

## Regulation of Cardiac Muscle $\text{Ca}^{2+}$ Release Channel by Sarcoplasmic Reticulum Lumenal $\text{Ca}^{2+}$

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**ABSTRACT** The cardiac muscle sarcoplasmic reticulum  $\text{Ca}^{2+}$  release channel (ryanodine receptor) is a ligand-gated channel that is activated by micromolar cytoplasmic  $\text{Ca}^{2+}$  concentrations and inactivated by millimolar cytoplasmic  $\text{Ca}^{2+}$  concentrations. The effects of sarcoplasmic reticulum lumenal  $\text{Ca}^{2+}$  on the purified release channel were examined in single channel measurements using the planar lipid bilayer method. In the presence of caffeine and nanomolar cytosolic  $\text{Ca}^{2+}$  concentrations, lumenal-to-cytosolic  $\text{Ca}^{2+}$  fluxes  $\geq 0.25$  pA activated the channel. At the maximally activating cytosolic  $\text{Ca}^{2+}$  concentration of 4  $\mu\text{M}$ , lumenal  $\text{Ca}^{2+}$  fluxes of 8 pA and greater caused a decline in channel activity. Lumenal  $\text{Ca}^{2+}$  fluxes primarily increased channel activity by increasing the duration of mean open times. Addition of the fast  $\text{Ca}^{2+}$ -complexing buffer 1,2-bis(2-aminophenoxy)ethanetetraacetic acid (BAPTA) to the cytosolic side of the bilayer increased lumenal  $\text{Ca}^{2+}$ -activated channel activities, suggesting that it lowered  $\text{Ca}^{2+}$  concentrations at cytosolic  $\text{Ca}^{2+}$ -inactivating sites. Regulation of channel activities by lumenal  $\text{Ca}^{2+}$  could be also observed in the absence of caffeine and in the presence of 5 mM MgATP. These results suggest that lumenal  $\text{Ca}^{2+}$  can regulate cardiac  $\text{Ca}^{2+}$  release channel activity by passing through the open channel and binding to the channel's cytosolic  $\text{Ca}^{2+}$  activation and inactivation sites.

### INTRODUCTION

The release and sequestration of  $\text{Ca}^{2+}$  ions by an intracellular membrane compartment, the sarcoplasmic reticulum (SR), is essential to the process of cardiac muscle contraction and relaxation. In cardiac muscle, the influx of  $\text{Ca}^{2+}$  via a voltage-sensitive dihydropyridine receptor (DHPR)/ $\text{Ca}^{2+}$  channel (L-type) triggers the massive release of  $\text{Ca}^{2+}$  by opening SR  $\text{Ca}^{2+}$  release channels (CRCs) (for review see Wier, 1990). The CRC binds the plant alkaloid ryanodine with high affinity and specificity and hence is also known as the ryanodine receptor (for reviews see Franzini-Armstrong and Protasi, 1997; Sutko et al., 1997; Meissner, 1994). CRCs are ligand-gated channels with  $\text{Ca}^{2+}$  as a major regulator. High-affinity activating and low-affinity inactivating  $\text{Ca}^{2+}$  binding sites have been identified (Liu et al., 1998; Fruen et al., 1996; Xu et al., 1996; Laver et al., 1995; Chu et al., 1993; Zimanyi and Pessah, 1991; Meissner and Henderson, 1987). Rapid activation and inactivation by cytosolic  $\text{Ca}^{2+}$  has suggested that these sites are located on the large cytosolic foot region of the channels (Laver and Curtis, 1996; Schiefer et al., 1995; Sitsapesan et al., 1995; Gyorke and Fill, 1993). Various other endogenous effectors of CRCs have been identified including  $\text{Mg}^{2+}$ , ATP, and calmodulin (Meissner, 1994).

In addition to cytosolic  $\text{Ca}^{2+}$ , SR lumenal  $\text{Ca}^{2+}$  may affect CRC activity. The most direct evidence for a regulation by SR lumenal  $\text{Ca}^{2+}$  has been obtained in single

channel measurements using the planar lipid bilayer technique. SR lumenal  $\text{Ca}^{2+}$  activated the skeletal muscle CRC in the presence of cytosolic ATP (Sitsapesan and Williams, 1995; Tripathy and Meissner, 1996) but no or only a modest activation was observed in the absence of ATP (Sitsapesan and Williams, 1995; Tripathy and Meissner, 1996; Herrmann-Frank and Lehmann-Horn, 1996). These results have raised the interesting possibility that skeletal CRCs have SR intralumenal  $\text{Ca}^{2+}$  binding sites that interact with cytosolic regulatory sites (Sitsapesan and Williams, 1995). An alternative suggestion has been that SR lumenal  $\text{Ca}^{2+}$  flowing through the channel regulates the skeletal muscle CRC by having access to cytosolic activation and inactivation sites (Tripathy and Meissner, 1996; Herrmann-Frank and Lehmann-Horn, 1996). In support of the latter suggestion, high concentrations of the "fast"  $\text{Ca}^{2+}$  buffer 1,2-bis(2-aminophenoxy)ethanetetraacetic acid (BAPTA) increased cytosolic ATP-activated, lumenal  $\text{Ca}^{2+}$ -activated skeletal muscle channel activities. This result suggested that lumenal  $\text{Ca}^{2+}$  passing through the skeletal CRC regulates the channel by having access to "BAPTA-inaccessible"  $\text{Ca}^{2+}$  activation and "BAPTA-accessible"  $\text{Ca}^{2+}$  inactivation sites (Tripathy and Meissner, 1996).

An increase in lumenal  $\text{Ca}^{2+}$  concentration also resulted in an increase in cardiac CRC open probability. The presence of another cytosolic channel activator such as sulmazole (Sitsapesan and Williams, 1994a) or ATP (Lukyanenko et al., 1996) was required to observe activation by lumenal  $\text{Ca}^{2+}$ . These results were considered to be inconsistent with the idea that lumenal  $\text{Ca}^{2+}$  ions flowing through the channel have direct access to cytosolic  $\text{Ca}^{2+}$  activation sites.

The cardiac CRC represents a classical example of a  $\text{Ca}^{2+}$ -regulated  $\text{Ca}^{2+}$  release mechanism (Wier, 1990). Its regulation by  $\text{Ca}^{2+}$  and other endogenous effectors differs from that of the skeletal CRC (Franzini-Armstrong and

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Protasi, 1997; Sutko et al., 1997; Meissner, 1994). It is therefore conceivable that the two channel isoforms are regulated differently by luminal  $\text{Ca}^{2+}$ . To clarify the ways in which luminal  $\text{Ca}^{2+}$  ions regulate the cardiac CRC, we have investigated their effects on single canine cardiac muscle CRCs, using the planar lipid bilayer method. Our results indicate that luminal  $\text{Ca}^{2+}$  flowing through the channel regulates the cardiac  $\text{Ca}^{2+}$  release channel via direct feedback by binding to cytosolic  $\text{Ca}^{2+}$  activation and inactivation sites. An activation of channel activity by luminal  $\text{Ca}^{2+}$  was observed at  $\text{Mg}^{2+}$  and ATP concentrations corresponding to those in myocardium. These results suggest that activation of cardiac CRCs by luminal  $\text{Ca}^{2+}$  fluxes may be a physiologically relevant mechanism. A preliminary report of this work has been presented in abstract form (Xu and Meissner, 1997).

## EXPERIMENTAL PROCEDURES

### Materials

Phospholipids were obtained from Avanti Polar Lipids (Birmingham, AL). All other chemicals were of analytical grade.

### Preparation of sarcoplasmic reticulum vesicles and purification of $\text{Ca}^{2+}$ release channels

Canine cardiac SR vesicle fractions enriched in [ $^3\text{H}$ ]ryanodine binding and  $\text{Ca}^{2+}$  release channel activities were prepared in the presence of protease inhibitors as described (Xu et al., 1993). The CHAPS (3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate)-solubilized canine heart 30S  $\text{Ca}^{2+}$  release channel complex was isolated by rate density gradient centrifugation and reconstituted into proteoliposomes by removal of CHAPS by dialysis (Lee et al., 1994).

### Single channel measurements

Single channel measurements were performed by fusing proteoliposomes containing the purified cardiac muscle  $\text{Ca}^{2+}$  release channel with Mueller-Rudin-type bilayers containing phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine in the ratio 5:3:2 (25 mg total phospholipid/ml *n*-decane) (Lee et al., 1994). The side of the bilayer to which the proteoliposomes were added was defined as the *cis* side. A strong dependence of channel activity on micromolar *cis*  $\text{Ca}^{2+}$  concentrations suggested that the *cis* side corresponded to the SR cytosolic side in a majority (>98%) of our recordings. The *trans* side of the bilayer was defined as ground. Single channels were recorded in a symmetrical KCl buffer solution (0.25 M KCl, 20 mM KHepes, pH 7.4) containing the additions indicated in the text. Electrical signals were filtered at 2 kHz, digitized at 10 kHz, and analyzed. Data acquisition and analysis were performed with a commercially available software package (pClamp 6.0.3., Axon Instruments, Burlingame, CA) using an IBM-compatible Pentium computer and 12-bit A/D-D/A converter (Digidata 1200, Axon Instruments) (Xu et al., 1996).

### Determination of free $\text{Ca}^{2+}$ concentrations

Free  $\text{Ca}^{2+}$  concentrations of  $>1 \mu\text{M}$  were determined with a  $\text{Ca}^{2+}$ -selective electrode (World Precision Instruments, Inc., Sarasota, FL). Free  $\text{Ca}^{2+}$  concentrations of  $<1 \mu\text{M}$  were obtained by including in the solutions the appropriate amounts of  $\text{Ca}^{2+}$  and EGTA as determined using the

stability constants and computer program published by Schoenmakers et al. (1992).

### Statistics

Results are given as means  $\pm$  SE. Significance of differences of data was analyzed with Student's *t*-test. Differences were regarded to be statistically significant at  $P < 0.05$ .

## RESULTS

Purified cardiac  $\text{Ca}^{2+}$  release channels reconstituted into proteoliposomes were incorporated into planar lipid bilayers and recorded in symmetrical 0.25 M KCl buffer. The use of  $\text{K}^+$  rather than  $\text{Ca}^{2+}$  as a current carrier avoided the buildup of a large  $\text{Ca}^{2+}$  gradient near the mouth of the channel, thus simplifying analysis of regulation of the cardiac CRC by  $\text{Ca}^{2+}$ . Single channel conductance with 0.25 M  $\text{K}^+$  as current carrier was 770 pS (Xu et al., 1993). The effects of cytosolic and luminal  $\text{Ca}^{2+}$  on channel activity were examined in the presence and absence of 10 mM cytosolic caffeine. Caffeine increases the apparent  $\text{Ca}^{2+}$  affinity of the  $\text{Ca}^{2+}$  activation sites (Liu et al., 1998; Zucchi and Ronca-Testoni, 1997), which allows the use of low cytosolic  $\text{Ca}^{2+}$  concentrations in testing the effects of luminal  $\text{Ca}^{2+}$ . Channels were also recorded in the presence of 5 mM cytosolic MgATP (0.7 mM free  $\text{Mg}^{2+}$ ) to better simulate the intracellular conditions in myocardium.

### Regulation of cardiac $\text{Ca}^{2+}$ release channels by cytosolic and luminal $\text{Ca}^{2+}$ in the presence of 10 mM caffeine

In Fig. 1 *A*, a single cardiac CRC was recorded in the presence of 10 mM cytosolic (*cis*) caffeine at three different cytosolic  $\text{Ca}^{2+}$  concentrations and holding potentials of  $-35$  and  $+35$  mV. Short, often not fully resolved channel events were observed with  $0.1 \mu\text{M}$  free  $\text{Ca}^{2+}$  in the cytosolic bilayer chamber (Fig. 1 *A*, *top traces*). Elevation of cytosolic  $\text{Ca}^{2+}$  concentration to  $1 \mu\text{M}$  increased channel open probability ( $P_o$ ) (*middle traces*) at both holding potentials. In the presence of  $10 \mu\text{M}$  cytosolic  $\text{Ca}^{2+}$ , long open events interrupted by brief closings were observed at both holding potentials, resulting in a nearly fully activated channel (*bottom traces*).

Fig. 1 *B* shows that channels in the presence of 10 mM cytosolic caffeine were half-maximally activated at  $\sim 1 \mu\text{M}$  cytosolic  $\text{Ca}^{2+}$ , and half-maximally inhibited at  $\geq 10 \text{ mM}$  cytosolic  $\text{Ca}^{2+}$ . In agreement with previous studies (Liu et al., 1998; Fruen et al., 1996; Xu et al., 1996; Laver et al., 1995; Zimanyi and Pessah, 1991; Meissner and Henderson, 1987), data of Fig. 1 *B* suggest that the cardiac CRC has both high-affinity  $\text{Ca}^{2+}$  activation and low-affinity  $\text{Ca}^{2+}$  inactivation sites. Furthermore, Fig. 1 shows that the cardiac CRC exhibits no significant voltage dependence when activated and inactivated by cytosolic  $\text{Ca}^{2+}$  in the presence of caffeine.

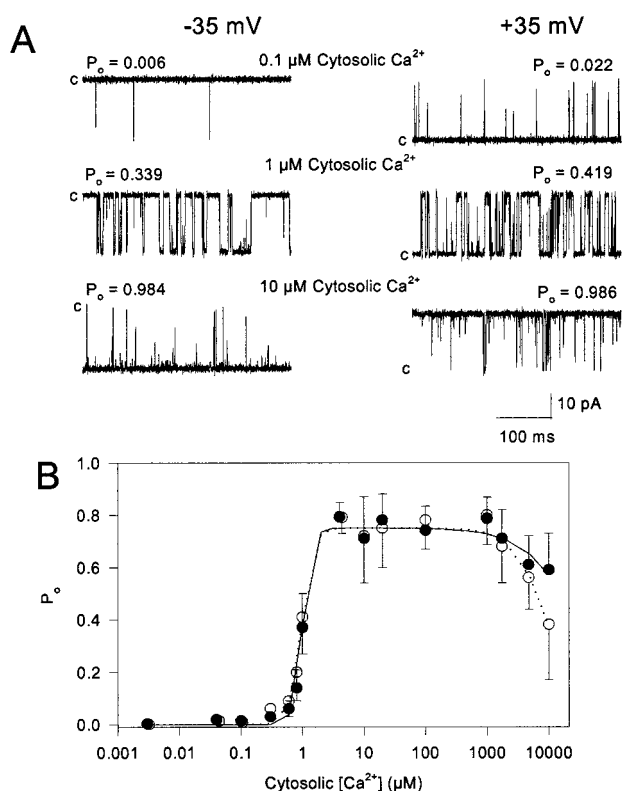


FIGURE 1 Dependence of single channel activities on cytosolic  $[Ca^{2+}]$  in the presence of 10 mM caffeine. (A) Single channel currents were recorded at  $-35$  mV (downward deflections, left current traces) (c = closed) and  $+35$  mV (upward deflections, right current traces) (c = closed) in symmetrical 0.25 M KCl, 20 mM KHepes, pH 7.4 media containing 10 mM cytosolic caffeine, 200  $\mu M$  EGTA, and  $[Ca^{2+}]$  to yield the indicated free cytosolic  $[Ca^{2+}]$ . The *trans* (SR luminal) solution contained  $<2$   $\mu M$   $Ca^{2+}$ . (B) Channel open probabilities ( $P_o$ ) were determined at  $-35$  mV ( $\circ$ ) and  $+35$  mV ( $\bullet$ ) as in (A). Values are the mean  $\pm$  SE of 5–12 experiments. Continuous lines were obtained assuming that the CRC possesses cooperatively interacting high-affinity  $Ca^{2+}$  activation and low-affinity  $Ca^{2+}$ -inactivation sites (Scheme 1 and Eq. 1 of Liu et al., 1998). At  $-35$  mV, Hill constants and coefficients were  $K_{Ha} = 1$   $\mu M$ ,  $n_{Ha} = 4.5$ ,  $K_{Hi} = 10$  mM, and  $n_{Hi} = 1.6$ .

The CRC showed a strong voltage dependence when the luminal instead of cytosolic  $Ca^{2+}$  concentration was elevated. In Fig. 2 A (top traces), a single cardiac CRC was initially recorded under conditions similar to those in Fig. 1 A (top traces), i.e., at a low cytosolic  $[Ca^{2+}]$  ( $<0.01$   $\mu M$ ) in the presence of 10 mM cytosolic caffeine. The luminal  $Ca^{2+}$  concentration was  $<2$   $\mu M$  and the holding potentials were  $-50$  mV and  $+50$  mV. As in Fig. 1, brief, often not fully resolved channel events were observed at both holding potentials. An increase of luminal  $Ca^{2+}$  concentration from  $<2$   $\mu M$  to 1 mM increased  $P_o$   $>100$ -fold at  $-50$  mV, but was essentially without an effect at  $+50$  mV (middle traces).

Fig. 2 B describes the dependence of mean  $P_o$  of minimally ( $<0.01$   $\mu M$  cytosolic  $Ca^{2+}$ ) and close to maximally (4  $\mu M$  cytosolic  $Ca^{2+}$ ) activated CRCs on luminal  $Ca^{2+}$  concentrations of 2  $\mu M$  to 10 mM. For the minimally activated channels, a significant increase in channel open probability was observed at a luminal  $Ca^{2+}$  concentration

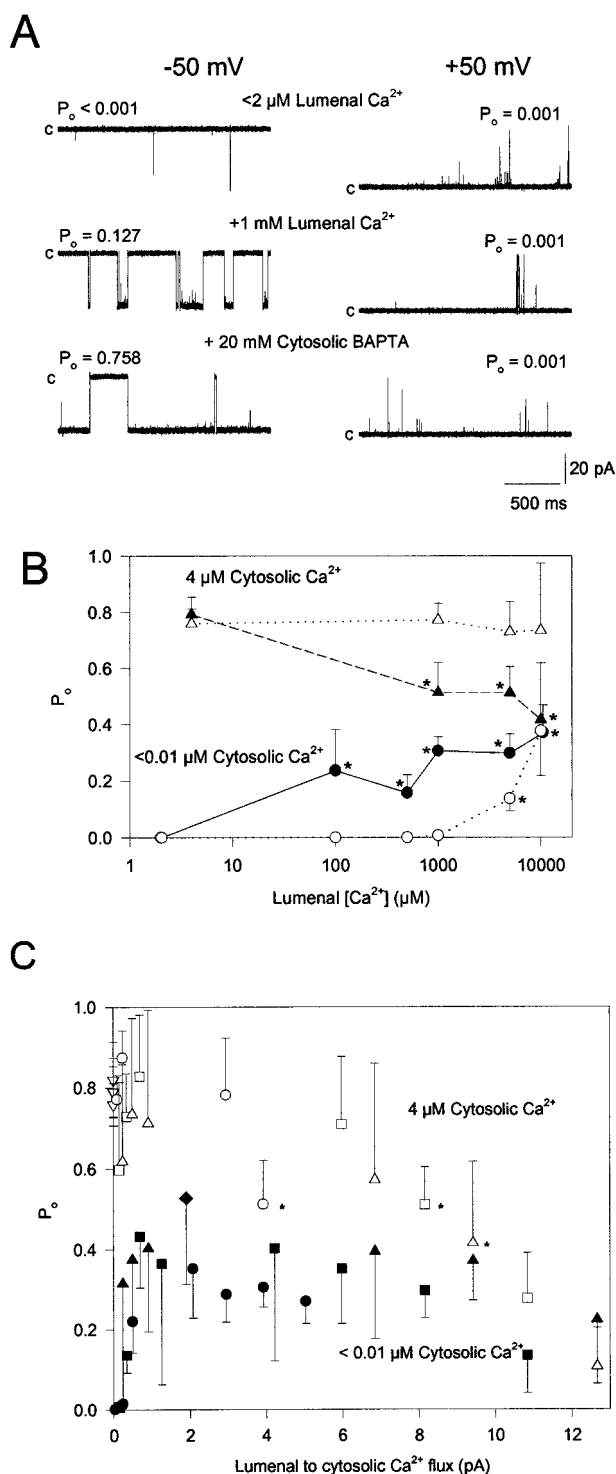
of 100  $\mu M$  and holding potential of  $-50$  mV. To obtain a similar increase in  $P_o$  at  $+50$  mV, a luminal  $Ca^{2+}$  concentration of 5 mM and greater was required. A different response was observed for channels that were close to maximally activated by 4  $\mu M$  cytosolic  $Ca^{2+}$ . In this case, an increase in luminal  $Ca^{2+}$  concentration lowered  $P_o$  at  $-50$  mV. No significant changes in  $P_o$  were observed at  $+50$  mV. Data of Fig. 2 B suggest that three parameters must be taken into account to understand the way in which luminal  $Ca^{2+}$  activates and inactivates the cardiac CRC. These are the extents to which channels are activated by cytosolic effectors such as  $Ca^{2+}$ , the luminal  $Ca^{2+}$  concentration, and the holding potential.

A negative holding potential favors, whereas a positive holding potential disfavors, the movement of cations from the SR luminal (*trans*) side to the cytosolic (*cis*) side of the bilayer. The different  $Ca^{2+}$  activation/inactivation curves of Fig. 2 B suggest, therefore, that luminal  $Ca^{2+}$  flowing through the open channel affects channel activity by having access to cytosolic  $Ca^{2+}$  regulatory sites. Luminal  $Ca^{2+}$  fluxes could not be directly measured (except at 0 mV, see below) because of the presence of  $K^+$  as the major current carrier in our recording solutions. Luminal-to-cytosolic  $Ca^{2+}$  fluxes were therefore calculated according to a barrier model that describes the ionic conduction of the sheep cardiac CRC (Tinker et al., 1992). Fig. 2 C shows the dependence of mean  $P_o$  of minimally ( $<0.01$   $\mu M$  cytosolic  $Ca^{2+}$ ) and close to maximally (4  $\mu M$  cytosolic  $Ca^{2+}$ ) activated CRCs on the calculated luminal  $Ca^{2+}$  fluxes. Luminal  $Ca^{2+}$  fluxes were calculated at six holding potentials ranging from  $-65$  mV to  $+65$  mV and four luminal  $Ca^{2+}$  concentrations ranging from  $<2$   $\mu M$  to 10 mM. Fig. 2 C shows that at 0.01  $\mu M$  cytosolic  $Ca^{2+}$  channels were maximally activated at a luminal-to-cytosolic  $Ca^{2+}$  flux of  $\sim 1$  pA.  $Ca^{2+}$  fluxes of  $>10$  pA appeared to be slightly inhibitory.

The SR membrane is highly permeable to  $K^+$  and  $Cl^-$  and the membrane potential across the SR membrane is therefore generally believed to be close to 0 mV (Meissner, 1983). The effects of luminal  $Ca^{2+}$  on  $P_o$  were therefore also determined at a holding potential of 0 mV in a symmetric 0.25 M KCl buffer containing 3 mM luminal free  $Ca^{2+}$  and a low cytosolic  $Ca^{2+}$  concentration ( $<0.01$   $\mu M$  free  $Ca^{2+}$  plus 10 mM caffeine). Under these conditions, the  $Ca^{2+}$  current could be measured directly. The measured  $Ca^{2+}$  current of  $1.9 \pm 0.1$  pA ( $n = 5$ ) was close to a calculated value of 2.1 pA. The averaged  $P_o$  of  $0.53 \pm 0.21$  ( $n = 5$ ) was close to values that yielded luminal-to-cytosolic  $Ca^{2+}$  fluxes of  $\sim 2$  pA at negative and positive holding potentials (2.1 and 1.3 pA at  $-20$  and  $+20$  mV and luminal  $[Ca^{2+}]$  of 1 and 5 mM, respectively).

Channels recorded at a close to maximally activating cytosolic  $Ca^{2+}$  concentration of 4  $\mu M$  were not further activated by luminal  $Ca^{2+}$  (Fig. 2 C). However, these channels were significantly inactivated at luminal  $Ca^{2+}$  fluxes of 8 pA and greater.

An intriguing finding was that at a low cytosolic  $Ca^{2+}$  concentration luminal  $Ca^{2+}$  fluxes were less effective than



**FIGURE 2** Activation of the cardiac  $\text{Ca}^{2+}$  release channel by luminal  $\text{Ca}^{2+}$  in the presence of  $10$  mM caffeine. (A) Single channel currents were recorded at  $-50$  mV (downward deflections, left panels) and  $+50$  mV (upward deflections, right panels) in symmetrical  $0.25$  M KCl,  $20$  mM KHepes, pH  $7.4$  media containing  $<0.01$   $\mu\text{M}$  free cytosolic  $\text{Ca}^{2+}$  ( $200$   $\mu\text{M}$  EGTA and  $<2$   $\mu\text{M}$  contaminating  $\text{Ca}^{2+}$ ) and  $10$  mM cytosolic caffeine. Bottom traces were obtained after the addition of  $20$  mM cytosolic BAPTA. SR luminal  $[\text{Ca}^{2+}]$  was  $<2$   $\mu\text{M}$  (top traces) and  $1$  mM (middle and bottom traces). Note: Negative holding potentials favor luminal-to-cytosolic  $\text{Ca}^{2+}$  fluxes. (B) Dependence of  $P_o$  on cytosolic and luminal  $[\text{Ca}^{2+}]$ . Holding potentials were  $-50$  mV ( $\bullet$ ,  $\blacktriangle$ ) and  $+50$  mV ( $\circ$ ,  $\triangle$ ). (C) Dependence of  $P_o$  on luminal-to-cytosolic  $\text{Ca}^{2+}$  fluxes. Luminal-to-

cytosolic  $[\text{Ca}^{2+}]$  in activating the CRC ( $P_{o,\text{max}} = \sim 0.8$  in Fig. 1 B vs.  $P_{o,\text{max}}$  of  $\sim 0.5$  in Fig. 2 C). This result can be rationalized if luminal  $\text{Ca}^{2+}$  inactivates before fully activating the release channel. We tested this idea using the “fast” complexing  $\text{Ca}^{2+}$  buffer BAPTA. Modeling studies have indicated that the free  $\text{Ca}^{2+}$  concentration near the release sites may reach values in excess of  $10$  mM (see Fig. 8). Because of its high association rate, BAPTA is more effective than the “slow” complexing  $\text{Ca}^{2+}$  buffer EGTA in suppressing such a rise in  $\text{Ca}^{2+}$  concentration (Stern, 1992). In the middle traces of Fig. 2 A, a single luminal  $\text{Ca}^{2+}$ -activated channel was recorded under standard conditions; that is, in the presence of  $<0.01$   $\mu\text{M}$  cytosolic  $\text{Ca}^{2+}$  and  $10$  mM cytosolic caffeine. Luminal  $\text{Ca}^{2+}$  was  $1$  mM. Bottom traces of Fig. 2 A show that the addition of  $20$  mM cytosolic BAPTA increased  $P_o$  at  $-50$  mV, but not at  $+50$  mV. Fig. 3 B (top panel) summarizes the effects of  $20$  mM BAPTA on several luminal  $\text{Ca}^{2+}$ -activated single channels. At luminal  $\text{Ca}^{2+}$  fluxes of  $0.25$ – $4$  pA,  $20$  mM cytosolic BAPTA increased  $P_o$ . At a flux of  $3$  pA, a  $P_o$  value close to those observed in the presence of  $0.01$ – $1$  mM cytosolic  $\text{Ca}^{2+}$  was obtained (Fig. 3 A, top panel). This result suggested that BAPTA was apparently able to prevent luminal  $\text{Ca}^{2+}$ -mediated channel inactivation by minimizing the buildup of a high inactivating  $\text{Ca}^{2+}$  concentration near the cytosolic  $\text{Ca}^{2+}$  inactivation sites. However, BAPTA did not prevent channel activation, which suggested that at a concentration of  $20$  mM BAPTA did not lower the  $\text{Ca}^{2+}$  concentration below a maximally activating  $\text{Ca}^{2+}$  concentration of  $\sim 5$   $\mu\text{M}$  (Fig. 1 B) at the cytosolic  $\text{Ca}^{2+}$  activation sites. A direct pharmacological activation of CRCs by BAPTA appeared to be unlikely because none was observed when luminal  $\text{Ca}^{2+}$  fluxes were  $\leq 0.1$  pA (Fig. 3 B, top panel).

In the case of cytosolic  $\text{Ca}^{2+}$ -activated CRCs, both the  $\text{Ca}^{2+}$ -activating and -inactivating sites see the same  $[\text{Ca}^{2+}]$ . In contrast, luminal  $\text{Ca}^{2+}$  has access only to cytosolic regulatory sites when the channel is open. In addition, the  $\text{Ca}^{2+}$  activation and inactivation sites may see different  $[\text{Ca}^{2+}]$ , depending on their relative location with respect to the release site. It was therefore of interest to compare the kinetic parameters of cytosolic  $\text{Ca}^{2+}$ -activated and luminal  $\text{Ca}^{2+}$ -activated channels (Fig. 3, A and B). An increase in cytosolic  $\text{Ca}^{2+}$  concentration from  $<0.01$   $\mu\text{M}$  to  $100$   $\mu\text{M}$

cytosolic  $\text{Ca}^{2+}$  fluxes were calculated according to the barrier model and parameters of Tinker et al. (1992) at  $<0.01$   $\mu\text{M}$  cytosolic (closed symbols) and  $4$   $\mu\text{M}$  cytosolic (open symbols)  $\text{Ca}^{2+}$  in the presence of  $<2$   $\mu\text{M}$  luminal  $\text{Ca}^{2+}$  ( $\nabla$ , masked by the other symbols at the origin,  $\nabla$ ) at  $\pm 35$  and  $\pm 50$  mV,  $1$  mM luminal  $\text{Ca}^{2+}$  at  $+65$ ,  $+50$ ,  $+35$ ,  $+20$ ,  $-20$ ,  $-35$  and  $-65$  mV ( $\bullet$ , from left to right) and  $+50$ ,  $+35$ ,  $-35$ , and  $-50$  mV ( $\circ$ , from left to right),  $3$  mM luminal  $\text{Ca}^{2+}$  ( $\blacklozenge$ ) at  $0$  mV,  $5$  mM luminal  $\text{Ca}^{2+}$  ( $\blacksquare$ ,  $\square$ ) at the membrane potentials indicated for  $1$  mM luminal  $\text{Ca}^{2+}$  except that the effects of  $5$  mM luminal  $\text{Ca}^{2+}$  at  $4$   $\mu\text{M}$  cytosolic  $\text{Ca}^{2+}$  were also determined at  $\pm 65$  mV, and in the presence of  $10$  mM luminal  $\text{Ca}^{2+}$  at ( $\blacktriangle$ ,  $\triangle$ ) at  $+65$ ,  $+50$ ,  $+35$ ,  $-35$ ,  $-50$  and  $-65$  mV (from left to right). (B) and (C) Values are the mean  $\pm$  SE of  $3$ – $19$  experiments. (B) \*Significantly different from  $P_o$  at  $\leq 4$   $\mu\text{M}$  luminal  $\text{Ca}^{2+}$ . (C) \*Significantly different from  $P_o$  at luminal  $\text{Ca}^{2+}$  flux of  $<0.1$  pA.



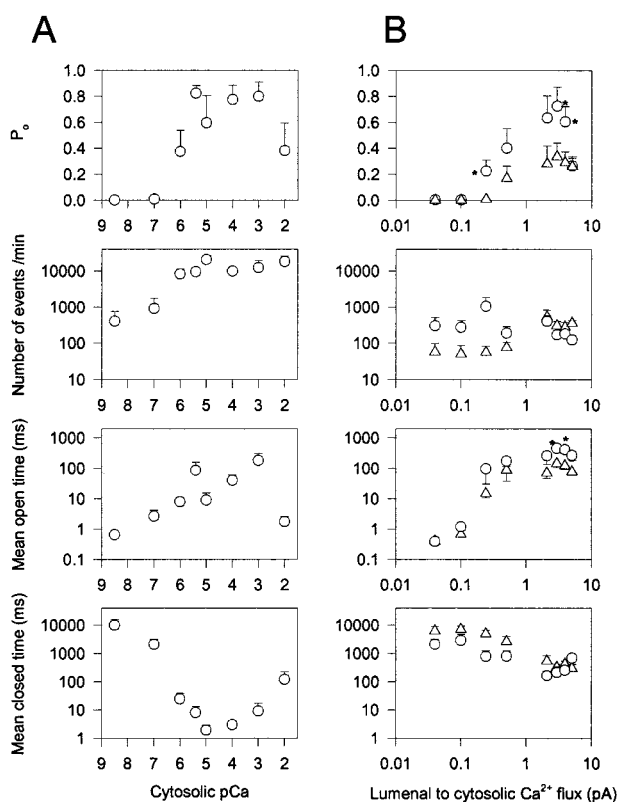


FIGURE 3 Single channel parameters of cytosolic and luminal Ca<sup>2+</sup>-activated channels in presence of 10 mM caffeine. (A) Single channel parameters were obtained from recordings at  $-35$  mV at indicated free cytosolic Ca<sup>2+</sup> concentrations as described in Fig. 1. Values are the mean  $\pm$  SE of 5–7 experiments. (B) Single channel parameters were obtained from recordings at holding potentials of  $+65$ ,  $+50$ ,  $+35$ ,  $+20$ ,  $-20$ ,  $-35$ ,  $-50$  and  $-65$  mV (from left to right) at luminal Ca<sup>2+</sup> concentration of 1 mM with (○) or without (△) 20 mM cytosolic BAPTA, as described in Fig. 2. Values are the mean  $\pm$  SE of 4–7 experiments. \*Significantly different from parameters in the absence of 20 mM BAPTA.

increased P<sub>o</sub> from close to zero to 0.8 by increasing the number of channel events by more than 10-fold, and the duration of mean open events by  $\sim 100$ -fold (Fig. 3 A). The duration of mean closed events was maximally decreased by  $\sim 10,000$ -fold. A further increase of cytosolic Ca<sup>2+</sup> to 10 mM decreased P<sub>o</sub> by shortening the duration of mean open events and increasing the duration of mean closed events, without having an appreciable effect on the number of channel events. In Fig. 3 B, channel parameters are plotted against the luminal Ca<sup>2+</sup> fluxes. Channels were recorded at eight holding potentials ranging from  $-65$  to  $+65$  mV and 1 mM luminal Ca<sup>2+</sup> and cytosolic Ca<sup>2+</sup> concentration of  $<0.01$   $\mu$ M in the presence and absence of 20 mM cytosolic BAPTA. In the absence of BAPTA, luminal Ca<sup>2+</sup> fluxes were less effective than cytosolic Ca<sup>2+</sup> in activating cardiac CRCs (top panels of Fig. 3, A and B). Luminal Ca<sup>2+</sup> opened and closed channels less frequently than cytosolic Ca<sup>2+</sup> (second panels). In both cases, mean open times were increased as channels were maximally activated by raising cytosolic [Ca<sup>2+</sup>] from  $\sim 0.003$   $\mu$ M to 10  $\mu$ M, and luminal Ca<sup>2+</sup> fluxes from 0.04 to 3 pA (third panels). However,

they showed major differences in the durations of mean closed times. An increase in cytosolic Ca<sup>2+</sup> from  $\sim 0.003$  to 10  $\mu$ M decreased the mean closed times from 10,000 ms to close to 1 ms (Fig. 3 A, bottom panel). By comparison, an increase in luminal Ca<sup>2+</sup> fluxes from 0.04 to 3 pA decreased the mean closed times by  $<100$ -fold (Fig. 3 B, bottom panel).

Cytosolic BAPTA significantly increased P<sub>o</sub> at elevated luminal Ca<sup>2+</sup> fluxes. This increase could be accounted for mostly by an increase in mean open times (Fig. 3 B, third panel). Some changes in the number of events and mean closed times were observed as well; however, none of these was significant.

### Regulation of cardiac Ca<sup>2+</sup> release channel by luminal Ca<sup>2+</sup> and Mg<sup>2+</sup> in the absence of caffeine

The effects of luminal Ca<sup>2+</sup> on CRCs were also investigated in the absence of caffeine. In Fig. 4 A a single channel was recorded with 10  $\mu$ M and 1 mM luminal Ca<sup>2+</sup>. Cytosolic Ca<sup>2+</sup> was 1  $\mu$ M, which was higher than in the recordings of Fig. 2 because preliminary experiments indicated that luminal Ca<sup>2+</sup> concentrations as high as 10 mM were ineffective in activating the CRC at cytosolic Ca<sup>2+</sup> concentrations of  $<0.1$   $\mu$ M (not shown). At such low Ca<sup>2+</sup> concentrations, channels rarely opened in the absence of caffeine. To observe appreciable channel activity in the absence of caffeine, a cytosolic Ca<sup>2+</sup> concentration of  $\geq 1$   $\mu$ M was required.

Fig. 4 A shows that an increase in luminal Ca<sup>2+</sup> concentration from 10  $\mu$ M to 1 mM caused an  $\sim 5$ -fold increase in P<sub>o</sub> at  $-35$  mV. By comparison, an only minimal increase in channel activity was evident at  $+35$  mV. Fig. 4 B compares the dependence of CRC activity on luminal Ca<sup>2+</sup> concentrations at cytosolic Ca<sup>2+</sup> concentrations that resulted in either a minimum (1  $\mu$ M Ca<sup>2+</sup>) or close to maximum (10  $\mu$ M Ca<sup>2+</sup>) channel activity in the absence of caffeine. In the presence of 1  $\mu$ M cytosolic Ca<sup>2+</sup>, lower luminal [Ca<sup>2+</sup>] was required at negative than positive holding potentials to observe a significant increase in P<sub>o</sub> ( $\geq 0.2$  mM at  $-35$  mV vs.  $\geq 5$  mM at  $+35$  mV; corresponding luminal Ca<sup>2+</sup> fluxes were  $\geq 0.8$  pA and  $\geq 0.7$  pA). In the presence of 10  $\mu$ M cytosolic Ca<sup>2+</sup>, P<sub>o</sub> decreased at the negative holding potential at [Ca<sup>2+</sup>]  $\geq 1$  mM, whereas only a small (not significant) increase was obtained at  $+35$  mV at luminal [Ca<sup>2+</sup>] as high as 10 mM (corresponding Ca<sup>2+</sup> fluxes were  $\geq 3$  pA and 0.9 pA, respectively). These results suggest that Ca<sup>2+</sup>-activated CRCs can be activated or inactivated in a voltage-dependent manner by luminal Ca<sup>2+</sup> in the absence of caffeine.

The inhibitory effects of luminal Ca<sup>2+</sup> on P<sub>o</sub> of maximally activated channels were also determined at a holding potential of 0 mV in a symmetric 0.25 M KCl buffer containing 20 mM luminal Ca<sup>2+</sup> and 10  $\mu$ M cytosolic Ca<sup>2+</sup>. The measured Ca<sup>2+</sup> current of  $2.7 \pm 0.4$  pA ( $n = 4$ ) was close to a calculated value of 3.1 pA. P<sub>o</sub> was signifi-

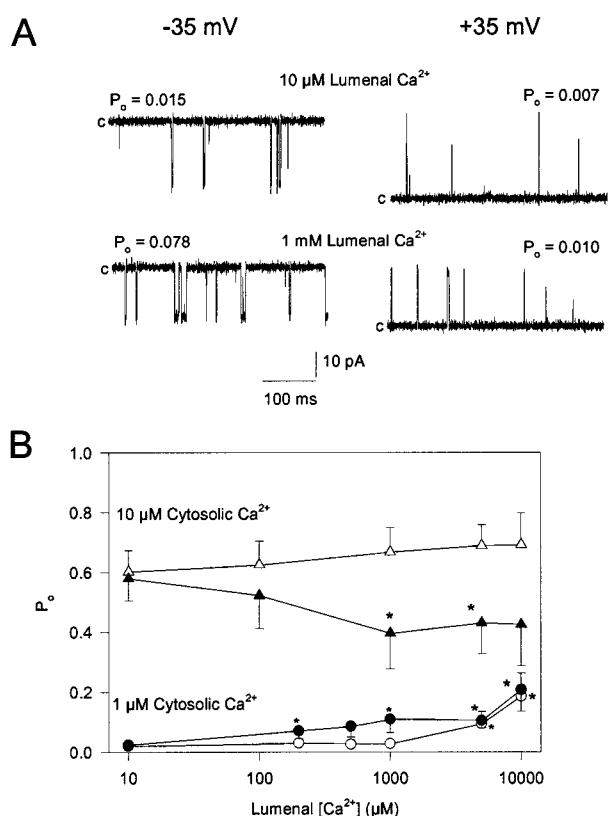


FIGURE 4 Dependence of  $P_o$  on luminal  $[\text{Ca}^{2+}]$  in absence of caffeine. (A) Single channel currents were recorded at  $-35$  mV (downward deflections, left panels) and  $+35$  mV (upward deflections, right panels) in symmetrical  $0.25$  M KCl,  $20$  mM KHepes, pH  $7.4$  media containing  $1$   $\mu\text{M}$  free cytosolic  $\text{Ca}^{2+}$  and indicated concentrations of luminal  $\text{Ca}^{2+}$ . (B) Single channels were recorded as in (A) in presence of indicated concentrations of cytosolic and luminal  $\text{Ca}^{2+}$ . Holding potentials were  $-35$  mV (●, ▲) and  $+35$  mV (○, △). Calculated  $\text{Ca}^{2+}$  fluxes at  $-35$  and  $+35$  mV were, respectively,  $0.8$  and  $0.1$  pA ( $200$   $\mu\text{M}$  luminal  $\text{Ca}^{2+}$ ),  $3.0$  and  $0.2$  pA ( $1$  mM luminal  $\text{Ca}^{2+}$ ),  $6.0$  and  $0.7$  pA ( $5$  mM luminal  $\text{Ca}^{2+}$ ), and  $6.9$  and  $0.9$  pA ( $10$  mM luminal  $\text{Ca}^{2+}$ ). Values are the mean  $\pm$  SE of  $8$ – $14$  experiments. \*Significantly different from  $P_o$  values at  $10$   $\mu\text{M}$  luminal  $\text{Ca}^{2+}$ .

cantly decreased by  $32 \pm 7\%$  ( $n = 4$ ) compared to control values obtained at  $\pm 5$  mV at luminal  $\text{Ca}^{2+}$  concentration of  $<2$   $\mu\text{M}$  (not shown). We conclude that the maximally  $\text{Ca}^{2+}$ -activated CRCs can be inactivated at  $0$  mV by a directly measured  $\text{Ca}^{2+}$  flux in the absence of caffeine.

CRC conducts  $\text{Mg}^{2+}$  (Meissner, 1994) and cytosolic  $\text{Mg}^{2+}$  inactivates the cardiac CRC by binding to  $\text{Ca}^{2+}$  activation and inactivation sites with micromolar and millimolar affinity, respectively (Liu et al., 1998; Laver et al., 1997). We rationalized that a voltage-independent inhibition of luminal  $\text{Mg}^{2+}$  would suggest the existence of  $\text{Mg}^{2+}$  inhibitory sites that reside on the SR luminal site, whereas a voltage-dependent inhibition would favor the idea of an access of luminal  $\text{Mg}^{2+}$  to the cytosolic  $\text{Ca}^{2+}$  regulatory sites. The effects of luminal  $\text{Mg}^{2+}$  ( $0$ – $50$  mM) on single cytosolic  $\text{Ca}^{2+}$ -activated channels were tested at holding potentials of  $\pm 5$ ,  $\pm 35$ , and  $\pm 50$  mV. In the presence of  $\sim 4$   $\mu\text{M}$   $\text{Ca}^{2+}$  in both bilayer chambers, a strong inhibition of

channel activity was observed at negative holding potentials that favored the movement of luminal  $\text{Mg}^{2+}$  to the cytosolic side of CRC, and yielded luminal-to-cytosolic  $\text{Mg}^{2+}$  fluxes of  $\geq 2.0$  pA. No appreciable inhibition was noted at positive holding potentials that disfavored the movement of luminal  $\text{Mg}^{2+}$  to the cytosolic side of the bilayer and yielded  $\text{Mg}^{2+}$  fluxes of  $<1$  pA (not shown). We also measured the  $\text{Mg}^{2+}$  current at  $0$  mV in a symmetrical  $0.25$  M KCl solution containing  $10$  mM luminal  $\text{Mg}^{2+}$ . Addition of  $10$  mM luminal  $\text{Mg}^{2+}$  decreased  $P_o$  to  $22 \pm 10\%$  of the control at  $\pm 5$  mV in the absence of  $\text{Mg}^{2+}$  ( $n = 5$ ). The directly measured  $\text{Mg}^{2+}$  current of  $2.1 \pm 0.1$  pA ( $n = 5$ ) agreed well with calculated value of  $2.2$  pA. We conclude from these observations that luminal  $\text{Mg}^{2+}$  fluxes affected cardiac CRC activity by having access to the channel's cytosolic  $\text{Ca}^{2+}$  regulatory sites. In frog skeletal muscle, the  $\text{Mg}^{2+}$  levels in the SR lumen near the  $\text{Ca}^{2+}$  release sites increase rather than decrease during tetanus (Somlyo et al., 1985). An in vivo regulation by luminal-to-cytosolic  $\text{Mg}^{2+}$  fluxes appears, therefore, to be unlikely.

#### Regulation of cardiac $\text{Ca}^{2+}$ release channel by cytosolic and luminal $\text{Ca}^{2+}$ in the presence of $5$ mM MgATP

The total ATP and free  $\text{Mg}^{2+}$  concentrations in myocardium have been estimated to range from  $5$  to  $10$  mM (Koretsune et al., 1991; Hohl et al., 1992) and  $0.7$ – $1.0$  mM (Murphy et al., 1989), respectively. Figs. 5 and 6 compare the voltage-dependence of cytosolic and luminal  $\text{Ca}^{2+}$ -activated channel activities recorded in the presence of  $5$  mM cytosolic MgATP ( $\sim 0.7$  mM free  $\text{Mg}^{2+}$ ), but in the absence of caffeine. An  $\sim 10\times$  higher cytosolic  $\text{Ca}^{2+}$  concentration was required to half-maximally activate the cardiac CRC ( $K_{\text{Ha}} = 14.4$   $\mu\text{M}$  vs.  $1$   $\mu\text{M}$ , Figs. 5 B and 1 B, respectively). As observed in the presence of caffeine (Fig. 1), no significant voltage-dependence in channel activity was noted for cardiac release channels activated by cytosolic  $\text{Ca}^{2+}$  in the presence of  $5$  mM cytosolic MgATP (Fig. 5, A and B).

In contrast to cytosolic  $\text{Ca}^{2+}$ -activated channels, CRC activities indicate a voltage-dependence when recorded at elevated luminal  $\text{Ca}^{2+}$  concentrations. In Fig. 6 A, a single cardiac CRC was recorded in the presence of  $10$   $\mu\text{M}$  cytosolic  $\text{Ca}^{2+}$  and  $5$  mM cytosolic MgATP at luminal  $\text{Ca}^{2+}$  concentrations of  $4$   $\mu\text{M}$  and  $200$   $\mu\text{M}$ . Elevation of luminal  $\text{Ca}^{2+}$  resulted in increased channel activity at  $-50$  mV but not  $+50$  mV. Fig. 6 B shows that at  $-50$  mV channels were significantly activated at luminal  $[\text{Ca}^{2+}]$  of  $\sim 200$ – $1000$   $\mu\text{M}$ . Higher luminal  $\text{Ca}^{2+}$  concentrations resulted in (not significant) inactivation of channel activities. At  $+50$  mV, higher luminal  $\text{Ca}^{2+}$  concentrations were required to observe an increase in channel activity; however, these were not significant.

Fig. 7, A and B compares the kinetic parameters of cytosolic and luminal  $\text{Ca}^{2+}$ -activated channel activities re-

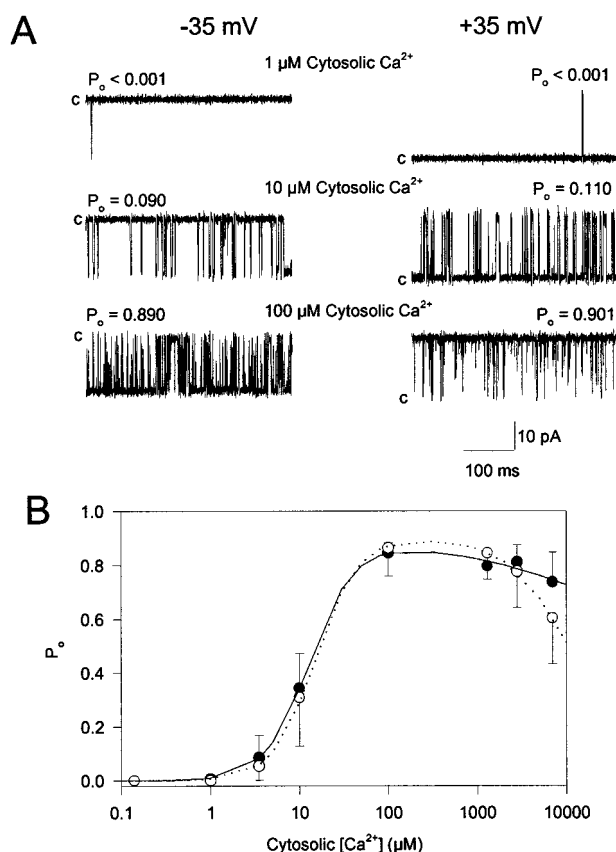


FIGURE 5 Dependence of single channel activities on cytosolic  $[\text{Ca}^{2+}]$  in the presence of 5 mM MgATP. (A) Single channel currents were recorded at  $-35$  mV (downward deflections, left current traces) and  $+35$  mV (upward deflections, right current traces) in symmetrical 0.25 M KCl, 20 mM KHepes, pH 7.4 media containing 5 mM cytosolic MgATP and indicated free cytosolic  $[\text{Ca}^{2+}]$ . The *trans* (SR luminal) solution contained 4  $\mu\text{M}$   $\text{Ca}^{2+}$ . (B) Dependence of  $P_o$  on cytosolic  $[\text{Ca}^{2+}]$ .  $P_o$  values were determined at  $-35$  mV ( $\circ$ ) and  $+35$  mV ( $\bullet$ ) as in (A). Values are the mean  $\pm$  SE of five experiments. Continuous lines were obtained assuming that CRC possesses cooperatively interacting high-affinity  $\text{Ca}^{2+}$  activation and low-affinity  $\text{Ca}^{2+}$ -inactivation sites (Scheme 1 and Eq. 1 of Liu et al., 1998). At  $-35$  mV, Hill constants and coefficients were  $K_{\text{Ha}} = 14.4$   $\mu\text{M}$ ,  $n_{\text{Ha}} = 1.8$ ,  $K_{\text{Hi}} = 12.6$  mM, and  $n_{\text{Hi}} = 1.2$ .

corded in the presence of 5 mM cytosolic MgATP. An increase in cytosolic  $\text{Ca}^{2+}$  concentration from  $\sim 0.1$  to 100  $\mu\text{M}$  increased  $P_o$  from nearly zero to  $\sim 1.0$ . This increase could be largely accounted for by an  $\sim 100$ -fold increase in the number of channel events and  $\sim 1000$ -fold increase in mean open times (Fig. 7 A). Mean closed events were decreased by  $\sim 100$ -fold. A further increase of cytosolic  $\text{Ca}^{2+}$  to 10 mM decreased  $P_o$  by decreasing mean open times and by slightly increasing the duration of mean closed events, without having an appreciable effect on the number of events. In Fig. 7 B, mean  $P_o$ , number of channel events, and mean open and closed times are plotted against the luminal  $\text{Ca}^{2+}$  fluxes. The latter were less effective in activating cardiac CRCs than cytosolic  $\text{Ca}^{2+}$  ( $P_{o,\text{max}} = \sim 1$  at cytosolic  $\text{Ca}^{2+}$  of  $\sim 100$   $\mu\text{M}$  vs.  $\sim 0.15$  at luminal  $\text{Ca}^{2+}$  flux of  $\sim 3$  pA). Small increases in  $P_o$  could be largely

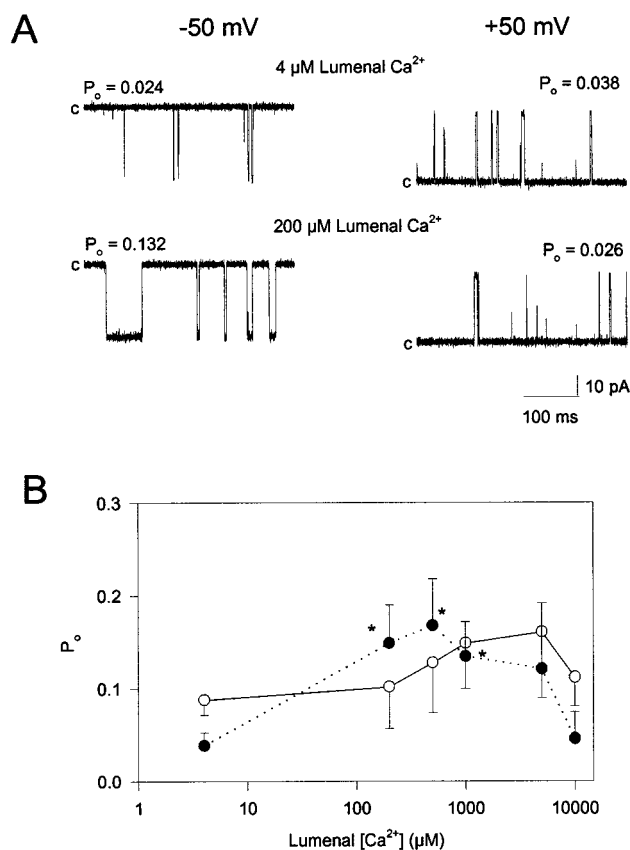


FIGURE 6 Activation of the cardiac  $\text{Ca}^{2+}$  release channel by luminal  $\text{Ca}^{2+}$  in the presence of 5 mM MgATP. (A) Single channel currents were recorded at  $-50$  mV (downward deflections, left panels) and  $+50$  mV (upward deflections, right panels) in symmetrical 0.25 M KCl, 20 mM KHepes, pH 7.4 media containing 10  $\mu\text{M}$  free cytosolic  $\text{Ca}^{2+}$  and 5 mM cytosolic MgATP, and indicated concentrations of luminal  $\text{Ca}^{2+}$ . (B) Dependence of  $P_o$  on luminal  $[\text{Ca}^{2+}]$  in the presence of 2–10  $\mu\text{M}$  free cytosolic  $\text{Ca}^{2+}$  and 5 mM cytosolic MgATP. Holding potentials were  $-50$  mV ( $\bullet$ ) and  $+50$  mV ( $\circ$ ). Values are the mean  $\pm$  SE of three to nine experiments. \*Significantly different from  $P_o$  at 4  $\mu\text{M}$  luminal  $\text{Ca}^{2+}$  (B).

accounted for by small (significant) increases in duration of mean open times. Few, if any, changes were observed in the number of channel events and duration of mean closed events, as luminal  $\text{Ca}^{2+}$  fluxes increased from 0.003 to 10 pA. Taken together, the data of Fig. 7, A and B suggest that luminal-to-cytosolic  $\text{Ca}^{2+}$  fluxes can regulate the cardiac CRC in the presence of physiologically relevant concentrations of  $\text{Mg}^{2+}$  and ATP.

## DISCUSSION

The results of this study suggest that luminal  $\text{Ca}^{2+}$  flowing through the open cardiac  $\text{Ca}^{2+}$  release channel can regulate the channel by having access to cytosolic activation and inactivation sites. Activation in the presence of  $\text{Mg}^{2+}$  and ATP suggests that regulation of CRC by luminal  $\text{Ca}^{2+}$  fluxes may be physiologically relevant.

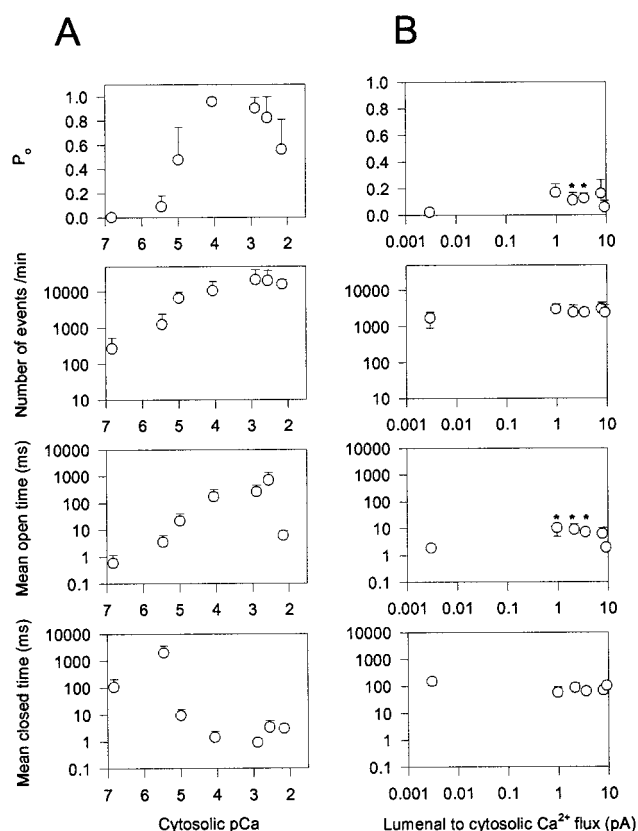


FIGURE 7 Single channel parameters of cytosolic and luminal  $\text{Ca}^{2+}$ -activated channels in the presence of 5 mM MgATP. Single channel parameters in (A) and (B) were obtained from recordings (A) at  $-35$  mV and (B) at  $-50$  mV at luminal  $\text{Ca}^{2+}$  concentrations of 4  $\mu\text{M}$ , 200  $\mu\text{M}$ , 500  $\mu\text{M}$ , 1 mM, 5 mM, and 10 mM (from left to right) as described in Figs. 5 and 6, respectively. Values are the mean  $\pm$  SE of three experiments (A) and three to nine experiments (B). \*Significantly different from luminal  $\text{Ca}^{2+}$  flux of 0.003 pA (B).

### Regulation of cardiac CRC activity by cytosolic and luminal $\text{Ca}^{2+}$

To distinguish between the effects of SR cytosolic and luminal  $\text{Ca}^{2+}$  on channel activity, single purified channels were recorded in symmetric KCl media at different holding potentials and with varying  $\text{Ca}^{2+}$  concentrations in the *trans* (SR luminal) and *cis* (cytosolic) chambers of the bilayer apparatus. As previously observed for the skeletal muscle CRC (Tripathy and Meissner, 1996), a strong voltage-dependence of channel activities was observed in the presence of elevated levels of luminal, but not cytosolic,  $\text{Ca}^{2+}$ . A voltage-dependent activation by luminal  $\text{Ca}^{2+}$  was observed in the absence of caffeine provided sufficiently high cytosolic  $[\text{Ca}^{2+}]$  was used to partially open the channel, which suggested that other channel activators such as sulmazole (Sitsapesan and Williams, 1994a) or ATP (Lukyanenko et al., 1996) were not required for cardiac channel activation by luminal  $\text{Ca}^{2+}$ . In the absence of caffeine and with cytosolic  $[\text{Ca}^{2+}]$  of  $<0.1$   $\mu\text{M}$  in the presence (Fig. 7) and absence (Fig. 4) of 5 mM MgATP, the cardiac CRC rarely opened. Under these recording conditions, luminal

$[\text{Ca}^{2+}]$  as high as 10 mM was not able to significantly activate the channel. In agreement with this finding, cellular SR luminal  $[\text{Ca}^{2+}]$ , which is thought to be close to 1 mM, does not activate the "closed" CRC. As in cells, where  $\text{Ca}^{2+}$  ions entering the cells activates the cardiac CRC, the presence of a cytosolic activator such as  $\text{Ca}^{2+}$  or caffeine was required before an activation and inactivation of the CRC by luminal  $\text{Ca}^{2+}$  could be observed. Lack of an activation of the "closed" CRC by luminal  $\text{Ca}^{2+}$  argues against a low-affinity  $\text{Ca}^{2+}$  regulatory site that resides on the luminal site of the channel.

The luminal-to-cytosolic  $\text{Ca}^{2+}$  fluxes were calculated using a four-barrier model that describes the ionic conduction of the sheep cardiac CRC (Tinker et al., 1992). In general, barrier models are inadequate to explain ion fluxes through channels over a large range of membrane potential (Chen et al., 1997). This limitation was also pointed out by Tinker et al. (1992) who could not fit their data by a four-barrier model at potentials  $>\pm 80$  mV. Recently, the flow of  $\text{K}^{+}$  through cardiac CRC has been modeled by diffusion theory using a combination of the Nernst-Planck and Poisson (PNP) equations (Chen et al., 1997). The model predicts a high  $\text{K}^{+}$  concentration ( $\sim 4$  M) in the selectivity filter at bath concentrations as low as 25 mM, thus providing an explanation for the high conductances of the CRCs. However, in contrast to the Tinker model, the PNP model has not yet been extended to mixed solutions containing  $\text{Ca}^{2+}$ . Tinker et al. (1992) measured and modeled ion conductances in bionic and mixed solutions, including  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ , and  $\text{K}^{+}$ . We directly measured  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  currents and their effects at 0 mV in symmetric KCl solutions. Good agreement with the calculated values suggests that at the membrane potentials used in our study, the Tinker model serves as a useful "curve-fitting" tool to predict ion fluxes in mixed solutions.

### Parameters determining the extent of CRC activation and inactivation by luminal $\text{Ca}^{2+}$

The extent of CRC activation by luminal  $\text{Ca}^{2+}$  was dependent on the presence of  $\text{Ca}^{2+}$ , MgATP, and caffeine in the cytosolic (*cis*) chamber of the bilayer apparatus. In agreement with observations of an increased  $\text{Ca}^{2+}$  affinity of  $\text{Ca}^{2+}$  activation sites by caffeine (Zucchi and Ronca-Testoni, 1997; Liu et al., 1998), channels could be more effectively activated at lower luminal  $\text{Ca}^{2+}$  fluxes in the presence of caffeine (100  $\mu\text{M}$  luminal  $\text{Ca}^{2+}$  at  $-50$  mV corresponds to luminal  $\text{Ca}^{2+}$  flux of 0.6 pA, Fig. 2 B) than in the absence of caffeine (200  $\mu\text{M}$  luminal  $\text{Ca}^{2+}$  at  $-35$  mV corresponds to luminal  $\text{Ca}^{2+}$  flux of 0.8 pA, Fig. 4 B). Addition of 5 mM cytosolic MgATP increases the Hill constant of  $\text{Ca}^{2+}$  activation by cytosolic  $\text{Ca}^{2+}$  by 3–4-fold (Xu et al., 1996; Fig. 6). In reasonable agreement with this result, CRCs were activated by lower luminal  $\text{Ca}^{2+}$  fluxes in the absence of MgATP (0.8 pA in Fig. 4 B; in Fig. 6, 200  $\mu\text{M}$  luminal  $\text{Ca}^{2+}$  at  $-50$  mV corresponds to luminal  $\text{Ca}^{2+}$  flux of 1.0 pA).



Luminal  $\text{Ca}^{2+}$  fluxes lead to the buildup of a high cytosolic  $\text{Ca}^{2+}$  concentration near the release sites (Stern, 1992; Fig. 8), which raised the possibility that luminal  $\text{Ca}^{2+}$  fluxes inactivated the channels before they could be fully activated. We tested this idea using the “fast”  $\text{Ca}^{2+}$ -complexing buffer BAPTA. Because of its high association rate BAPTA can suppress the rise in  $\text{Ca}^{2+}$  concentration at locations several nanometers away from the release site (Stern, 1992; Fig. 8). Fig. 3 *B* (top panel) shows that 20 mM cytosolic BAPTA increased channel activities close to those observed in the presence of micromolar-to-millimolar cytosolic  $[\text{Ca}^{2+}]$  (Fig. 3 *A*, top panel), thus supporting the idea that luminal  $\text{Ca}^{2+}$  fluxes cannot only activate but also inactivate the cardiac CRC. Channel activation by cytosolic effectors was required to observe the effects of luminal  $\text{Ca}^{2+}$ . This finding limited the conditions that could be used to test the effects of BAPTA. Specifically, BAPTA could not be used to test the effects of luminal  $\text{Ca}^{2+}$  fluxes in the presence of 5 mM MgATP because, in agreement with the *in vivo* function of the CRC, only few, if any, channel openings could be observed at cytosolic  $\text{Ca}^{2+}$  concentrations of  $\leq 0.1 \mu\text{M}$ .

### Kinetics of CRC activation and inactivation by cytosolic and luminal $\text{Ca}^{2+}$

Kinetics of cytosolic  $\text{Ca}^{2+}$ -mediated channel activation and inactivation are, at least in principle, more straightforward than those by luminal  $\text{Ca}^{2+}$  and will therefore be discussed first. At low cytosolic  $\text{Ca}^{2+}$  concentrations, channels opened infrequently and long-closed/short-open channel events predominated, resulting in a low channel open probability (Figs. 1 *A* and 5 *A*). An increase in the number of channel events and a decrease in closed mean times with increasing  $\text{Ca}^{2+}$  concentration indicated that cytosolic  $\text{Ca}^{2+}$  increased  $P_o$  by increasing the transition rates from the closed to open state(s). A second effect of increasing cytosolic  $[\text{Ca}^{2+}]$  was to increase the mean open times.  $\text{Ca}^{2+}$  activated CRCs by a cooperative mechanism in the presence of caffeine and MgATP, and the increase in mean open time may have been therefore due to the cooperative binding of  $\text{Ca}^{2+}$  to the tetrameric channel complex. An increase in open times by cytosolic  $\text{Ca}^{2+}$  was also observed for the sheep cardiac CRC (Sitsapasan and Williams, 1994b). This increase was explained by assuming a  $\text{Ca}^{2+}$ -dependent pathway between two open states. High  $\text{Ca}^{2+}$  concentrations inactivate the channel by binding to low-affinity sites (Liu et al., 1998; Laver et al., 1995). In our single channel recordings, 10 mM cytosolic  $\text{Ca}^{2+}$  decreased  $P_o$  by decreasing the mean open times and increasing the mean closed times, without appreciably affecting the number of single channel events. These changes suggest that  $\text{Ca}^{2+}$  binding to the  $\text{Ca}^{2+}$ -inactivation sites affects both the transition rates from the open-to-closed and from the closed-to-open states, increasing the former and decreasing the latter.

According to our model, luminal  $\text{Ca}^{2+}$  is only available to cytosolic  $\text{Ca}^{2+}$  regulatory sites when a channel opens.

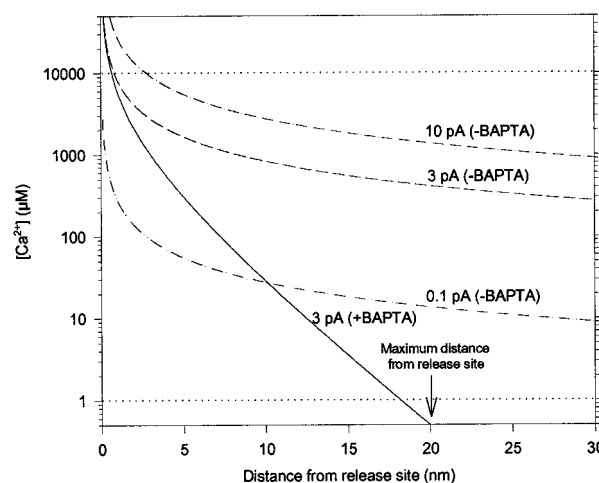


FIGURE 8 Cytosolic  $[\text{Ca}^{2+}]$  profiles at 0.1, 3, and 10 pA luminal  $\text{Ca}^{2+}$  fluxes in the presence 0.2 mM EGTA and absence of BAPTA, and in the presence of 20 mM BAPTA. Also shown (dotted lines) are  $[\text{Ca}^{2+}]$  that half-maximally activated ( $1 \mu\text{M}$   $\text{Ca}^{2+}$ ) and inactivated ( $10 \text{ mM}$   $\text{Ca}^{2+}$ ) cytosolic  $\text{Ca}^{2+}$ -activated CRCs in the presence of 10 mM caffeine (Fig. 1 *B*). Luminal-to-cytosolic  $\text{Ca}^{2+}$  fluxes were calculated using the barrier model and parameters of Tinker et al. (1992). Cytosolic  $\text{Ca}^{2+}$  gradients were derived according to Eq. 13 of Stern (1992) using the following constants for  $\text{Ca}^{2+}$  and BAPTA:  $k_{\text{on}} = 1.7 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ ;  $K_d = 4 \times 10^{-7} \text{ M}$ ;  $D_{\text{Ca}} = 3 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ ;  $D_{\text{BAPTA}} = D_{\text{CaBAPTA}} = 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ . EGTA has an  $\sim 1000$ -fold lower  $\text{Ca}^{2+}$  on-rate constant than BAPTA. The presence of 0.2 mM EGTA therefore did not significantly affect cytosolic  $[\text{Ca}^{2+}]$  profiles.

$\text{Ca}^{2+}$  gradients formed by  $\text{Ca}^{2+}$  fluxes build up and dissipate in  $\sim 50 \mu\text{s}$  as channels open and close (Simon and Llinas, 1985). Accordingly, cytosolic  $\text{Ca}^{2+}$  gradients formed by luminal  $\text{Ca}^{2+}$  fluxes likely had a lifetime that was less than that of the shortest channel events seen in the bilayers ( $\sim 0.2 \text{ ms}$ ). One would then expect that the frequency of channel openings is being set mainly by the cytosolic  $\text{Ca}^{2+}$ , caffeine, and MgATP concentrations, while luminal  $\text{Ca}^{2+}$  does not noticeably affect the number of events and duration of mean closed events. In agreement with this prediction, luminal  $\text{Ca}^{2+}$  did not significantly affect the number of channel events and duration of mean closed events. Much of the increase in  $P_o$  observed for luminal  $\text{Ca}^{2+}$ -activated channels could be accounted for by an increase in mean open times. This increase was likely due to the rapid buildup of a cytosolic  $\text{Ca}^{2+}$  gradient because a similar prolongation in mean open times was observed with increasing cytosolic  $[\text{Ca}^{2+}]$  (Figs. 3 *A* and 7 *A*, third panels). We conclude that luminal  $\text{Ca}^{2+}$  ions flowing through open channels may increase the duration of channel open events by elevating cytosolic  $[\text{Ca}^{2+}]$  at  $\text{Ca}^{2+}$  activation sites. The frequency of these regulatory events is mainly set by cytosolic factors that determine the frequency of channel openings such as cytosolic  $\text{Ca}^{2+}$  or MgATP.

### Location of activation and inactivation sites

Cryoelectron microscopy and image analysis have indicated that the skeletal muscle CRC consists of a large  $29 \times 29 \times$

12 nm cytosolic “foot” region and a smaller transmembrane region that extends  $\sim 7$  nm toward the SR lumen and likely contains a centrally located  $\text{Ca}^{2+}$  channel pore (Radermacher et al., 1994; Serysheva et al., 1995). A very similar architecture has been deduced for the cardiac CRC (Sharma et al., 1997). The cardiac CRC is thought to have at least two classes of  $\text{Ca}^{2+}$  binding sites, a high-affinity activation and a low-affinity inactivation site. The location of these sites, however, has not been established. Although our single channel measurements cannot pinpoint the location of the  $\text{Ca}^{2+}$  regulatory sites on the large cardiac CRC complex, our data can provide tentative information with respect to their distance from the cytosolic  $\text{Ca}^{2+}$  release site. Fig. 3 B shows that luminal  $\text{Ca}^{2+}$  fluxes of 0.1 pA did not significantly activate the cardiac CRC in the presence of 10 mM caffeine. By comparison,  $\text{Ca}^{2+}$  fluxes of 1 pA and greater caused a nearly maximum activation of channels that were recorded in the presence of 10 mM caffeine and 20 mM BAPTA. The cytosolic  $\text{Ca}^{2+}$  concentration profiles that were obtained at luminal  $\text{Ca}^{2+}$  fluxes of 0.1 pA and 3 pA are included in Fig. 8. Also indicated in Fig. 8 is the cytosolic  $\text{Ca}^{2+}$  concentration (1  $\mu\text{M}$ , dotted line) that resulted in half-maximum activation of CRCs in the presence of 10 mM caffeine (Fig. 1 B). Together these data show that luminal  $\text{Ca}^{2+}$  fluxes as low as 0.1 pA should have been sufficient to maximally activate the CRC, even if the activation sites would have been located 30 nm away from the release site, which is more than the dimensions of the cardiac CRC. Another argument against a distance  $\geq 20$  nm between the  $\text{Ca}^{2+}$  activation and release sites is that 20 mM BAPTA at luminal flux of 3 pA would have been expected to lower channel activity, which clearly was not the case. A similar paradoxical situation between the measured cytosolic  $\text{Ca}^{2+}$ -activating concentrations and calculated effects of luminal  $\text{Ca}^{2+}$  fluxes was obtained for the skeletal muscle CRC (Tripathy and Meissner, 1996). To explain the paradox, skeletal muscle cytosolic  $\text{Ca}^{2+}$  activation sites were placed within the foot region at BAPTA “inaccessible” sites. It was further suggested that these sites see a minor portion, whereas  $\text{Ca}^{2+}$  inactivation sites see a major portion of luminal  $\text{Ca}^{2+}$ . We propose a similar model for the cardiac CRC. The model suggests that luminal  $\text{Ca}^{2+}$  fluxes increase  $\text{Ca}^{2+}$  concentrations to a lesser extent at the  $\text{Ca}^{2+}$  activation than  $\text{Ca}^{2+}$  inactivation sites, thus explaining that, as observed in the present study,  $\text{Ca}^{2+}$  inactivation sets in before the cardiac CRC can be fully activated by luminal  $\text{Ca}^{2+}$ .

The distance between the  $\text{Ca}^{2+}$  release and  $\text{Ca}^{2+}$  inactivation sites of the cardiac CRC was estimated as follows. Fig. 2 C shows that channels activated by 4  $\mu\text{M}$  cytosolic  $\text{Ca}^{2+}$  in the presence of 10 mM caffeine were half-maximally inactivated at a luminal  $\text{Ca}^{2+}$  flux of  $\sim 10$  pA. This flux resulted in a half-maximally inactivating cytosolic  $\text{Ca}^{2+}$  concentration of 10 mM (Fig. 1 B) at a distance of  $\sim 3$  nm from the release site (Fig. 8). Single channel measurements with the fast  $\text{Ca}^{2+}$ -complexing buffer BAPTA suggest that a distance of 3 nm between the release and  $\text{Ca}^{2+}$

inactivation sites may be an upper limit. BAPTA increased channel activities at a luminal  $\text{Ca}^{2+}$  flux of 3 pA to close a maximum value (Fig. 3 B). At a distance of 3 nm, a cytosolic  $[\text{Ca}^{2+}]$  of  $\sim 3$  mM is calculated (Fig. 8), which appears to be too low to cause substantial  $\text{Ca}^{2+}$  inactivation (Fig. 1 B). Higher cytosolic  $[\text{Ca}^{2+}]$  exists closer to the release site (Fig. 8). However, placement of  $\text{Ca}^{2+}$  inactivation sites too close to the release site is problematic because it renders BAPTA ineffective in lowering  $[\text{Ca}^{2+}]$ . According to Fig. 8, a compromise is reached at a distance of 1 nm from the release site. At this distance and at a luminal  $\text{Ca}^{2+}$  flux of 3 pA, a cytosolic  $[\text{Ca}^{2+}]$  of  $\sim 9$  mM is calculated, which is lowered by 20 mM BAPTA to  $\sim 6$  mM (Fig. 8). Such a decrease can account, at least in principle, for the activating effects of BAPTA (Fig. 3 B). Taken together, these results suggest that the  $\text{Ca}^{2+}$  inactivation site(s) lie(s) at a distance of  $\leq 3$  nm from the release site. This distance is reasonably close to the distances of 3–6 nm estimated between the two sites of the skeletal muscle CRC (Tripathy and Meissner, 1996).

### Physiological implications

In mammalian ventricular muscle, clusters of  $\text{Ca}^{2+}$  release channels are located near the surface membrane and tubular infoldings (T-tubule) of the surface membrane (Franzini-Armstrong and Protasi, 1997). Immunolocalization studies suggest a co-distribution of CRCs with surface dihydropyridine receptors ( $\text{Ca}^{2+}$  channels, L-type), which provides a morphological basis for the  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release (CICR) mechanism (Carl et al., 1995). Recent studies suggest that the opening of a single L-type  $\text{Ca}^{2+}$  channel may be sufficient to evoke a localized  $\text{Ca}^{2+}$  release event (“ $\text{Ca}^{2+}$  spark”) by activating one or more CRCs (Santana et al., 1996). During L-type  $\text{Ca}^{2+}$  channel opening the  $\text{Ca}^{2+}$  concentration can reach millimolar values (Langer and Peskoff, 1996), which are more than enough to activate closely apposed  $\text{Ca}^{2+}$  release channels. The present study shows that SR luminal  $\text{Ca}^{2+}$  can contribute to the regulation of cardiac SR  $\text{Ca}^{2+}$  release via direct feedback by binding to channels that release  $\text{Ca}^{2+}$ . In myocardium these events may involve more than one channel because, in addition to its own channel, luminal  $\text{Ca}^{2+}$  fluxes may activate and inactivate closely located  $\text{Ca}^{2+}$  release channels.

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