Effect of Luminal Calcium on Ca^{2+} Release Channel Activity of Sarcoplasmic Reticulum in Situ

Nagomi Kurebayashi and Yasuo Ogawa
Department of Pharmacology, Juntendo University School of Medicine, Tokyo 113-8421, Japan

ABSTRACT Ca^{2+} influx into empty SR in the absence of Ca^{2+} pump activity was determined in skinned frog skeletal muscle fibers and compared with Ca^{2+} efflux from loaded SR (i.e., Ca^{2+} release) to deepen our understanding of the properties of the Ca^{2+} release channel (CRC). Calcium content in SR increased approximately in a first-order kinetics and finally reached the equilibrium level determined by cytoplasmic Ca^{2+} ($[Ca^{2+}]_C$). Because AMP caused an increase in the rate of Ca^{2+} influx, and procaine, Mg^{2+}, and high concentrations of Ca^{2+} caused a characteristic decrease, the major Ca^{2+} influx pathway was concluded to be the CRC, as is true of Ca^{2+} release. The apparent rate constant (k_{app}) of Ca^{2+} efflux did not significantly change when the loading level was decreased to one-third. At a given $[Ca^{2+}]_C$, the same equilibrium level of calcium in SR was attained with a similar k_{app} by both Ca^{2+} influx and Ca^{2+} efflux. The relationship between $[Ca^{2+}]_C$ and calcium in SR indicated the Ca^{2+} binding sites in SR. These results, together with the anticipated effects of these Ca^{2+} buffer sites on kinetics, are consistent with the idea that luminal Ca^{2+} inhibits the CRC.

INTRODUCTION

The Ca^{2+} release channel/ryanodine receptor in sarcoplasmic reticulum (SR) plays a critical role in excitation-contraction coupling in vertebrate skeletal muscles by opening in response to sarcolemmal depolarization. Ca^{2+}-induced Ca^{2+} release (CICR) is also observed with the Ca^{2+} release channel, and its properties have been extensively investigated. Cytoplasmic Ca^{2+} has a dual action on the channel activity, i.e., it is stimulating between 10^{-6} to 10^{-4} M and inhibitory at higher concentrations, by acting on activating and inactivating Ca^{2+} sites of the Ca^{2+} release channel, respectively (Endo, 1981; Kurebayashi and Ogawa, 1986; Meissner et al., 1986; Ogawa and Harafuji, 1990).

The properties of CICR have been investigated with several methods using different preparations, such as Ca^{2+} efflux measurement from Ca^{2+}-loaded SR in skinned fiber preparations (Endo, 1981; Kurebayashi and Ogawa, 1986; Murayama et al., 1997) or isolated vesicles (Meissner et al., 1986), [^{3}H]ryanodine binding to the Ca^{2+} release channel proteins (Ogawa and Harafuji, 1990; Murayama and Ogawa, 1996), and single-channel measurements with ryanodine receptor incorporated into planer lipid bilayers (Smith et al., 1986, 1988). Among them, skinned fiber preparations have the advantage that the organization of various biological components including SR is well maintained and that it is easy to modulate the cytoplasmic milieu by changing the composition of incubation solutions repeatedly with the same preparation. In the preparation, the channel activity has usually been determined as a rate of Ca^{2+} efflux into cytoplasmic solution from SR loaded with a specified level of Ca^{2+} according to a downhill gradient. The rate of Ca^{2+} efflux depends on both the Ca^{2+} gradient across SR membrane and permeability of the channel. It is easy to measure Ca^{2+} efflux under the condition where the Ca^{2+} gradient is large, but it would be difficult to determine the efflux rate in the presence of high concentrations of $[Ca^{2+}]_C$ where the Ca^{2+} concentration gradient is small or may be reversed. It is also impossible to determine Ca^{2+} efflux from empty SR. Kitazawa and Endo (1976) have shown that Ca^{2+} influx into empty SR with a reversed gradient was stimulated by caffeine and suppressed by Mg^{2+} or procaine, and claimed that the influx was a reversal in direction of Ca^{2+} release.

Recently modulatory effects of luminal Ca^{2+} of SR ($[Ca^{2+}]_L$) on the Ca^{2+} release channel activity have been claimed. The reported results, however, are somewhat controversial and variable: increased Ca^{2+} release channel activity by increased $[Ca^{2+}]_L$ (Ikemoto et al., 1989; Donoso et al., 1995; Sitsapesan and Williams, 1995), decreased activity (Fill et al, 1990; Ma et al., 1988), or biphasically affected activity (Tripathy and Meissner, 1996). Various putative underlying mechanisms for those findings are also proposed: direct action of Ca^{2+} itself on ryanodine receptors from the luminal side (Sitsapesan and Williams, 1995) or from the cytosolic side accessed by Ca^{2+} coming out (Ma et al., 1988; Fill et al., 1990; Tripathy and Meissner, 1996), and indirect action via luminal proteins such as calsequestrin (Ikemoto et al., 1991; Kawasaki and Kasai, 1994).

The measurement of Ca^{2+} influx may be useful for determining the channel activity at high concentrations of $[Ca^{2+}]_C$ and/or low concentrations of luminal free Ca^{2+} to elucidate these discrepancies. In this paper we determined the properties of Ca^{2+} influx into SR in skinned fibers under a reversed Ca^{2+} gradient and showed that the Ca^{2+} influx as well as the Ca^{2+} efflux occurred mainly through the Ca^{2+} release channel. We also estimated the size of luminal Ca^{2+}

Received for publication 11 August 1997 and in final form 13 January 1998.

Address reprint requests to Dr. Nagomi Kurebayashi, Department of Pharmacology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan. Tel.: +81-3-5802-1035; Fax: +81-3-5802-0419; E-mail: nagomik@med.juntendo.ac.jp.

0006-3495/98/04/1795/13 $2.00

binding sites in SR from the equilibrium level of calcium content (free Ca^{2+} plus bound Ca^{2+}) in SR at various levels of $[Ca^{2+}]_C$. The effect of luminal Ca^{2+} on the Ca^{2+} permeability of SR in situ was examined by comparing time courses of Ca^{2+} efflux from loaded SR and Ca^{2+} influx into empty SR and by modeling of Ca^{2+} fluxes by using the estimated parameters for the size of Ca^{2+} binding sites in SR.

MATERIALS AND METHODS

Preparations

Frog (*Rana japonica*) was killed by decapitation, and the iliofibularis muscle was excised. A single skinned fiber (50–85 μm in diameter), isolated and mechanically split in a relaxing solution (see Table 1 for composition), was mounted in an experimental chamber at a sarcomere length of 2.6 μm (Kurebayashi and Ogawa, 1986; Murayama et al., 1997). All of the experiments were performed at a temperature of 16°C.

Solutions

Table 1 shows the composition of experimental solutions. A relaxing solution (RS) and Ca^{2+} loading solutions (L1 and L2) contained 4 mM MgATP and 1 mM free Mg^{2+}, and 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 detection of Ca^{2+} release from SR. A Ca^{2+} concentration in TS was achieved by mixing EGTA and $CaCl_2$ as described in Table 1. AMP at 4 mM was added to TS in most experiments, except where stated otherwise (cf. Fig. 5). Ionic strengths of all of the solutions were adjusted to 0.16 with KCl, and pHs were adjusted to 6.8 with KOH. Free Ca^{2+} concentration in TS was calculated using 1.048×10^6 M^{-1} for the apparent binding constant of EGTA for Ca^{2+} (Harafuji and Ogawa, 1980). All solutions contained 2 μg ml^{-1} leupeptin, which kept the Ca^{2+}-releasing and Ca^{2+}-accumulating activities of SR in good conditions during the entire course of experiments.

TABLE 1 Composition of experimental solutions

Solution	EGTA/K_2 (mM)	$CaCl_2$ (mM)	$MgCl_2$ (mM)	Fura 2/K_5 (μM)	ATP/Na_2 (mM)	Others (mM)
RS	2	—	5	—	4.3	—
L1	2	0.5	5	—	4.3	—
L2	2	0.2	5	—	4.3	—
W1	10	—	1	—	—	—
W2	10	—	—	1.0	—	—
W3	0.1	—	—	1.0	—	—
TS	0–10*	0–30*	#	—	—	#
DS	0.1	—	—	1.0	—	25 caffeine 1 AMP

All solutions contained 20 mM MOPS and 2 μg ml^{-1} leupeptin. Their pHs were adjusted to 6.8 with KOH and ionic strengths to 0.16 with KCl.

* Free Ca^{2+} concentration was made by mixing EGTA and $CaCl_2$ as follows: $[Ca^{2+}] \leq 0.1$ mM, 10 mM EGTA plus calculated $CaCl_2$; 0.1 mM $< [Ca^{2+}] <$ 10 mM, 10 mM $CaCl_2$ plus calculated EGTA as $(10 - [Ca^{2+}])$; $[Ca^{2+}] \geq 10$ mM, $CaCl_2$ alone.

CICR modulators such as Mg^{2+}, AMP and procaine were added if necessary.

Experimental setup for Ca^{2+} determination and calibration of fura 2 ratio signals

The previous experimental setup (Kurebayashi and Ogawa, 1991) was modified to enable us to measure fura 2 fluorescence (Murayama et al., 1997). Briefly, the experimental chamber (2-mm width × 30-mm length × 1-mm depth) had a bottom of clear thin glass and was fixed on a stage of an inverted epifluorescent microscope (Nikon TMD, Tokyo), which was equipped with a Spex spectrofluorometer (model CM1T11I; Spex, Edison, NJ). The skinned fiber and the surrounding solution in the chamber were illuminated with an alternating beam of 340 nm and 380 nm, and epifluorescent light longer than 420 nm within a rectangular field of 350 × 350 μm was detected by a photomultiplier. The ratio of fluorescent light of fura 2 excited at 340 nm to that at 380 nm was determined to detect Ca^{2+} concentration changes within the field. The increase in fluorescence ratio (ΔR) that was caused by the increase in Ca^{2+} in DS was normalized by the maximum ratio difference, $R_{max\text{-}min}$, ($= R_{max} - R_{min}$, where R_{min} and R_{max} denote ratios on the addition of 10 mM EGTA and 10 mM $CaCl_2$, respectively). The ratio for a Ca^{2+}-unloaded fiber in DS (R_{DS}) was close to R_{min} ($(R_{DS} - R_{min})/R_{max\text{-}min} \leq 0.002$).

To mimic determinations with skinned fibers, we determined a relationship between $\Delta R/R_{max\text{-}min}$ and the amount of Ca^{2+} that was added to DS in the experimental chamber through a micropipette containing 10 mM $CaCl_2$ solution, which was connected to a pressure system Picospritzer II (General Valve Co., Fairfield, NJ). The tip (inner diameter of 1–3 μm) was placed at the left side of the monitored rectangular field in the experimental chamber. When a given period of pressure pulse was applied at a constant pressure, the ratio signal increased to reach the peak and then slowly decreased (Fig. 1 *A*), which was due to diffusion of Ca^{2+}. The peak amplitude of $\Delta R/R_{max\text{-}min}$ increased with the pulse duration. The $\Delta R/R_{max\text{-}min}$ signal within 0.15 was linear to the pulse duration with an x intercept of 15 ms (Fig. 1 *B*). Similar results were obtained with two other micropipettes of different diameters (data not shown). The slope was steeper with a larger diameter of micropipette, but the x intercept was the same. Because a linear relation between ejected volume and pulse duration with a particular mechanical lag time is an expected performance for this pressure system as the manufacturer describes, we concluded that $\Delta R/R_{max\text{-}min}$ signal up to 0.15 is linearly related to the amount of Ca^{2+} added to DS. The ejected volumes from the pipette indicated in Fig. 1 *B* were determined as follows. The change in the excitation spectrum (320–420 nm) of 50 μl W3 solution after ejection of 20–50 pulses followed by complete mixing was compared with that of W3 solutions containing calibrated amounts of Ca^{2+}. The result indicated that a single pressure pulse of 500 ms duration ejected 600 pl of the solution through the pipette. The ejected volumes that were calculated from the varied durations of the pulse by the linear relationship were plotted on the second x axis in Fig. 1 *B*. Note that the lag time of 15 ms was adjusted in the second axis. In this relation, 10 pmol of $CaCl_2$ corresponded to $\Delta R/R_{max\text{-}min}$ of 0.13. Similar relations were obtained with the other micropipettes.

Ca^{2+} flux measurements

Experimental protocols for determination of Ca^{2+} fluxes were similar to those described previously (Murayama et al., 1997). Before a series of experiments, a skinned fiber was treated with DS to empty the SR of Ca^{2+} and was kept in RS until use. A series of steps for Ca^{2+} influx protocol were carried out as shown in Fig. 2 *A*. After removal of ATP by washing in succession with solution W1 for 60 s and solution W2 for 30 s, the skinned fiber was treated with a TS containing a specified concentration of Ca^{2+} for a specified period. The fiber was then washed again with solutions W1, W2, and W3 for 30 s, 15 s, and 15 s, respectively. During the washing procedure, the amount of calcium accumulated in SR did not significantly decrease. The fiber was then challenged with DS to discharge completely releasable calcium in SR. The peak $\Delta R/R_{max\text{-}min}$ in DS is indicative of the amount of calcium accumulated in SR. The treatment with DS was enough to discharge all calcium in SR, because further application of 5 μM A23187 or 1% Triton X-100 to solution W3 did not show further

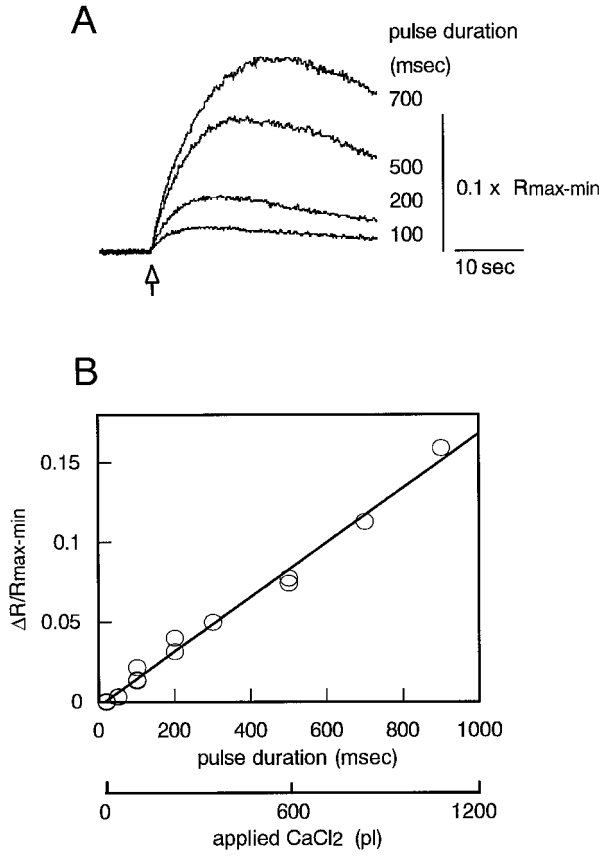

FIGURE 1 Calibration of Ca^{2+} signal of fura 2 in our experimental setup. (*A*) Typical ratio signals recorded when 10 mM $CaCl_2$ was ejected from the micropipette into DS solution, which mimics Ca^{2+} release from SR in skinned fiber. The ejected volume was controlled by applying pressure pulses for 100, 200, 500, or 700 ms at a constant pressure. The fluorescence ratio signal increased to the peak value and then decreased. This decrease is due to spreading out of Ca^{2+} from the focus, not to fluorescent bleaching. The absolute ejected volume was determined in separate experiments (see text for details). (*B*) Relationship of the peak level of the fura 2 ratio signal ($\Delta R/R_{max\text{-}min}$) versus the amount of Ca^{2+} applied from the micropipette. Note that $\Delta R/R_{max\text{-}min}$ is proportional to the amount of applied Ca^{2+} within the value of 0.15.

change in the Ca^{2+} signal (data not shown). The magnitude of $\Delta R/R_{max\text{-}min}$ was at its highest (0.12) for the Ca^{2+} release from SR in DS in our experiments, which was within a linear range of the Ca^{2+}-$\Delta R/R_{max\text{-}min}$ relation, as indicated in Fig. 1 *B*.

For Ca^{2+} efflux measurements, SR in a skinned fiber was at first actively loaded to a constant level by incubation with solution L1 of pCa 6.5 for 2 min (prescriptive loading) (see Fig. 2 *B*). The following successive steps, including washing, treatment in TS, washing, and discharging Ca^{2+}, were the same as those for influx measurement, and the amount of remaining calcium in the SR after test treatment for a specified period was determined (Fig. 2 *B*, *traces*). These influx or efflux protocols were repeated with the same fiber. In some experiments, the time courses of Ca^{2+} efflux from lower loading levels were compared to that from the prescriptive loading level. To obtain a lower loading level, the skinned fiber was incubated in solution L2 of pCa 7.0 for 60 s (for 1/3 loading) or 120 s (1/2 loading).

The prescriptive loading levels without any releasing stimulus were determined every three to five series of determinations and used as a control (prescriptive loading). In the text, the amount of calcium in SR was expressed as a value relative to the amount of the prescriptive loading level, except where stated otherwise. The prescriptive loading level amounted to 90% of the maximum accumulation obtained in solution L1. The level was between two and three times higher than the amount of calcium in the SR determined just after the skinning procedure, which nearly corresponds to the calcium content in the SR in intact fiber (data not shown).

The amount of calcium in SR at the prescriptive loading in a skinned fiber of 80-μm diameter would correspond to an impulse of ~5.3 pmol Ca^{2+} in Fig. 1, on the basis of the following findings: the monitored muscle length was 350 μm; the total calcium concentration in SR would be 30 mM (see Fig. 9); the SR volume is estimated to be 10% of the total muscle (Ogawa, 1970; Baylor et al., 1983) (0.04^2 * Π * 0.35 * 10^{-6} * 0.1 * 0.03 mol). The amount of Ca^{2+} would give a $\Delta R/R_{max\text{-}min}$ of 0.065 (see Fig. 1), which is comparable to the actual $\Delta R/R_{max\text{-}min}$ observed for the prescriptive loadings in our skinned fiber experiments (see Fig. 2 *B*).

RESULTS AND DISCUSSION

Identification of Ca^{2+} influx pathway

Initially we examined the basic properties of passive Ca^{2+} influx into SR to confirm that the involved main pathway is the Ca^{2+} release channel. Traces in Fig. 2 *A* show ratio signals of fura 2 in DS after a skinned fiber with empty SR was incubated in a TS containing 3 mM cytoplasmic Ca^{2+} ($[Ca^{2+}]_C$) and 4 mM AMP for various periods. The amplitude of the Ca^{2+} signal, which indicates the amount of calcium accumulated in SR, increased with prolongation of the incubation period. Fig. 2 *B*, the counterpart of Fig. 2 *A*, shows ordinary CICR experiments with the same preparation prescriptively loaded. We would like to point out that the remaining amounts of calcium in SR after 600 s of stimuli were similar in Ca^{2+} influx and efflux (see also Fig. 8).

Examples of time courses of Ca^{2+} influx into SR at 1 mM (*open squares*) and 5 mM $[Ca^{2+}]_C$ (*closed circles*) are plotted in Fig. 3. The amount of calcium in SR is expressed relative to the prescriptive loading level. The accumulated calcium in SR became larger with a longer incubation period and finally reached a steady level, depending on $[Ca^{2+}]_C$. The time courses of the Ca^{2+} influxes could be fitted with an exponential equation, $A * \{1 - \exp(-k_{app} * t)\}$, where A is the amount of calcium in SR at steady state and k_{app} is the apparent rate constant of the Ca^{2+} influx. It should be noted that no clear lag or delay was observed in the rising phase of the influx, indicating no sign of change in Ca^{2+} permeability through the SR membrane during Ca^{2+} influx into the empty SR. The steady loading level at 5 mM $[Ca^{2+}]_C$ was higher than that at 1 mM $[Ca^{2+}]_C$, whereas k_{app} was ~2.5 times smaller in 5 mM $[Ca^{2+}]_C$.

The steady level of calcium in SR (A) and the apparent rate constant (k_{app}) are plotted as a function of $[Ca^{2+}]_C$ (Fig. 4). The steady amount of calcium in SR monotonically increased with an increase in $[Ca^{2+}]_C$. The passive loading level at pCa 2.0 was similar to the prescriptive loading level, and a higher Ca^{2+} loading level was attained with incubation in 30 mM $[Ca^{2+}]_C$. The apparent rate constant, k_{app}, on the other hand, decreased with an increase in $[Ca^{2+}]_C$. When $[Ca^{2+}]_C$ was 30 mM, k_{app} was as small as 0.2 min^{-1}, and it took more than 300 s to reach a plateau. This negative

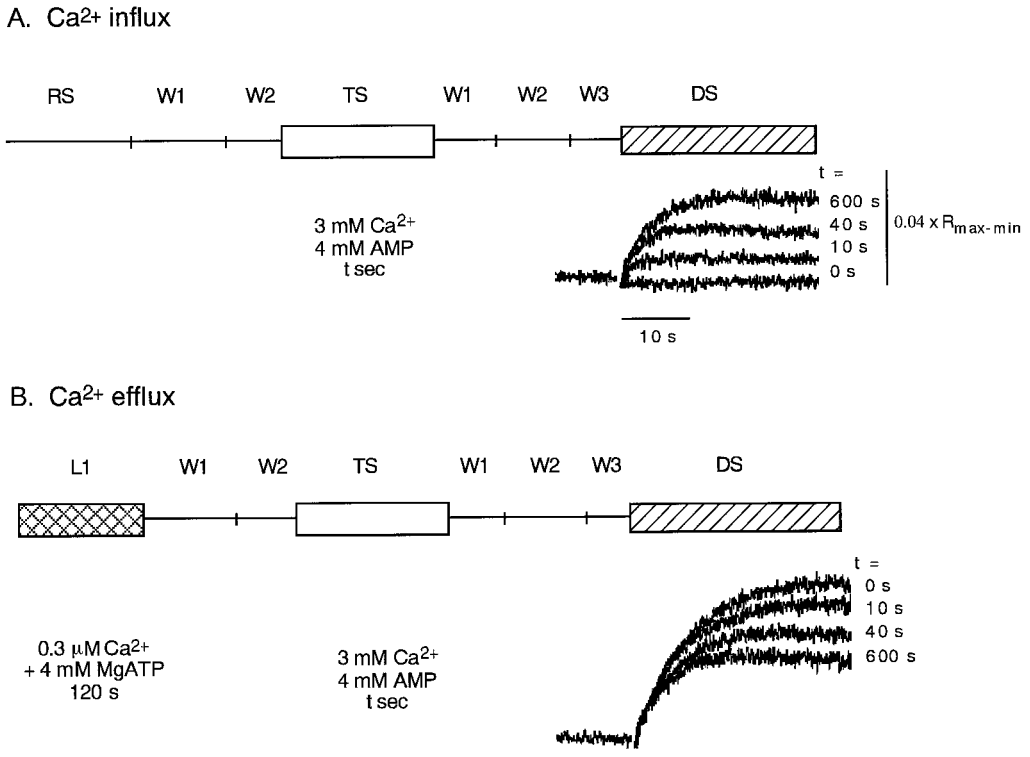

FIGURE 2 Experimental protocols for Ca^{2+} influx into empty SR and for Ca^{2+} efflux from loaded SR. (*A*) Procedure for Ca^{2+} influx into empty SR, together with superimposed traces of Ca^{2+} ratio signal in DS after incubations for 0, 10, 40, and 600 s with a TS containing 3 mM Ca^{2+} and 4 mM AMP. (*B*) Procedure for Ca^{2+} efflux measurements with traces of ratio signal, showing the remaining Ca^{2+} in SR after incubation with TS for the same periods. SR was actively loaded with Ca^{2+} to a constant level at first, and the remaining calcium in SR was determined after TS treatment. Note that the amounts of calcium in SR achieved by either procedure reached an almost identical level after 600 s of incubation in TS.

dependence of the rate constant, which corresponds to the Ca^{2+} permeability, on high concentrations of $[Ca^{2+}]_C$ was similar to the characteristics shared by CICR activities in skinned fibers, SR vesicles, and purified proteins (Endo, 1981; Meissner et al., 1986; Ogawa and Harafuji, 1990; Murayama and Ogawa, 1996; Murayama et al., 1997).

It is well known that the Ca^{2+} release channel is activated by adenine nucleotides and inhibited by procaine or Mg^{2+}. We examined the effects of these modulators on Ca^{2+}

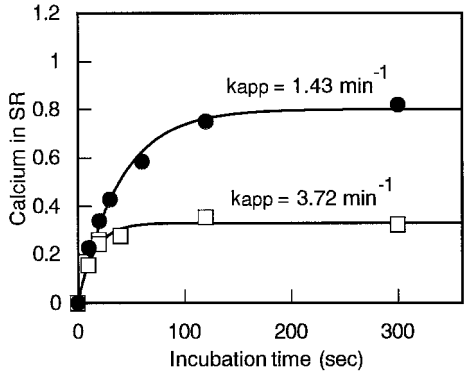

FIGURE 3 Time course of Ca^{2+} influx into SR. The amount of calcium in SR was determined after incubation of empty SR for various periods with a TS containing 4 mM AMP and 1 mM Ca^{2+} (□) or 5 mM Ca^{2+} (●). Solid lines correspond to the expression $Y = A * \{1 - \exp(-k_{app} * t)\}$. A and k_{app} for the best fit were 0.33 and 3.72 min^{-1} for 1 mM $[Ca^{2+}]_C$ and 0.80 and 1.43 min^{-1} for 5 mM $[Ca^{2+}]_C$, respectively. The unity in "Calcium in SR" corresponds to the amount of calcium loading at pCa 6.5 for 2 min at 16°C.

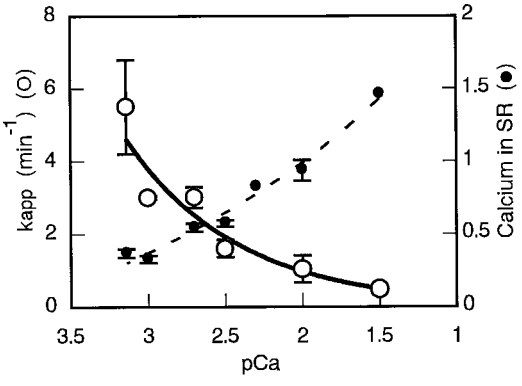

FIGURE 4 Steady amount of calcium in SR (●) and apparent rate constants (○) of Ca^{2+} influx into SR as a function of $[Ca^{2+}]_C$. The apparent rate constants were obtained by fitting time courses of Ca^{2+} influx into SR as shown in Fig. 3. Note that the amount of calcium in SR at the steady state increased with increase in $[Ca^{2+}]_C$, whereas the rate constants decreased. Each point and bar represents the mean ± SE of up to six independent determinations in different fibers.

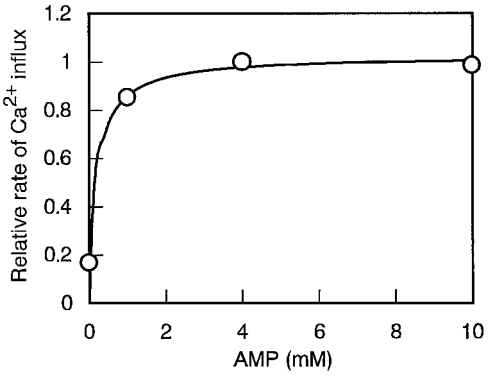

FIGURE 5 Effect of AMP on Ca^{2+} influx into SR. Rates of Ca^{2+} influx were normalized by the value in the presence of 4 mM AMP.

influx. The dependence of the Ca^{2+} influx rate on AMP concentration was plotted in Fig. 5. The rate of Ca^{2+} influx was very slow in the absence of AMP, although the steady level attained was similar (data not shown). The rate increased with increase in AMP concentration and reached the maximum at 4 mM or higher. The experiments described below were carried out in the presence of 4 mM AMP.

Fig. 6 *A* shows the effect of procaine on Ca^{2+} influx at various levels of $[Ca^{2+}]_C$ and compares it with the effect on Ca^{2+} release, i.e., Ca^{2+} efflux. Ca^{2+} efflux from the prescriptive loading was examined at pCa 5.3 and 3.0, and Ca^{2+} influx into empty SR was examined at pCa 3.0 and 2.0. The apparent rate constants in the presence of procaine were normalized to that in the absence of procaine at each condition. The dose dependence of Ca^{2+} influx on procaine at pCa 3.0 was very similar to that of efflux at the same pCa, and half-inhibitory concentrations were ~4 mM. Therefore the effect of procaine on Ca^{2+} fluxes was independent of the direction of flux. Furthermore, the extent of inhibition by procaine was similar regardless of $[Ca^{2+}]_C$. Even at very high $[Ca^{2+}]_C$, such as 10 mM, where very strong inhibition by Ca^{2+} was already obvious, procaine was as effective as at a lower $[Ca^{2+}]_C$, at pCa 5.3, where no inactivating effect of Ca^{2+} was observed. This indicates that the inhibitory effect of procaine occurs independently of the inactivating effect of high concentrations of $[Ca^{2+}]_C$. This result is consistent with the results of experiments with skinned fibers in that procaine suppressed bell-shaped Ca^{2+}-dependent CICR activity without changing Ca^{2+} dependence (Endo, 1981) .

Mg^{2+} is another well-known CICR inhibitor, and the inhibition is due to both the competition with Mg^{2+} for activating Ca^{2+} sites and the synergistic effect of Mg^{2+} on inactivating Ca^{2+} sites (Meissner et al., 1986, 1997). Consistently, Endo (1981) has reported that Mg^{2+} not only reduces CICR activity, but also shifts this Ca^{2+} dependence to higher $[Ca^{2+}]_C$. As expected, the extent of inhibition will be dependent on $[Ca^{2+}]_C$. In accordance with the prediction, Ca^{2+} efflux at pCa 5.3 was strongly suppressed by Mg^{2+}, with a half-inhibitory concentration (IC_{50}) of less than 0.2 mM, whereas the efflux at pCa 3.0 was moderately inhibited, with an IC_{50} of 1.5 mM (Fig. 6 *B*). At the same $[Ca^{2+}]_C$ of pCa 3.0, the IC_{50} for Ca^{2+} influx into SR was 1.2 mM, which was similar to that for Ca^{2+} efflux. At pCa 2.0, the influx was clearly much less sensitive to Mg^{2+}, probably indicating the same inactivation site for Ca^{2+} and Mg^{2+} in the Ca^{2+} release channel (Meissner et al., 1986). This is in contrast to procaine, which was completely independent of the inhibitory effect of $[Ca^{2+}]_C$.

As described above, CICR modulators such as procaine and Mg^{2+} suppressed the rates of Ca^{2+} influx and Ca^{2+} efflux to a similar extent when determinations were made at the same $[Ca^{2+}]_C$. Those inhibitors could reduce the rates of Ca^{2+} influx and Ca^{2+} efflux to less than 10% of the original activity. These results indicate that the major pathway of Ca^{2+} influx is the Ca^{2+} release channel, as is true of Ca^{2+} efflux. The measurement of the rate of Ca^{2+} influx can be a useful tool for the study of in situ activity of the Ca^{2+} release channel, especially at high $[Ca^{2+}]_C$ or at very low $[Ca^{2+}]_L$.

Comparison of the time courses of Ca^{2+} fluxes

Ca^{2+} efflux from SR at various loading levels

To study the effect of luminal calcium on Ca^{2+} release channel activity, we have approached this problem in the following two ways: to examine the time courses of Ca^{2+}

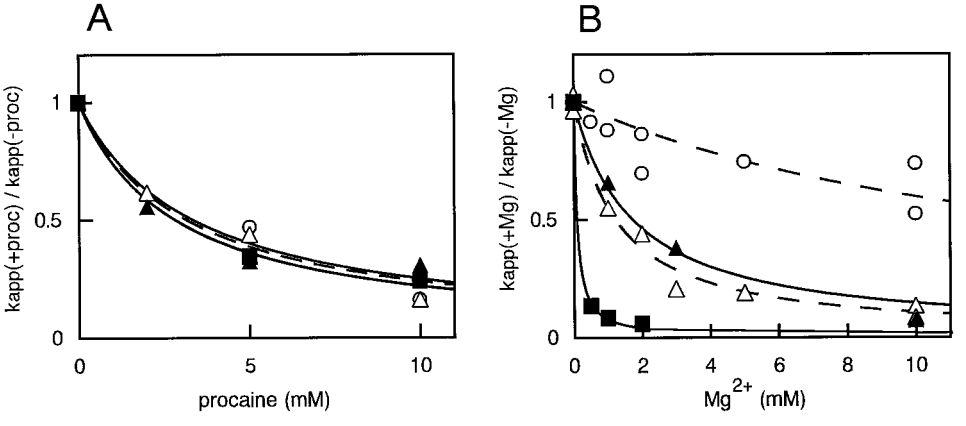

FIGURE 6 Effects of procaine (*A*) and Mg^{2+} (*B*) on Ca^{2+} influx and Ca^{2+} efflux at various concentrations of $[Ca^{2+}]_C$. Time courses of Ca^{2+} efflux were determined at pCa 5.3 (■) and 3.0 (▲); influx was determined at pCa 3.0 (△) and 2.0 (○). Each rate constant of fluxes was normalized to that in the absence of procaine or Mg^{2+}.

effluxes from different loading levels and to compare the time courses between Ca^{2+} influx into empty SR and Ca^{2+} efflux from Ca^{2+} loaded SR. Initially, we carried out the measurements of Ca^{2+} effluxes from SR at different loading levels in the presence of a low concentration of $[Ca^{2+}]_C$, 1.6 μM $[Ca^{2+}]_C$ (Fig. 7). The lower loading level in SR was obtained by incubation with a lower Ca^{2+} concentration (L2) and/or for a shorter period. As shown in Fig. 7 *A*, the normalized time course of the efflux from 1/2 loading was similar to that from the prescriptive loading (k_{app} values were 1.67 min^{-1} and 1.32 min^{-1}, respectively). Fig. 7 *B* shows the comparison between the Ca^{2+} effluxes from the prescriptive loading and 1/3 loading, which were determined in another skinned fiber. The efflux rate constant from 1/3 loading (1.87 min^{-1}) became slightly larger than the control value (1.39 min^{-1}). Similar results were obtained in three different fibers, and the average apparent rate constant was 1.31 $\pm$ 0.09 (mean $\pm$ SD, $p = 0.03$) times greater at 1/3 loading than that at the prescriptive loading. A lower loading level appears to cause a greater rate constant for the Ca^{2+} release. A similar observation was reported in isolated vesicles from rabbit skeletal muscle (Ikemoto et al., 1989). A possible heterogeneity of the store site for loaded Ca^{2+}, i.e., longitudinal tubules and/or terminal cisternae, might be another explanation for the faster release from a lower loading level. The two potential explanations are not exclusive, but may be complementary to each other.

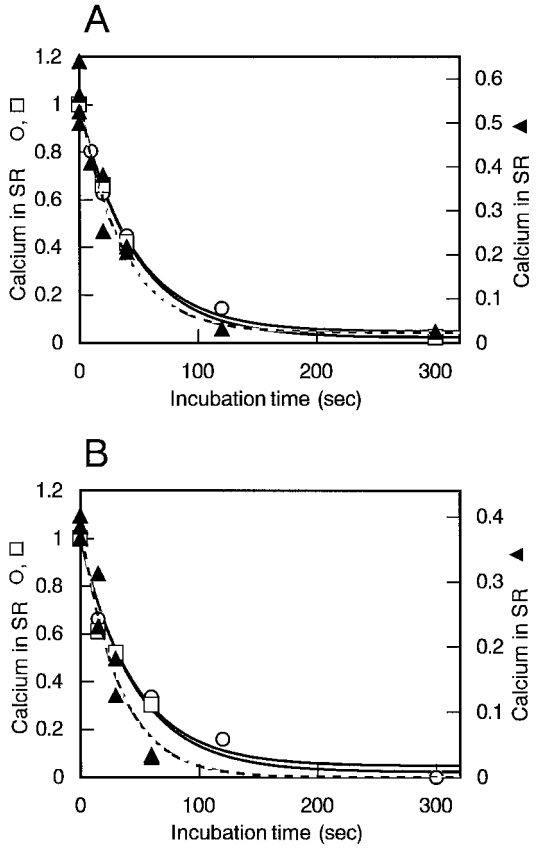

FIGURE 7 Comparison of Ca^{2+} effluxes from SR at various loading levels. (*A*) Time courses of Ca^{2+} efflux from SR of prescriptive loading (○, □) and of half the loading (▲) were determined at pCa 5.7 using a single skinned fiber. (*B*) Experiments similar to those in *A*, except that one-third loading was carried out on a different single fiber. In both panels, data points were obtained in the order ○, ▲, and □.

Ca^{2+} influx into empty SR and Ca^{2+} efflux from loaded SR

We could not detect a marked difference between rate constants of Ca^{2+} effluxes from the prescriptive loading and from 1/3 loading as shown above. The Ca^{2+} concentration in SR at these loading levels, however, still seems to be on the order of mM, even at 1/3 loading. Because the stimulating effect of luminal Ca^{2+} was reported to be almost saturated at mM concentrations of Ca^{2+} in previous reports (Ikemoto et al., 1989; Donoso et al., 1995; Sitsapesan and Williams, 1995), it may be difficult to detect the effect of luminal Ca^{2+} from the efflux measurements by varying loading levels in our experimental conditions. Then we compared the time courses of Ca^{2+} influx into empty SR with those of Ca^{2+} efflux from loaded SR at a given $[Ca^{2+}]_C$ in the same skinned fibers, because the initial luminal Ca^{2+} concentrations in SR should be very different between them. Fig. 8 shows an example of the time courses determined in the same skinned fiber. When a skinned fiber was incubated at 2 mM $[Ca^{2+}]_C$, total calcium in SR increased exponentially with empty SR, whereas it decreased exponentially from loaded SR. In either direction of flux, calcium in SR reached an identical steady level. The apparent rate constants fitted to a first-order kinetics were 2.05 min^{-1} for the influx and 1.99 min^{-1} for the efflux in this experiment. Similar experiments were performed with different fibers at various pCas; their results were summarized in Table 2. In nine experiments out of 13, the difference in apparent rate constants of influx were within 70–130% of that of efflux. A twofold difference between influx and efflux rate constants was found in only one experiment. No

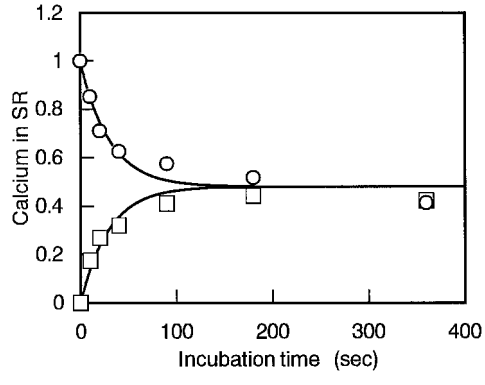

FIGURE 8 Time courses of Ca^{2+} influx into empty SR (□) and Ca^{2+} efflux from SR at prescriptive loading (○) at 2 mM $[Ca^{2+}]_C$, which were determined in the same single fiber. The best fits (*solid lines*) to the experimental points were given by $Y = 0.48 * \{1 - \exp(-2.05 * t)\}$ for influx and $Y = 0.52 * \exp(-1.99 * t) + 0.48$ for efflux.

TABLE 2 Comparison between the apparent rate constants for Ca^{2+} fluxes through SR membrane in frog skinned fibers

Preparations	pCa	Calcium in SR at equilibrium	Apparent rate constants (min^{-1}) Influx (k_i)	Efflux (k_e)	k_i/k_e
061996	4.24	0.211	4.14	4.28	0.97
061396	3.62	0.275	3.79	3.73	1.02
061496		0.230	3.70	7.50	0.49
053196	3.14	0.289	7.02	6.06	1.16
062796		0.377	2.68	3.58	0.75
070496		0.438	3.57	3.52	1.01
062896	3.00	0.370	4.00	6.40	0.63
112196		0.272	2.27	2.97	0.76
062796	2.70	0.585	3.65	2.78	1.31
071296		0.550	3.37	3.80	0.89
072496		0.480	2.05	1.99	1.03
072596		0.480	3.23	2.21	1.46
062896	2.50	0.603	1.13	1.24	0.91
Mean ± SD					0.95 ± 0.27

significant difference was found between the two rate constants for fluxes when analyzed with Student's paired *t*-test at $p < 0.01$.

The relationship between equilibrated luminal calcium level and $[Ca^{2+}]_C$: detection of Ca^{2+} binding sites in SR

As shown in Fig. 8, the final steady level of calcium in SR during the Ca^{2+} influx experiment was identical to that during the Ca^{2+} efflux experiment, and the level was determined by $[Ca^{2+}]_C$, as shown in Table 2. The results shown in Fig. 4 and Table 2 are consistent with one another, suggesting the presence of Ca^{2+} binding sites, as already reported with isolated SR vesicles (Ikemoto et al., 1989; Volpe and Simon, 1991). To improve accuracy and precision in the results, similar determinations in varied $[Ca^{2+}]_C$ were carried out in the same skinned fiber, and those results with three different fibers are compiled in Fig. 9 for analysis of the Ca^{2+} binding sites. When the fiber with the prescriptively loaded SR was incubated with 10 mM $[Ca^{2+}]_C$, the level of calcium in SR changed only slightly, whereas the final loading level became close to the prescriptive loading when the empty SR was incubated with the same solution. Therefore, the free $[Ca^{2+}]$ inside SR ($[Ca^{2+}]_L$) at the prescriptive loading appeared to be ~10 mM. For direct comparison of amounts of calcium in SR, the equilibrated levels in SR at various pCas were normalized by the value at pCa 2.0 instead of the prescriptive loading level (Fig. 9).

To estimate the fraction of bound to total calcium in SR, we have fit the normalized calcium content in SR (NCC) to the following equation, by assuming that total calcium in SR ($[Ca]_{total}$) is the sum of free Ca^{2+} ($[Ca^{2+}]_L$) and bound Ca^{2+} ($[CaB]_L$), as shown in Fig. 9 *A*:

$$\begin{aligned} NCC &= F*[Ca]_{total} \\ &= F*\{[Ca^{2+}]_L + [CaB]_L\} \\ &= F*\{[Ca^{2+}]_L + B_{total}*[Ca^{2+}]_L^{\,n}/([Ca^{2+}]_L^{\,n} + K_d^{\,n})\} \end{aligned} \quad (1)$$

where F is a normalizing factor, and B_{total}, K_d, and n represent the total concentration, the dissociation constant for Ca^{2+}, and a Hill coefficient of Ca^{2+} binding sites, respectively. Here we assume that $[Ca^{2+}]_C = [Ca^{2+}]_L$ in the steady state, because the membrane potential across SR membrane would negligibly contribute to distribution of Ca^{2+} (Somlyo et al., 1981; Volpe and Simon, 1991). The solid curve in Fig. 9 *B* represents the best fitted curve, which was obtained when $n = 0.65$, $K_d = 5$ mM, and $B_{total} = 38$ mM. The Hill coefficient of 0.65 can be reasonably interpreted as the sum of multiple heterogeneous binding sites with different affinities, as discussed later in this section, although the possibility of negative cooperative binding cannot be excluded. We also made a second type of calculation by using the Hill coefficient of the unity under the assumption that the major Ca^{2+} binding sites would be homogeneous and independent in SR (Fig. 9 *C*). K_d and B_{total} obtained from the fitting were 1.0 mM and 14 mM, respectively.

In the two kinds of fittings, the square of regression coefficient, r^2, of 0.987 in the case of Fig. 9 *C* was not very different from that in the case of Fig. 9 *B* ($r^2 = 0.989$). It is difficult to differentiate the two alternatives from the present experimental results because of low affinities of Ca^{2+} binding sites and of unsaturating increase in free Ca^{2+}. The experimental data in Fig. 9 can also be explained by a combination of intermediate values for n, K_d, and B_{total}, which were obtained in the experiments in Fig. 9, *B* and *C*. The relationship between total calcium in SR and $[Ca^{2+}]_C$ indicates the presence of massive Ca^{2+} binding sites in SR in skinned fibers, although it did not give a set of decisive values for binding parameters.

One of the most probable candidates of the Ca^{2+} binding sites in SR is calsequestrin (MacLennan and Wong, 1971; Ikemoto et al., 1971, 1974; Volpe and Simon, 1991). Volpe and Simon (1991) reported that the K_d, B_{total}, and n for calsequestrin in frog SR vesicles were 1.1 mM, 6.1 mM, and 1, respectively, in a physiological condition. On the other hand, the Ca^{2+} pump in rabbit SR is reported to have three low-affinity Ca^{2+} binding sites per molecule with a K_d of ~1 mM (Ikemoto, 1975), and its concentration in frog SR is estimated to be 0.6 mM (Ogawa, 1970). The Ca^{2+} binding sites in Fig. 9 *C* can be interpreted to be calsequestrin and the Ca^{2+} pump protein. Then the estimated value for B_{total} is 8 mM (6.1 + 0.6 * 3), which is similar to the value of 14 mM in the simulation, considering experimental errors in the estimation.

Miyamoto and Kasai (1979) described several classes of Ca^{2+} binding sites in SR. Among them, $\beta 3$ sites had a K_d of 38 mM and a capacity of 144 nmol/mg protein of SR (see

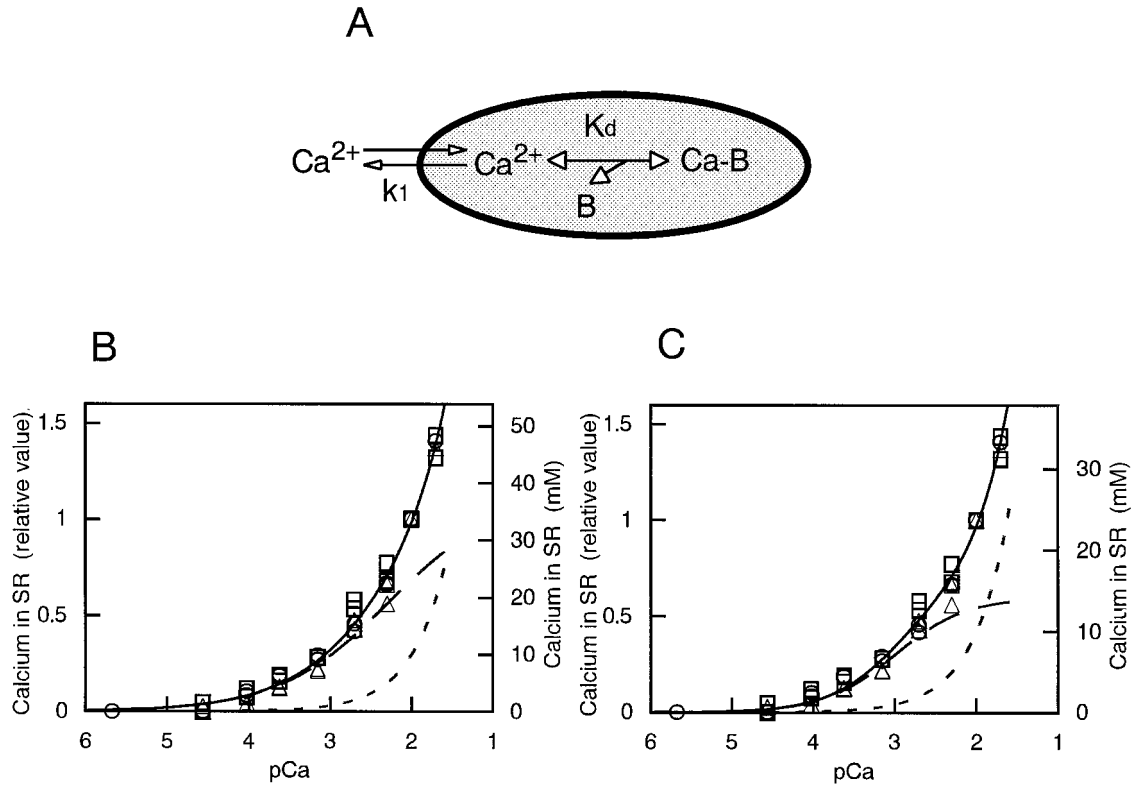

FIGURE 9 Ca^{2+} binding sites in SR. (*A*) Schematic model of distribution of calcium in SR at a cytoplasmic Ca^{2+} ($[Ca^{2+}]_C$). (*B* and *C*) The relationship between equilibrated levels of calcium in SR and $[Ca^{2+}]_C$ in skinned fibers. Calcium in SR was determined after incubation of skinned fibers in a specified concentration of $[Ca^{2+}]_C$ for 300 or 600 s when the steady state was attained from either direction of influx or efflux. Different symbols represent different preparations. Data points are the same in *B* and *C*. Solid lines represent $Y = F * \{B_{total} * [Ca^{2+}]_C{}^n/(K_d{}^n + [Ca^{2+}]_C{}^n) + [Ca^{2+}]_C\}$, assuming that total calcium in SR is the sum of free and bound calcium. When the fitting was performed with variable n, the best fit was obtained with parameters of B_{total} = 38 mM, K_d = 5.0 mM, n = 0.65 and F = 29.8 mM^{-1}, and the regression coefficient, r^2, was 0.989 (*B*). When n was fixed at 1.0, the best fit values were B_{total} = 14 mM, K_d = 1.0 mM, and F = 42.2 mM^{-1}, and r^2 was 0.986 (*C*). Total calcium, solid lines; free Ca^{2+}, short dashed lines; bound Ca^{2+}, long dashed lines.

also Volpe and Simon, 1991). Probable Ca^{2+} binding sites may also include phospholipids such as phosphatidylserine or phosphatidylinositol, which were noticed as nonspecific binding sites (Philipson et al., 1980). These heterogeneous binding sites with various K_d values, in addition to calsequestrin and Ca^{2+}-ATPase, may explain the binding profile shown in Fig. 9 *B*.

By using Fig. 9, we can convert a relative value of calcium in SR into total and free calcium concentration in SR. The prescriptive loading was similar to the steady state after an equilibration in pCa 2.0, when $[Ca^{2+}]_L$ = 10 mM. At the prescriptive loading, therefore, free and total calcium concentrations in SR are estimated to be ~10 mM and 24 mM (Fig. 9 *C*) to 33 mM (Fig. 9 *B*), respectively. The fraction of free to total calcium would become higher with a higher calcium loading level. For example, when the amount of total calcium in SR was decreased to one-third of the prescriptive loading, estimated free calcium and total calcium concentrations would be ~1 mM and 8–10 mM, respectively.

In intact muscle, the calcium loading level was two- to threefold lower than the prescriptive uptake level. Then the total calcium concentration in SR in intact muscle fiber is estimated to be 8–16 mM. Because the fractional volume of SR and muscle water space were estimated to be 0.11 and 0.7 of the whole fiber volume (Ogawa, 1970; Baylor et al., 1983), respectively, the value of 8–16 mM would correspond to 1.1–2.3 mmol/liter muscle water in frog skeletal muscle, which was close to the reported values in intact or cut frog muscle (1–4 mmol/liter) (Somlyo et al., 1981; Pape et al., 1995; Shirokova et al., 1996; Owen et al., 1997)

In empty SR, on the other hand, total calcium concentration should be very small, because no increase in fura 2 fluorescence signal was detected in Triton X-100 or A23187 containing solution W3. The free Ca^{2+} concentration in empty SR should be less than 20 μM, because it is the lowest Ca^{2+} concentration detectable by our experimental system.

The effects of luminal Ca^{2+} binding sites on the time course of Ca^{2+} fluxes

Ca^{2+} influx into empty SR and Ca^{2+} efflux from loaded SR

The results described in Fig. 9 indicate the presence of massive Ca^{2+} binding sites in SR, although parameters

were not definitely determined. We simulate the effect of luminal Ca^{2+} binding sites on the time courses of Ca^{2+} fluxes in the cases corresponding to the Ca^{2+} binding sites in Fig. 9, *B* and *C*. Fig. 10, *A* and *B*, shows simulated time courses of Ca^{2+} influx into empty SR and Ca^{2+} efflux from loaded SR at a constant $[Ca^{2+}]_C$, using the binding parameters obtained in the experiments in Fig. 9, *B* and *C*, respectively. The assumptions here are the following. First, only free Ca^{2+} permeates the channel, depending on the free Ca^{2+} gradient across SR membrane with a rate constant, k_1 (min^{-1}), which is independent of direction or time. The previous results already showed no rectifying characteristics in purified ryanodine receptors (Smith et al., 1988; Tinker and Williams, 1992). Second, an equilibrium between free Ca^{2+} and Ca^{2+} binding sites occurs instantaneously in the SR lumen, and finally, luminal Ca^{2+} does not affect k_1 itself. The relationships used for the simulation are Eqs. 1 and 2:

$$d[Ca]_{total,L}/dt = k_1*([Ca^{2+}]_C - [Ca^{2+}]_L) \quad (2)$$

where $d[Ca]_{total,L}/dt$ is the rate of change in total calcium concentration that flows into or out of SR. For the simulation, we adopted 10 and 0 mM as initial values of $[Ca^{2+}]_L$ for Ca^{2+} efflux and Ca^{2+} influx, respectively, and 1 mM of $[Ca^{2+}]_L$ ($= [Ca^{2+}]_C$) for the final steady state, where about one-third of the prescriptive loading was obtained as shown in Fig. 9 (see also Table 2). A k_1 of 6 min^{-1} was arbitrarily chosen for all calculations. If no Ca^{2+} binding sites exist in SR, the time courses would follow the expression $Y = A * \{1 - \exp(-k_1 * t)\}$ for Ca^{2+} influx and $Y = (B - A) * \exp(-k_1 * t) + A$ for Ca^{2+} efflux, where *A* and *B* denote the steady and initial levels of calcium in SR, respectively.

The simulation based on the set of parameters in Fig. 9 *B* ($n = 0.65$, $K_d = 5$ mM, and $B_{total} = 38$ mM) is shown in Fig. 10 *A*, where the calculated changes in free, bound, and total calcium are plotted. The computed time course of total Ca^{2+} influx or efflux in the presence of Ca^{2+} binding sites did not follow a simple first-order kinetics, although it appeared to. It is difficult to differentiate the predicted time

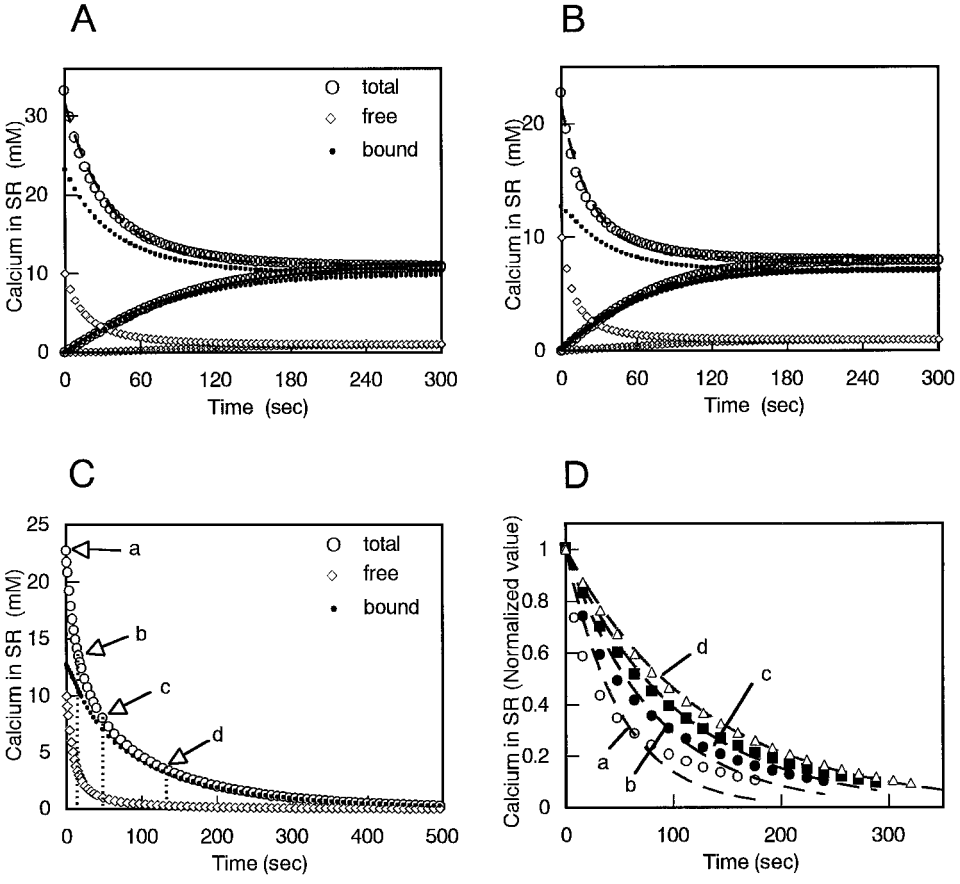

FIGURE 10 Computed time course of change in calcium in SR during Ca^{2+} fluxes. *A* and *B* demonstrate time courses of Ca^{2+} influx into empty SR and Ca^{2+} efflux from SR at prescriptive loading in the presence of 1 mM $[Ca^{2+}]_C$. Parameters for Ca^{2+} binding sites correspond to the results in Fig. 9, *B* and *C*, respectively: $B_{total} = 38$ mM, $K_d = 5$ mM, and $n = 0.65$ (*A*) or $B_{total} = 14$ mM, $K_d = 1$ mM and $n = 1$ (*B*). The intrinsic rate constant (k_1) used in those simulations was 6 min^{-1}, and luminal initial free $[Ca^{2+}]_L$ was set to zero for empty SR and 10 mM for SR at prescriptive loading. The calculated time courses of total calcium in SR were then refitted to a first-order reaction (*broken lines*) to obtain apparent rate constants. ○, ◇, ●: Total, free, and bound calcium, respectively. (*C*) Simulated time courses of total, free, and bound Ca^{2+} in SR during Ca^{2+} efflux from the prescriptive loading (10 mM $[Ca^{2+}]_L$) into 0 mM $[Ca^{2+}]_C$. The parameters used for the simulation were $B_{total} = 14$ mM, $K_d = 1$ mM, $n = 1$, and $k_1 = 6$ min^{-1}. Normalized time courses of Ca^{2+} effluxes from SR at the prescriptive, 1/2, 1/3, and 1/6 loading levels (which correspond to the points indicated by *arrows a–d*) were plotted in *D*. The calculated values for k_{app} are 1.19, 0.73, 0.56, and 0.46 min^{-1} for *a* (○), *b* (●), *c* (■), and *d* (△), respectively.

course from an exponential one in our determinations. Therefore the computed time course of change in total calcium in SR was again expressed by a first-order reaction to give an apparent rate constant, k_{app} (*broken line*). The calculated values for k_{app} were much smaller than k_1, 1.9 min^{-1} and 0.7 min^{-1} for efflux and influx, respectively. In this case, there is an approximately threefold difference between influx and efflux rate constants. Fig. 10 *B* represents a similar simulation, using the second set of parameters, $n = 1.0$, $K_d = 1$ mM, $B_{total} = 14$ mM, and $k_1 = 6$ min^{-1}, corresponding to Fig. 9 *C*. In this case the calculated values for k_{app} were 2.7 min^{-1} and 0.9 min^{-1} for efflux and influx, respectively. Here again, the calculated k_{app} for efflux was larger than the counterpart for influx. Thus the effects of presence of intravesicular Ca^{2+} binding sites on k_{app} values for Ca^{2+} influx and Ca^{2+} efflux are different in magnitude. This conclusion was the same even if the size of Ca^{2+} binding sites was twofold smaller than our assumption. When the calculation was made using 7 mM instead of 14 mM for B_{total}, computed values of k_{app} for Ca^{2+} efflux and influx were 3.9 min^{-1} and 1.6 min^{-1}, respectively.

Recently Chen et al. (1994) and Ma et al. (1995) showed that the addition of exogenous FK506 binding protein (FKBP12) to the ryanodine receptor from rabbit SR blocked the flow of monovalent cation (K^+ or Cs^+) from the cytoplasmic to the luminal side (i.e., "influx" in our terminology), but had no effect on the flow in the opposite direction. Ma et al. (1995) also reported that this rectification property was observed in a population of 20% of SR vesicle preparations used for the experiment, whereas the rest of them were nonrectifying. If this type of rectification occurred in our skinned fiber SR, the difference in the simulated rate constants between Ca^{2+} efflux and influx would be much larger. Therefore this type of rectification, if any, cannot explain our present experimental results.

Ca^{2+} efflux from SR at various loading levels

We have also modeled the time courses of Ca^{2+} efflux from SR at different calcium loading levels by using Eqs. 1 and 2 (Fig. 10, *C* and *D*). Fig. 10 *C* shows the simulated time course of total, free, and bound calcium in SR during Ca^{2+} efflux from 10 mM $[Ca^{2+}]_L$ into zero $[Ca^{2+}]_C$, using the second set of parameters, i.e., 14 mM for B_{total}, 1 mM for K_d, and 1 for n. A k_1 of 6 min^{-1} was adopted for the simulation. The time course of the simulated efflux does not seem to follow a first-order kinetics. The calculated rate of the Ca^{2+} efflux appeared to be much faster than the rate expected from a single exponential kinetics at the initial phase, whereas the reversed relation was obtained in the later phase. This is because free and total calcium concentrations can easily be changed at the higher Ca^{2+} concentrations, where Ca^{2+} binding sites are highly saturated. To see how the apparent rate constant is affected by a decrease in the loading level, the time courses of fractional change in calcium content in SR during Ca^{2+} efflux from the levels shown by *marks a–d* in Fig. 10 *C* were replotted in Fig. 10 *D*. Calcium contents in SR indicated by *arrows a–d* correspond to the prescriptive (10 mM $[Ca^{2+}]_L$), 1/2 (3 mM $[Ca^{2+}]_L$), 1/3 (1 mM $[Ca^{2+}]_L$), and 1/6 (0.3 mM $[Ca^{2+}]_L$) loading levels, respectively. The time courses were refitted with the expression of $Y = \exp(-k_1 * t)$ $(0.1 \le Y \le 1)$. The extents of deviation of the simulated time course from the exponential one were greater in 10 mM (*a*) and 3 mM $[Ca^{2+}]_L$ (*b*) than those in 1 mM (*c*) and 0.3 mM $[Ca^{2+}]_L$ (*d*). At a lower calcium loading level of SR, where a fraction of bound Ca^{2+} is larger, the time course of efflux is slower and closer to the first order kinetics. Within lower Ca^{2+} loading levels, therefore, the effect of change in the Ca^{2+} loading level on k_{app} becomes smaller. The efflux from the prescriptive loading (= 10 mM $[Ca^{2+}]_L$) is calculated to be about twice as fast as that from 1/3 loading (= 1 mM $[Ca^{2+}]_L$). On the basis of results in Table 2, it would be difficult to detect a difference of 30% in the k_{app} value in our determinations, but we can definitely detect a twofold difference. The determinations are at variance with the simulated predictions.

A model that can explain our results

To get better agreements between our determinations and simulations, in the next model we assumed that the intrinsic rate constant k_1 is affected by $[Ca^{2+}]_L$ in the relationship, as shown in Eq. 3, but is otherwise unchanged:

$$\begin{aligned} k' &= k_1 * \{1 - [Ca^{2+}]_L/(K_i + [Ca^{2+}]_L)\} \\ &= k_1 * \{K_i/(K_i + [Ca^{2+}]_L)\} \end{aligned} \quad (3)$$

where K_i is an inhibitory constant for Ca^{2+}. Other parameters used were all the same as those in Fig. 10, *B* and *C*. Fig. 11 *A* shows a simulation for time courses of Ca^{2+} efflux from the prescriptive loading and Ca^{2+} influx into empty SR at $[Ca^{2+}]_C = 1$ mM, corresponding to Fig. 10 *B*. When K_i was 10 mM or larger, the results were similar to those in Fig. 10 *B*. As K_i became smaller, the efflux k_{app} (i.e., k_{app} for the Ca^{2+} efflux) decreased, whereas the influx k_{app} did not change much. When a K_i of 2 mM was used, the efflux k_{app} (0.78 min^{-1}) was very close to the influx k_{app} (0.80 min^{-1}). With a K_i smaller than 2 mM, the efflux k_{app} became smaller than the influx k_{app}. Therefore 2 mM is acceptable for K_i in the case of 1 mM $[Ca^{2+}]_C$ in Table 2 (see also Fig. 11). With the successful value of 2 mM for K_i, we made similar calculations at various levels of $[Ca^{2+}]_C$, as shown in Table 2; the results were summarized in Table 3. Within the accuracy of our determinations, this model with $K_i = 2$ mM satisfactorily explains the results shown in Table 2.

We also examined whether time courses of Ca^{2+} efflux from different loading levels as shown in Fig. 7 can be explained. When K_i was 2 mM, a time course of the efflux was very close to a single exponential, giving similar efflux k_{app} values from any Ca^{2+} loading level (Fig. 11 *B*). The calculated values of k_{app} were 0.51, 0.50, and 0.46 min^{-1} for the initial levels of the prescriptive, 1/2, and 1/3 loading

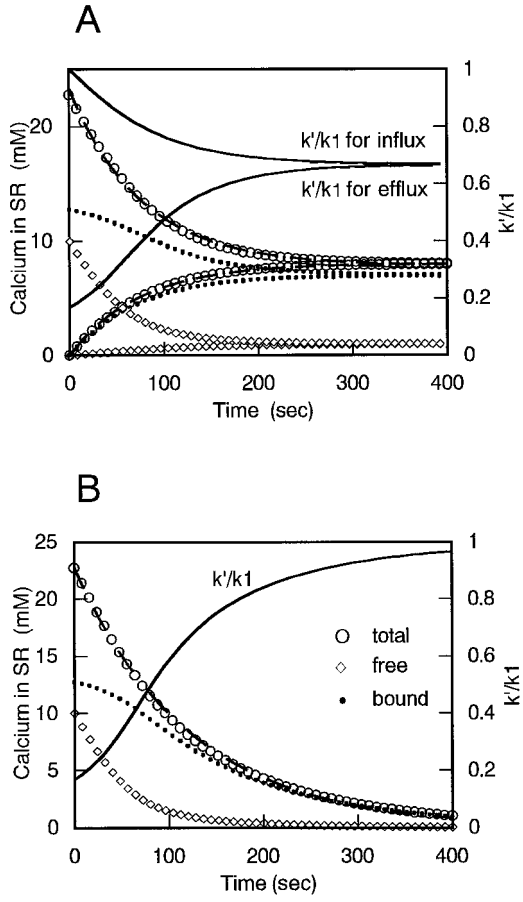

FIGURE 11 Inhibitory effects of luminal Ca^{2+} on the simulated time course of change in calcium in SR during Ca^{2+} fluxes. Simulation was performed by assuming that k_1, the intrinsic rate constant, is negatively modulated by $[Ca^{2+}]_L$ according to Eq. 3 (see the text for details). The effective coefficients (k'/k_1) are indicated by solid lines in each panel. Parameters used for the simulations were $B_{total} = 14$ mM, $K_d = 1$ mM, $n = 1$, $k_1 = 6$ min^{-1}, and $K_i = 2$ mM. (*A*) Time courses of Ca^{2+} influx into empty SR (initial $[Ca^{2+}]_L = 0$ mM) and Ca^{2+} efflux from SR at prescriptive loading (initial $[Ca^{2+}]_L = 10$ mM) in the presence of 1 mM $[Ca^{2+}]_C$. The calculated time courses of total calcium were refitted to a first-order reaction (*broken lines*) to obtain apparent rate constants (see also Table 3). (*B*) Simulated time course of total, free, and bound calcium during Ca^{2+} efflux from the prescriptive loading (10 mM $[Ca^{2+}]_L$) into 0 mM $[Ca^{2+}]_C$. Note that the time course is well fitted to a first-order reaction, with a k_{app} of 0.5 min^{-1} (*broken line*).

(Table 3). When a K_i of 1 mM was used, the calculated efflux k_{app} from the prescriptive loading level (0.32 min^{-1}) was smaller than that from 1/2 loading levels (0.38 min^{-1}).

We have shown that a K_i of 2 mM gave a good fitting. We should mention, however, that a value in the range of ~2 mM for K_i may also be satisfactory. It should also be pointed out that there may be many possible ways other than this model for luminal Ca^{2+} to have an inhibitory effect. The effect of Ca^{2+} may be cooperative; the extent of the inhibition may be partial, unlike the case proposed here (100% inhibition) and others. In any event, a negative modulation of Ca^{2+} permeability by luminal Ca^{2+} is strongly suggested. It is interesting that a satisfactory value

TABLE 3 Simulated inhibitory effect of luminal Ca^{2+} on k_{app} for Ca^{2+} fluxes

Initial $[Ca^{2+}]_L$ (mM)	$[Ca^{2+}]_C$ (mM)	$K_i = \infty$			$K_i = 2$ mM		
		k_{ec}	k_{ic}	k_{ic}/k_{ec}	k_{ec}	k_{ic}	$k_{ic/k_{ec}}$
Fig. 11 *A* (see also Table 2)							
0 or 10	0	1.19	—	—	0.51	—	—
0 or 10	0.3	1.41	0.56	0.40	0.63	0.53	0.84
0 or 10	1.0	2.73	0.88	0.32	0.78	0.80	1.02
0 or 10	2.0	3.64	1.37	0.38	0.93	1.17	1.25
0 or 10	3.0	4.10	1.80	0.44	0.96	1.45	1.51
Fig. 11 *B*							
10	0	1.19	—	—	0.51	—	—
3	0	0.73	—	—	0.50	—	—
1	0	0.56	—	—	0.46	—	—
0.3	0	0.46	—	—	0.46	—	—

k_{app} values at various $[Ca^{2+}]_C$ with ($K_i = 2$ mM) and without ($K_i = \infty$) the inhibition by luminal Ca^{2+} were calculated as shown in Figs. 10 and 11. All simulations were performed for Ca^{2+} fluxes using parameters of $B_{max} = 14$ mM, $K_d = 1$ mM, $n = 1$, and $k_1 = 6$ min^{-1}. k_{ec} and k_{ic} indicate calculated efflux k_{app} and influx k_{app}, respectively. For the upper rows where $[Ca^{2+}]_C$ was varied, the calculations of k_{ec} and k_{ic} were performed with a constant k_1 to show the differentiating effects of luminal Ca^{2+} on k_{ec} and k_{ic}. Here the ratio k_{ec}/k_{ic} is a good index for the evaluation of the model. It should be noted that k_1 is also decreased by a mM order of $[Ca^{2+}]_C$.

for K_i is similar to the dissociation constant for Ca^{2+} of luminal Ca^{2+} binding sites mentioned above. This suggests that some of the Ca^{2+} binding sites besides the Ca^{2+} release channel may serve the inhibitory function. The Ca^{2+} release channel would probably have two kinds of inhibitory Ca^{2+} sites, the conventional one on the cytoplasmic side and a new one on the luminal side.

CONCLUSIONS

In this article we have shown that the Ca^{2+} influx activity into empty SR reflects well Ca^{2+} release channel activity in SR in situ: it was stimulated by AMP and suppressed by procaine, Mg^{2+}, and high concentrations of Ca^{2+}, as was true of Ca^{2+} efflux activity in the same preparations. The effect of luminal Ca^{2+} on the Ca^{2+} release channel activity was investigated by comparing the time courses between Ca^{2+} influx into empty SR and Ca^{2+} efflux from calcium-loaded SR, and those among Ca^{2+} effluxes from SR at various loading levels. The apparent rate constant for the Ca^{2+} efflux from loaded SR was not significantly different from that for the Ca^{2+} influx into empty SR at a specified $[Ca^{2+}]_C$. We did not find any evidence for lowered permeability of SR membrane at very low $[Ca^{2+}]_L$ compared to that at high $[Ca^{2+}]_L$. This is different from the conclusion by Donoso et al. (1995) and Ikemoto et al. (1989), who reported lowered permeability at a low $[Ca^{2+}]_L$. A possible explanation for this discrepancy may be the difference in preparations: they used an isolated triad or heavy SR vesicle fraction, whereas we used skinned fibers, which maintain

the entire SR structure, including terminal cisternae and longitudinal tubules.

The time course of Ca^{2+} efflux from the prescriptive loading was not very different from that from one-third loading. Similar results were obtained with depolarization-induced Ca^{2+} release (DICR) from SR in intact and skinned fibers. Pape et al. (1995) reported that the relation between the rate of Ca^{2+} release by single action potential and the calcium content in SR appeared to be approximately linear in frog cut muscle fibers between 1 and 4 mmol total calcium/liter muscle water. Owen et al. (1997) reported that the depolarization of the T-system by ionic replacement could fully deplete SR and that the amplitude of depolarization-induced force response was dependent on the calcium content in SR of toad and rat peeled skeletal muscle fibers. Although CICR may be different from DICR in the mode of opening of the channel, a similar effect of luminal Ca^{2+} may be involved in the rate of Ca^{2+} release in vivo.

We have confirmed the existence of Ca^{2+} binding sites in SR from the relationship indicated in Fig. 9. Because we observed the amount of total (the sum of free and bound) calcium but not free Ca^{2+} in SR in skinned fiber preparations, we have to take the effect of Ca^{2+} buffering capacity of SR into account for analysis of the rate of Ca^{2+} fluxes. Under an assumption of no regulation by luminal Ca^{2+}, the computer simulation using parameters for luminal Ca^{2+} binding sites as obtained in Fig. 9 would lead to the following conclusions: 1) the apparent rate constants (k_{app}) of Ca^{2+} influx into empty SR would be smaller than that of Ca^{2+} efflux from loaded SR at a $[Ca^{2+}]_C$; 2) k_{app} of Ca^{2+} efflux from highly loaded SR would be larger than that from less loaded SR. These conclusions, however, are at variance with our findings. A conceivable explanation is that luminal Ca^{2+} may have a negative regulatory effect on the Ca^{2+} release channel. This is supported by simulation based on a model in which the luminal Ca^{2+} serves the inhibitory effect on the intrinsic rate constant k_1 for the Ca^{2+} release channels.

There are two possibilities for the negative effect of luminal Ca^{2+} on the Ca^{2+} release channel. It may affect the release channel from the luminal side directly or indirectly through interacting proteins. However, any evidence other than the findings presented here has not been shown for the effect of Ca^{2+} on the release channel from the luminal side. It is possible that Ca^{2+} flowing out of SR has access to cytoplasmic inactivating sites of the channel, as Tripathy and Meissner (1996) claimed, because the inactivation sites are not yet saturated in our experimental conditions. Other additional mechanisms, however, may be involved. Further investigation is required to elucidate the mechanisms of luminal regulation of Ca^{2+} release channel activity.

This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan; the Uehara Memorial Foundation; and the Suzuken Memorial Foundation.

REFERENCES

Baylor, S. M., W. K. Chandler, and M. W. Marshall. 1983. Sarcoplasmic reticulum calcium release in frog skeletal muscle fibres estimated from arsenazo III calcium transients. *J. Physiol. (Lond.).* 344:625–666.

Chen, S. R. W., L. Zhang, and D. H. MacLennan. 1994. Asymmetrical blockade of the Ca^{2+} release channel (ryanodine receptor) by 12-kDa FK506 binding protein. *Proc. Natl. Acad. Sci. USA.* 91:11953–11957.

Donoso, P., H. Prieto, and C. Hidalgo. 1995. Luminal calcium regulates calcium release in triads isolated from frog and rabbit skeletal muscle. *Biophys. J.* 68:507–515.

Endo, M. 1981. Mechanism of calcium-induced calcium release in the SR membrane. *In* The Mechanism of Gated Calcium Transport across Biological Membranes. Academic Press, New York. 257–264.

Fill, M., R. Coronado, J. R. Mickelson, J. Vilven, J. Ma, B. A. Jacobson, and C. F. Louis. 1990. Abnormal ryanodine receptor channels in malignant hyperthermia. *Biophys. J.* 57:471–475.

Harafuji, H., and Y. Ogawa. 1980. Re-examination of the apparent binding constant of ethylene glycol bis(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid with calcium around neutral pH. *J. Biochem.* 87: 1305–1312.

Ikemoto, N. 1975. Transport and Inhibitory Ca^{2+} binding sites on the ATPase enzyme isolated from the sarcoplasmic reticulum. *J. Biol. Chem.* 250:7219–7224.

Ikemoto, N., B. Antoniu, J. J. Kang, L. G. Meszaros, and M. Ronjat. 1991. Intravesicular calcium transient during calcium release from sarcoplasmic reticulum. *Biochemistry.* 30:5230–5237.

Ikemoto, N., G. M. Bhatnagar, and J. Gergely. 1971. Fractionation of solubilized sarcoplasmic reticulum. *Biochem. Biophys. Res. Commun.* 44:1510–1517.

Ikemoto, N., B. Nagy, G. M. Bhatnagar, and J. Gergely. 1974. Studies on a metal-binding protein of the sarcoplasmic reticulum. *J. Biol. Chem.* 249:2357–2365.

Ikemoto, N., M. Ronjat, L. G. Meszaros, and M. Koshita. 1989. Postulated role of calsequestrin in the regulation of calcium release from sarcoplasmic reticulum. *Biochemistry.* 28:6764–6771.

Kawasaki, T., and M. Kasai. 1994. Regulation of calcium channel in sarcoplasmic reticulum by calsequestrin. *Biochem. Biophys. Res. Commun.* 199:1120–1127.

Kitazawa, T., and M. Endo. 1976. Increase in passive calcium influx into sarcoplasmic reticulum by "depolarization" and caffeine. *Proc. Jpn. Acad.* 52:599–602.

Kurebayashi, N., and Y. Ogawa. 1986. Characterization of increased Ca^{2+} efflux by quercetin from the sarcoplasmic reticulum in frog skinned skeletal muscle fibres. *J. Muscle Res. Cell Motil.* 7:142–150.

Kurebayashi, N., and Y. Ogawa. 1991. Discrimination of Ca^{2+}-ATPase activity of the sarcoplasmic reticulum from actomyosin-type ATPase activity of myofibrils in skinned mammalian skeletal muscle fibres—distinct effects of cyclopiazonic acid on the two ATPase activities. *J. Muscle Res. Cell Motil.* 12:355–365.

Ma, J., M. B. Bhat, and J. Zhao. 1995. Rectification of skeletal muscle ryanodine receptor mediated by FK506 binding protein. *Biophys. J.* 69:2398–2404.

Ma, J., M. Fill, C. M. Knudson, K. P. Campbell, and R. Coronado. 1988. Ryanodine receptor of skeletal muscle is a gap junction-type channel. *Science.* 242:99–102.

MacLennan, D. H., and P. T. Wong. 1971. Isolation of a calcium-sequestering protein from sarcoplasmic reticulum. *Proc. Natl. Acad. Sci. USA.* 68:1231–1235.

Meissner, G., E. Darling, and J. Eveleth. 1986. Kinetics of rapid Ca^{2+} release by sarcoplasmic reticulum. Effects of Ca^{2+}, Mg^{2+}, and adenine nucleotides. *Biochemistry.* 25:236–244.

Meissner, G., E. Rios, A. Tripathy, and D. A. Pasek. 1997. Regulation of skeletal muscle Ca^{2+} release channel (ryanodine receptor) by Ca^{2+} and monovalent cations and anions. *J. Biol. Chem.* 272:1628–1638.

Miyamoto, H., and M. Kasai. 1979. Asymmetric distribution of calcium binding sites of sarcoplasmic reticulum fragments. *J. Biochem.* 85: 765–773.

Murayama, T., N. Kurebayashi, and Y. Ogawa. 1998. Stimulation by polyols of the two ryanodine receptor isoforms of frog skeletal muscle. *J. Muscle Res. Cell Motil.* 19:15–24.

Murayama, T., and Y. Ogawa. 1996. Similar Ca^{2+} dependence of [^{3}H]ryanodine binding to α- and β-ryanodine receptors purified from bullfrog skeletal muscle in an isotonic medium. *FEBS Lett.* 380:267–271.

Ogawa, Y. 1970. Some properties of fragmented frog sarcoplasmic reticulum with particular reference to its response to caffeine. *J. Biochem.* 67:667–683.

Ogawa, Y., and H. Harafuji. 1990. Effect of temperature on [^{3}H]ryanodine binding to sarcoplasmic reticulum from bullfrog skeletal muscle. *J. Biochem.* 107:887–893.

Owen, V. J., G. D. Lamb, D. G. Stephenson, and M. W. Fryer. 1997. Relationship between depolarization-induced force responses and Ca^{2+} content in skeletal muscle fibres of rat and toad. *J. Physiol. (Lond.).* 498:571–586.

Pape, P. C., D. S. Jong, and W. K. Chandler. 1995. Calcium release and its voltage dependence in frog cut muscle fibers equilibrated with 20 mM EGTA. *J. Gen. Physiol.* 106:259–336.

Philipson, K. D., D. M. Bers, and A. Y. Nishimoto. 1980. The role of phospholipids in the Ca^{2+} binding of isolated cardiac sarcolemma. *J. Mol. Cell. Cardiol.* 12:1159–1173.

Shirokova, N., J. Garcia, G. Pizarro, and E. Rios. 1996. Ca^{2+}-release from the sarcoplasmic reticulum compared in amphibian and mammalian skeletal muscle. *J. Gen. Physiol.* 107:1–18.

Sitsapesan, R., and A. J. Williams. 1995. The gating of the sheep skeletal sarcoplasmic reticulum Ca^{2+} release channel is regulated by luminal Ca^{2+}. *J. Membr. Biol.* 146:133–144.

Smith, J. S., R. Coronado, and G. Meissner. 1986. Single channel measurements of the calcium release channel from skeletal muscle sarcoplasmic reticulum. Activation by Ca^{2+} and ATP and modulation by Mg^{2+}. *J. Gen. Physiol.* 88:573–588.

Smith, J. S., T. Imagawa, J. Ma, M. Fill, K. P. Campbell, and R. Coronado. 1988. Purified ryanodine receptor from rabbit skeletal muscle is the calcium-release channel of sarcoplasmic reticulum. *J. Gen. Physiol.* 92:1–26.

Somlyo, A. V., H. G. Gonzalez-Serratos, H. Shuman, G. McClellan, and A. P. Somlyo. 1981. Calcium release and ionic changes in the sarcoplasmic reticulum of tetanized muscle: an electron-probe study. *J. Cell Biol.* 90:577–594.

Tinker, A., and A. J. Williams. 1992. Divalent cation conduction in the ryanodine receptor channel of sheep cardiac muscle sarcoplasmic reticulum. *J. Gen. Physiol.* 100:479–493.

Tripathy, A., and G. Meissner. 1996. Sarcoplasmic reticulum lumenal Ca^{2+} has access to cytosolic activation and inactivation sites of skeletal muscle Ca^{2+} release channel. *Biophys. J.* 70:2600–2615.

Volpe, P., and B. J. Simon. 1991. The bulk of Ca^{2+} released to the myoplasm is free in the sarcoplasmic reticulum and does not unbind from calsequestrin. *FEBS Lett.* 278:274–278.