from frog skeletal muscle (Ogawa and Harafuji, 1990; Ogawa et al., 1999). With purified isoforms, in contrast, the enhancement of [^{3}H]ryanodine binding was at most only ~20% (Table 2; see also Murayama and Ogawa, 1996). The presence of CHAPS, which potentiates the effect of adenine nucleotide (Ogawa et al., 1999), may be a possible explanation for the weak enhancement. Because effects of caffeine and adenine nucleotides are synergistic in the enhancement of k_{max} or B_{max}, this effect should also largely contribute to the Ca^{2+}-releasing action of caffeine in frog skeletal muscle. SR vesicles from a mammalian skeletal muscle that often fails to contract with caffeine showed a smaller enhancement of B_{max} than those from frogs, whereas there was no significant difference in the Ca^{2+}-sensitizing effect of caffeine between them (Ogawa et al., 1999). Thus the enhancement of k_{max} or B_{max} is also critically important for the induction of Ca^{2+} release in vivo.

The results presented in this study thus suggest that caffeine activates frog skeletal muscle RyRs by two different mechanisms: increasing the Ca^{2+} sensitivity of the A-site and enhancing k_{max}.

Relevant physiological significance

The affinities for Ca^{2+} and Mg^{2+} of each site of the Ca^{2+} release channel in the skinned fibers ranged in the presence of AMPPCP as follows: 2.5–3 μM for $K_{A,Ca}$; ~0.4 mM for $K_{I,Ca}$; 50–100 μM for $K_{A,Mg}$; and 0.18–0.36 mM for $K_{I,Mg}$ (Table 3). The physiological concentration of free Mg^{2+} has been estimated to be ~1 mM in skeletal muscle cells (Westerblad and Allen, 1992; Konishi et al., 1993). The myoplasmic Ca^{2+} concentration is 0.1–0.3 μM at rest (Harkins et al., 1993; Kurebayashi et al., 1993) and increases to ~10 μM during maximum activation (Konishi and Baylor, 1991). The k_{max} value in situ was estimated to be 35–50 min^{-1} from the results with 0.2 and 1 mM AMPPCP (Table 3 and Fig. 6), assuming that myoplasmic concentration of ATP is 4 mM. Fig. 8 *A* depicts the effect of 1 mM Mg^{2+} on the CICR activity in skinned fibers, using these parameters. The simulated probabilities of A- and I-sites that are occupied by Ca^{2+} or Mg^{2+} as a function of free Ca^{2+} concentration are shown in Fig. 8, *B* and *C*. An important conclusion is that nearly 80% of the I-sites are saturated with Mg^{2+}, irrespective of the myoplasmic Ca^{2+} concentration, from 0.1 to 100 μM Ca^{2+} (Fig. 8 *C*). This means that the fraction of potentially activatable channels for CICR (i.e., $(1 - f_I)$) will be only ~20% at most in the presence of 1 mM Mg^{2+} (see Fig. 8 *A*). In addition, at the resting level of submicromolar Ca^{2+}, more than 90% of the A-sites will be occupied by Mg^{2+}, and the vacant and Ca^{2+}-occupied sites are only 7% and less than 1%, respectively (Fig. 8 *B*). The fraction of A-sites occupied by Ca^{2+} increases with increase in free Ca^{2+}. Thus it appears that the exchange of Mg^{2+} with Ca^{2+} at the A-site may occur during activation of CICR by Ca^{2+}. The fraction of the A-sites occupied by Ca^{2+} (i.e., f_A) is estimated to be 3% at 1 μM Ca^{2+}, 8% at 3 μM Ca^{2+}, and 22% at 10 μM Ca^{2+}. The fractions of the CICR channel activated by Ca^{2+} (i.e., $f_A \times (1 - f_I)$) are 0.06–0.2, 0.6, 2, and 5% at 0.1–0.3, 1, 3, and 10 μM Ca^{2+}, respectively (see Fig. 8 *A*). These indicate that Mg^{2+} potently reduces the CICR channel activity in frog skeletal

muscle, not only in the resting state, but also during activation by interacting with both the A- and I-sites.

The rates of the physiological depolarization-induced Ca^{2+} release in frog intact and cut muscle fibers are reported to be 2–5%/ms, which corresponds to 1200–3000 min^{-1} (Csernoch et al., 1993; Pape et al., 1995). The CICR activity predicted by this study, on the other hand, is estimated to be not more than 10 min^{-1} in the presence of 4 mM ATP and 1 mM Mg^{2+} (see Figs. 6 *A* and 8 *A*). Because 5 mM of caffeine potentiates k_{max} by four- to sixfold, the rate of Ca^{2+} release induced can be estimated to be 50 min^{-1} or less, which would correspond to only a few percent of the rate of Ca^{2+} release on depolarization. The tension development by a frog intact single skeletal muscle fiber on exposure to 5 mM caffeine was traced by a mechanoelectrical transducer (RCA5734), while the isometric twitch tension was too fast to follow and had a spike-like appearance (figure 1 *C* of Lüttgau and Oetliker, 1968). This suggests that our speculation about the rate of the caffeine-induced Ca^{2+} release may be not very far from the truth. Therefore, we can conclude that CICR is much slower than depolarization-induced Ca^{2+} release. This will mean that the contribution of the CICR to the physiological Ca^{2+} release is minor, even at the optimal Ca^{2+} concentration.

Lamb and Stephenson (1991) found that a spontaneous Ca^{2+} release occurred at a resting level of free [Ca^{2+}] by lowering myoplasmic Mg^{2+} concentration from 1 mM to 0.05 mM, and they proposed a model for E-C coupling in which activation of the voltage sensor of T-tubule membrane might decrease the affinity for Mg^{2+} of the Ca^{2+} release channel by 10–20-fold, resulting in Ca^{2+} release during depolarization. Our results indicate that the CICR activity at a resting [Ca^{2+}] (0.1–0.3 μM) would be increased by 13-fold if the inhibition by Mg^{2+} at the A-site were canceled and by 60-fold if inhibition at both A- and I-sites disappeared in the presence of 1 mM Mg^{2+} (Fig. 8). The ratio of the increment is consistent with their prediction. In the absence of Mg^{2+}, however, the actual rate of CICR would be only ~1 min^{-1} at rest and ~40 min^{-1} at the optimal Ca^{2+} in the presence of 4 mM ATP (Fig. 8 *A*). These values are much lower than the rate of depolarization-induced Ca^{2+} release as discussed above. Therefore, the reduction of the affinity for Mg^{2+} in the process of the CICR cannot be the explanation for the rate of Ca^{2+} release on depolarization. A certain mechanism that causes enormous enhancement of the rate of release must be inevitable for physiological Ca^{2+} release. It is probable that an entirely different mode of opening of RyR may operate in the Ca^{2+} release.

Schneider and Simon (1988) reported the rapid Ca^{2+}-dependent inactivation of depolarization-induced Ca^{2+} release from the SR in frog skeletal muscle. They proposed a model in which Ca^{2+} rapidly binds to a site on the channel or a certain molecule, leading eventually to the inactivated state of the channel after the isomerization of the channel molecule. They claimed that it could be a high-affinity site with a micromolar value or less for the dissociation constant. Jong et al. (1995), on the other hand, interpreted the Ca^{2+}-dependent inactivation differently with a similar model. They claimed that the responsible inactivating sites must be near or within the mouth of the Ca^{2+} release channel. Although they did not state it explicitly, the affinity for Ca^{2+} appears to be low, because Ca^{2+} that comes out of the lumen of the SR should bind to the site before the ions are sequestered by EGTA or fura-2. Some groups have suggested the possibility that the luminal Ca^{2+} has access to the inactivation site and inhibits CICR activity (Tripathy and Meissner, 1996; Kurebayashi and Ogawa, 1998). It is therefore possible that the Ca^{2+} inactivation site of the depolarization-induced Ca^{2+} release might be the same as the I-site of CICR that is mentioned here. Our calculation suggests that the I-sites free of Mg^{2+} or Ca^{2+} (i.e., $1 - f_I$) can be ~20% at rest and that the increment of Ca^{2+} at 1–3 mM, which is the putative free Ca^{2+} concentration near the mouth of the channel during Ca^{2+} release, would decrease the $(1 - f_I)$ value to 8–15%. Therefore, the population of the active channel would be decreased by 25–60% by Ca^{2+} released from the SR. Although this consideration may support the possibility that the alleged site might be the I-site, this inactivation seems ineffective, because 80% of the channel has already been suppressed by Mg^{2+}. Further study is needed to identify the inactivation site in the depolarization-induced Ca^{2+} release and the effect of Ca^{2+} and Mg^{2+} on the physiological Ca^{2+} release.

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