Regulation of Cardiac Muscle Ca\(^{2+}\) Release Channel by Sarcoplasmic Reticulum Lumenal Ca\(^{2+}\)

Le Xu* and Gerhard Meissner*
Departments of *Biochemistry & Biophysics and #Physiology, University of North Carolina, Chapel Hill, North Carolina 27599-7260 USA

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

INTRODUCTION

The release and sequestration of 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 Ca\(^{2+}\) via a voltage-sensitive dihydropyridine receptor (DHPR)/Ca\(^{2+}\) channel (L-type) triggers the massive release of Ca\(^{2+}\) by opening SR 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 Ca\(^{2+}\) as a major regulator. High-affinity activating and low-affinity inactivating 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 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 Mg\(^{2+}\), ATP, and calmodulin (Meissner, 1994).

In addition to cytosolic Ca\(^{2+}\), SR lumenal Ca\(^{2+}\) may affect CRC activity. The most direct evidence for a regulation by SR lumenal Ca\(^{2+}\) has been obtained in single channel measurements using the planar lipid bilayer technique. SR lumenal 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 Ca\(^{2+}\) binding sites that interact with cytosolic regulatory sites (Sitsapesan and Williams, 1995). An alternative suggestion has been that SR lumenal 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” Ca\(^{2+}\) buffer 1,2-bis(2-aminoephenoxy)ethanetetraacetic acid (BAPTA) increased cytosolic ATP-activated, lumenal Ca\(^{2+}\)-activated skeletal muscle channel activities. This result suggested that lumenal Ca\(^{2+}\) passing through the skeletal CRC regulates the channel by having access to “BAPTA-inaccessible” Ca\(^{2+}\) activation and “BAPTA-accessible” Ca\(^{2+}\) inactivation sites (Tripathy and Meissner, 1996).

An increase in lumenal 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 Ca\(^{2+}\). These results were considered to be inconsistent with the idea that lumenal Ca\(^{2+}\) ions flowing through the channel have direct access to cytosolic Ca\(^{2+}\) activation sites.

The cardiac CRC represents a classical example of a Ca\(^{2+}\)-regulated Ca\(^{2+}\) release mechanism (Wier, 1990). Its regulation by Ca\(^{2+}\) and other endogenous effectors differs from that of the skeletal CRC (Franzini-Armstrong and...
Protasi, 1997; Sutko et al., 1997; Meissner, 1994). It is therefore conceivable that the two channel isoforms are regulated differently by lumenal Ca$^{2+}$. To clarify the ways in which lumenal 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 lumenal Ca$^{2+}$ flowing through the channel regulates the cardiac Ca$^{2+}$ release channel via direct feedback by binding to cytosolic Ca$^{2+}$ activation and inactivation sites. An activation of channel activity by lumenal Ca$^{2+}$ was observed at Mg$^{2+}$ and ATP concentrations corresponding to those in myocardium. These results suggest that activation of cardiac CRCs by lumenal 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 Ca$^{2+}$ release channels**

Canine cardiac SR vesicle fractions enriched in [3H]ryanodine binding and 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 Ca$^{2+}$ release channel complex was isolated by rate density 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 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 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 Ca$^{2+}$ concentrations**

Free Ca$^{2+}$ concentrations of >1 µM were determined with a Ca$^{2+}$-selective electrode (World Precision Instruments, Inc., Sarasota, FL). Free Ca$^{2+}$ concentrations of <1 µM were obtained by including in the solutions the appropriate amounts of 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 ± 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 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 K$^+$ rather than Ca$^{2+}$ as a current carrier avoided the buildup of a large Ca$^{2+}$ gradient near the mouth of the channel, thus simplifying analysis of regulation of the cardiac CRC by Ca$^{2+}$. Single channel conductance with 0.25 M K$^+$ as current carrier was 770 pS (Xu et al., 1993). The effects of cytosolic and lumenal Ca$^{2+}$ on channel activity were examined in the presence and absence of 10 mM cytosolic caffeine. Caffeine increases the apparent Ca$^{2+}$ affinity of the Ca$^{2+}$ activation sites (Liu et al., 1998; Zucchi and Ronca-Testoni, 1997), which allows the use of low cytosolic Ca$^{2+}$ concentrations in testing the effects of lumenal Ca$^{2+}$. Channels were also recorded in the presence of 5 mM cytosolic MgATP (0.7 mM free Mg$^{2+}$) to better simulate the intracellular conditions in myocardium.

**Regulation of cardiac Ca$^{2+}$ release channels by cytosolic and luminal 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 Ca$^{2+}$ concentrations and holding potentials of −35 and +35 mV. Short, often not fully resolved channel events were observed with 0.1 µM free Ca$^{2+}$ in the cytosolic bilayer chamber (Fig. 1 A, top traces). Elevation of cytosolic Ca$^{2+}$ concentration to 1 µM increased channel open probability (Po, middle traces) at both holding potentials. In the presence of 10 µM cytosolic 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 ~1 µM cytosolic Ca$^{2+}$ and half-maximally inhibited at ≥10 mM cytosolic 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 Ca$^{2+}$ activation and low-affinity Ca$^{2+}$ inactivation sites. Furthermore, Fig. 1 shows that the cardiac CRC exhibits no significant voltage dependence when activated and inactivated by cytosolic Ca$^{2+}$ in the presence of caffeine.
The CRC showed a strong voltage dependence when the lumenal 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 lumenal 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 lumenal Ca\(^{2+}\) concentration from <2 \(\mu\)M to 1 \(\mu\)M 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 lumenal 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 lumenal 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 lumenal 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 lumenal 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 lumenal 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 lumenal 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 lumenal (trans) side to the cytosolic (cis) side of the bilayer. The different Ca\(^{2+}\) activation/inactivation curves of Fig. 2 B suggest, therefore, that lumenal Ca\(^{2+}\) flowing through the open channel affects channel activity by having access to cytosolic Ca\(^{2+}\) regulatory sites. Lumenal 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. Lumenal-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 lumenal Ca\(^{2+}\) fluxes. Lumenal Ca\(^{2+}\) fluxes were calculated at six holding potentials ranging from −65 mV to +65 mV and four lumenal 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 lumenal-to-cytosolic Ca\(^{2+}\) flux of ~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 lumenal 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 lumenal 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 ± 0.1 pA (\(n = 5\)) was close to a calculated value of 2.1 pA. The averaged \(P_o\) of 0.53 ± 0.21 (\(n = 5\)) was close to values that yielded lumenal-to-cytosolic Ca\(^{2+}\) fluxes of ~2 pA at negative and positive holding potentials (2.1 and 1.3 pA at −20 and +20 mV and lumenal [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 lumenal Ca\(^{2+}\) (Fig. 2 C). However, these channels were significantly inactivated at lumenal Ca\(^{2+}\) fluxes of 8 pA and greater.

An intriguing finding was that at a low cytosolic Ca\(^{2+}\) concentration lumenal Ca\(^{2+}\) fluxes were less effective than...
FIGURE 2 Activation of the cardiac Ca\(_{\text{2+}}\) release channel by lumenal Ca\(_{\text{2+}}\) 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 μM free cytosolic Ca\(_{\text{2+}}\) (200 μM EGTA and <2 μM contaminating Ca\(_{\text{2+}}\)) and 10 mM cytosolic caffeine. Bottom traces were obtained after the addition of 20 mM cytosolic BAPTA. SR luminal [Ca\(_{\text{2+}}\)] was <2 μM (top traces) and 1 mM (middle and bottom traces). Note: Negative holding potentials favor luminal-to-cytosolic Ca\(_{\text{2+}}\) fluxes. (B) Dependence of \(P_o\) on cytosolic and luminal [Ca\(_{\text{2+}}\)]. Holding potentials were −50 mV (○, ▲) and +50 mV (○, △). (C) Dependence of \(P_o\) on luminal-to-cytosolic Ca\(_{\text{2+}}\) fluxes. Luminal-to-cytosolic [Ca\(_{\text{2+}}\)] in activating the CRC (\(P_{o,\text{max}} = \sim -0.8\) in Fig. 1 B vs. \(P_{o,\text{max}} = \sim 0.5\) in Fig. 2 C). This result can be rationalized if lumenal Ca\(_{\text{2+}}\) inactivates before fully activating the release channel. We tested this idea using the “fast” complexing Ca\(_{\text{2+}}\) buffer BAPTA. Modeling studies have indicated that the free Ca\(_{\text{2+}}\) 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 Ca\(_{\text{2+}}\) buffer EGTA in suppressing such a rise in Ca\(_{\text{2+}}\) concentration (Stern, 1992). In the middle traces of Fig. 2 A, a single lumenal Ca\(_{\text{2+}}\)-activated channel was recorded under standard conditions; that is, in the presence of <0.01 μM cytosolic Ca\(_{\text{2+}}\) and 10 mM cytosolic caffeine. Lumenal Ca\(_{\text{2+}}\) 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 lumenal Ca\(_{\text{2+}}\)-activated single channels. At luminal Ca\(_{\text{2+}}\) 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 Ca\(_{\text{2+}}\) was obtained (Fig. 3 A, top panel). This result suggested that BAPTA was apparently able to prevent lumenal Ca\(_{\text{2+}}\)-mediated channel inactivation by minimizing the buildup of a high inactivating Ca\(_{\text{2+}}\) concentration near the cytosolic Ca\(_{\text{2+}}\) inactivation sites. However, BAPTA did not prevent channel activation, which suggested that at a concentration of 20 mM BAPTA did not lower the Ca\(_{\text{2+}}\) concentration below a maximally activating Ca\(_{\text{2+}}\) concentration of ~5 μM (Fig. 1 B) at the cytosolic Ca\(_{\text{2+}}\) activation sites. A direct pharmacological activation of CRCs by BAPTA appeared to be unlikely because none was observed when luminal Ca\(_{\text{2+}}\) fluxes were ~0.1 pA (Fig. 3 B, top panel).

In the case of cytosolic Ca\(_{\text{2+}}\)-activated CRCs, both the Ca\(_{\text{2+}}\)-activating and -inactivating sites see the same [Ca\(_{\text{2+}}\)]. In contrast, luminal Ca\(_{\text{2+}}\) has access only to cytosolic regulatory sites when the channel is open. In addition, the Ca\(_{\text{2+}}\) activation and inactivation sites may see different [Ca\(_{\text{2+}}\)], depending on their relative location with respect to the release site. It was therefore of interest to compare the kinetic parameters of cytosolic Ca\(_{\text{2+}}\)-activated and luminal Ca\(_{\text{2+}}\)-activated channels (Fig. 3, A and B). An increase in cytosolic Ca\(_{\text{2+}}\) concentration from <0.01 μM to 100 μM Ca\(_{\text{2+}}\) fluxes were calculated according to the barrier model and parameters of Tinker et al. (1992) at ~0.01 μM cytosolic (closed symbols) and 4 μM cytosolic (open symbols) Ca\(_{\text{2+}}\) in the presence of <2 μM luminal Ca\(_{\text{2+}}\) (■, masked by the other symbols at the origin, Y) at ±35 and ±50 mV, 1 mM luminal Ca\(_{\text{2+}}\) at ±65, ±50, ±35, ±20, ±35–50 and −65 mV (○, from left to right) and +50, ±35, −35, and ±50 mV (○, from left to right), 3 mM luminal Ca\(_{\text{2+}}\) (•) at 0 mV, 5 mM luminal Ca\(_{\text{2+}}\) (■, □) at the membrane potentials indicated for 1 mM luminal Ca\(_{\text{2+}}\), except that the effects of 5 mM luminal Ca\(_{\text{2+}}\) at 4 μM cytosolic Ca\(_{\text{2+}}\) were also determined at ±65 mV, and in the presence of 10 mM luminal Ca\(_{\text{2+}}\) at (▲, △) at +65, ±50, ±35, ±35–50 and −65 mV (from left to right). (B) and (C) Values are the mean ± SE of 3–19 experiments. (B) *Significantly different from \(P_o\) at ±4 μM luminal Ca\(_{\text{2+}}\). (C) *Significantly different from \(P_o\) at luminal Ca\(_{\text{2+}}\) flux of <0.1 pA.
increased $P_0$ 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 ~100-fold (Fig. 3 A). The duration of mean closed events was maximally decreased by ~10,000-fold. A further increase of cytosolic $Ca^{2+}$ to 10 mM decreased $P_0$ 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 lumenal $Ca^{2+}$ fluxes. Channels were recorded at eight holding potentials ranging from −65 to +65 mV and 1 mM lumenal $Ca^{2+}$ and cytosolic $Ca^{2+}$ concentration of <0.01 μM in the presence and absence of 20 mM cytosolic BAPTA. In the absence of BAPTA, lumenal $Ca^{2+}$ fluxes were less effective than cytosolic $Ca^{2+}$ in activating cardiac CRCs (top panels of Fig. 3, A and B). Lumenal $Ca^{2+}$ opened and closed channels less frequently than cytosolic $Ca^{2+}$ (second panels). In both cases, mean open times were increased as channels were maximally activated by raising cytosolic $[Ca^{2+}]$ from ~0.003 μM to 10 μM, and lumenal $Ca^{2+}$ 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 $Ca^{2+}$ from ~0.003 to 10 μM decreased the mean closed times from 10,000 ms to close to 1 ms (Fig. 3 A, bottom panel). By comparison, an increase in lumenal $Ca^{2+}$ fluxes from 0.04 to 3 pA decreased the mean closed times by <100-fold (Fig. 3 B, bottom panel).

Cytosolic BAPTA significantly increased $P_0$ at elevated lumenal $Ca^{2+}$ 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 $Ca^{2+}$ release channel by lumenal $Ca^{2+}$ and $Mg^{2+}$ in the absence of caffeine

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

Fig. 4 A shows that an increase in lumenal $Ca^{2+}$ concentration from 10 μM to 1 mM caused an ~5-fold increase in $P_0$ 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 lumenal $Ca^{2+}$ concentrations at cytosolic $Ca^{2+}$ concentrations that resulted in either a minimum (1 μM $Ca^{2+}$) or close to maximum (10 μM $Ca^{2+}$) channel activity in the absence of caffeine. In the presence of 1 μM cytosolic $Ca^{2+}$, lower lumenal $[Ca^{2+}]$ was required at negative than positive holding potentials to observe a significant increase in $P_0$ (≥0.2 mM at −35 mV vs. ≥5 mM at +35 mV; corresponding lumenal $Ca^{2+}$ fluxes were ≥0.8 pA and ≥0.7 pA). In the presence of 10 μM cytosolic $Ca^{2+}$, $P_0$ decreased at the negative holding potential at $[Ca^{2+}]$ ≥1 mM, whereas only a small (not significant) increase was obtained at +35 mV at lumenal $[Ca^{2+}]$ as high as 10 mM (corresponding $Ca^{2+}$ fluxes were ≥3 pA and 0.9 pA, respectively). These results suggest that $Ca^{2+}$-activated CRCs can be activated or inactivated in a voltage-dependent manner by lumenal $Ca^{2+}$ in the absence of caffeine.

The inhibitory effects of lumenal $Ca^{2+}$ on $P_0$ 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 lumenal $Ca^{2+}$ and 10 μM cytosolic $Ca^{2+}$. The measured $Ca^{2+}$ current of 2.7 ± 0.4 pA ($n = 4$) was close to a calculated value of 3.1 pA. $P_0$ was signifi-
Regulation of cardiac Ca\(^{2+}\) release channel by cytosolic and luminal Ca\(^{2+}\) in the presence of 5 mM MgATP

The total ATP and free Mg\(^{2+}\) concentrations in myocardium have been estimated to range from 5 to 10 mM (Koretzune 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 Ca\(^{2+}\)-activated channel activities recorded in the presence of 5 mM cytosolic MgATP (~0.7 mM free Mg\(^{2+}\)) but in the absence of caffeine. An ~10× higher cytosolic Ca\(^{2+}\) concentration was required to half-maximally activate the cardiac CRC (\(K_{\text{Mg}} = 14.4 \, \mu\text{M}\) 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 Ca\(^{2+}\) in the presence of 5 mM cytosolic MgATP (Fig. 5, A and B).

In contrast to cytosolic Ca\(^{2+}\)-activated channels, CRC activities indicate a voltage-dependence when recorded at elevated luminal Ca\(^{2+}\) concentrations. In Fig. 6 A, a single cardiac CRC was recorded in the presence of 10 \(\mu\text{M}\) cytosolic Ca\(^{2+}\) and 5 mM cytosolic MgATP at luminal Ca\(^{2+}\) concentrations of 4 \(\mu\text{M}\) and 200 \(\mu\text{M}\). Elevation of luminal 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 [Ca\(^{2+}\)] of ~200–1000 \(\mu\text{M}\). Higher luminal Ca\(^{2+}\) concentrations resulted in (not significant) inactivation of channel activities. At +50 mV, higher luminal 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 Ca\(^{2+}\)-activated channel activities re-
corded in the presence of 5 mM cytosolic MgATP. An increase in cytosolic Ca\(^{2+}\) concentration from 0.1 to 100 \(\mu\)M increased \(P_o\) from nearly zero to 1.0. This increase could be largely accounted for by a 100-fold increase in the number of channel events and a 1000-fold increase in mean open times (Fig. 7A). Mean closed events were decreased by 100-fold. A further increase of cytosolic Ca\(^{2+}\) to 10 \(\mu\)M 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. 7B, mean \(P_o\) number of channel events, and mean open and closed times are plotted against the lumenal Ca\(^{2+}\) fluxes. The latter were less effective in activating cardiac CRCs than cytosolic Ca\(^{2+}\) (\(P_{o,max} = \sim 1\) at cytosolic Ca\(^{2+}\) of \(\sim 100 \mu\)M vs. \(\sim 0.15\) at lumenal Ca\(^{2+}\) flux of \(\sim 3\) pA). Small increases in \(P_o\) could be largely 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 lumenal Ca\(^{2+}\) fluxes increased from 0.003 to 10 pA. Taken together, the data of Fig. 7, A and B suggest that lumenal-to-cytosolic Ca\(^{2+}\) fluxes can regulate the cardiac CRC in the presence of physiologically relevant concentrations of Mg\(^{2+}\) and ATP.

**DISCUSSION**

The results of this study suggest that lumenal Ca\(^{2+}\) flowing through the open cardiac Ca\(^{2+}\) release channel can regulate the channel by having access to cytosolic activation and inactivation sites. Activation in the presence of Mg\(^{2+}\) and ATP suggests that regulation of CRC by lumenal Ca\(^{2+}\) fluxes may be physiologically relevant.
served in the absence of caffeine provided sufficiently high potentials and with varying Ca$^{2+}$ were recorded in symmetric KCl media at different holding potentials with cytosolic [Ca$^{2+}$]o of 200 μM, 500 μM, 1 mM, 5 mM, and 10 mM (from left to right) as described in Figs. 5 and 6, respectively. Values are the mean ± SE of three experiments (A) and three to nine experiments (B). *Significantly different from lumenal Ca$^{2+}$ of 0.003 pA (B).

FIGURE 7 Single channel parameters of cytosolic and lumenal 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 lumenal Ca$^{2+}$ concentrations of 4 μM, 200 μM, 500 μM, 1 mM, 5 mM, and 10 mM (from left to right) as described in Figs. 5 and 6, respectively. Values are the mean ± SE of three experiments (A) and three to nine experiments (B). *Significantly different from lumenal Ca$^{2+}$ flux of 0.003 pA (B).

Regulation of cardiac CRC activity by cytosolic and lumenal Ca$^{2+}$

To distinguish between the effects of SR cytosolic and lumenal Ca$^{2+}$ on channel activity, single purified channels were recorded in symmetric KCl media at different holding potentials and with varying Ca$^{2+}$ concentrations in the trans (SR lumenal) 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 lumenal, but not cytosolic, Ca$^{2+}$. A voltage-dependent activation by lumenal Ca$^{2+}$ was observed in the absence of caffeine provided sufficiently high cytosolic [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 lumenal Ca$^{2+}$. In the absence of caffeine and with cytosolic [Ca$^{2+}$] of <0.1 μM in the presence (Fig. 7) and absence (Fig. 4) of 5 mM MgATP, the cardiac CRC rarely opened. Under these recording conditions, lumenal [Ca$^{2+}$] as high as 10 mM was not able to significantly activate the channel. In agreement with this finding, cellular SR lumenal [Ca$^{2+}$], which is thought to be close to 1 mM, does not activate the “closed” CRC. As in cells, where Ca$^{2+}$ ions entering the cells activates the cardiac CRC, the presence of a cytosolic activator such as Ca$^{2+}$ or caffeine was required before an activation and inactivation of the CRC by lumenal Ca$^{2+}$ could be observed. Lack of an activation of the “closed” CRC by lumenal Ca$^{2+}$ argues against a low-affinity Ca$^{2+}$ regulatory site that resides on the lumenal site of the channel.

The lumenal-to-cytosolic 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 >±80 mV. Recently, the flow of K$^+$ through cardiac CRC has been modeled by diffusion theory using a combination of the Nernst-Plank and Poisson (PNP) equations (Chen et al., 1997). The model predicts a high K$^+$ concentration (~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 Ca$^{2+}$. Tinker et al. (1992) measured and modeled ion conductances in bionic and mixed solutions, including Ca$^{2+}$, Mg$^{2+}$, and K$^+$. We directly measured Ca$^{2+}$ and 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 lumenal Ca$^{2+}$

The extent of CRC activation by lumenal Ca$^{2+}$ was dependent on the presence of Ca$^{2+}$, MgATP, and caffeine in the cytosolic (cis) chamber of the bilayer apparatus. In agreement with observations of an increased Ca$^{2+}$ affinity of Ca$^{2+}$ activation sites by caffeine (Zucchi and Ronca-Testoni, 1997; Liu et al., 1998), channels could be more effectively activated at lower lumenal Ca$^{2+}$ fluxes in the presence of caffeine (100 μM lumenal Ca$^{2+}$ at −50 mV corresponds to lumenal Ca$^{2+}$ flux of 0.6 pA, Fig. 2 B) than in the absence of caffeine (200 μM lumenal Ca$^{2+}$ at −35 mV corresponds to lumenal Ca$^{2+}$ flux of 0.8 pA, Fig. 4 B). Addition of 5 mM cytosolic MgATP increases the Hill constant of Ca$^{2+}$ activation by cytosolic Ca$^{2+}$ by 3–4-fold (Xu et al., 1996; Fig. 6). In reasonable agreement with this result, CRCs were activated by lower lumenal Ca$^{2+}$ fluxes in the absence of MgATP (0.8 pA in Fig. 4 B; in Fig. 6, 200 μM lumenal Ca$^{2+}$ at −50 mV corresponds to lumenal Ca$^{2+}$ flux of 1.0 pA).
Lumenal Ca\(^{2+}\) fluxes lead to the buildup of a high cytosolic Ca\(^{2+}\) concentration near the release sites (Stern, 1992; Fig. 8), which raised the possibility that lumenal Ca\(^{2+}\) fluxes inactivated the channels before they could be fully activated. We tested this idea using the “fast” Ca\(^{2+}\)-complexing buffer BAPTA. Because of its high association rate BAPTA can suppress the rise in 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 [Ca\(^{2+}\)] (Fig. 3 A, top panel), thus supporting the idea that lumenal Ca\(^{2+}\) fluxes cannot only activate but also inactivate the cardiac CRC. Channel activation by cytosolic effectors was required to observe the effects of lumenal 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 lumenal 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 Ca\(^{2+}\) concentrations of ≤0.1 μM.

**Kinetics of CRC activation and inactivation by cytosolic and lumenal Ca\(^{2+}\)**

Kinetics of cytosolic Ca\(^{2+}\)-mediated channel activation and inactivation are, at least in principle, more straightforward than those by lumenal Ca\(^{2+}\) and will therefore be discussed first. At low cytosolic 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 Ca\(^{2+}\) concentration indicated that cytosolic Ca\(^{2+}\) increased \(P_\text{o}\) by increasing the transition rates from the closed to open state(s). A second effect of increasing cytosolic [Ca\(^{2+}\)] was to increase the mean open times. 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 Ca\(^{2+}\) to the tetrameric channel complex. An increase in open times by cytosolic Ca\(^{2+}\) was also observed for the sheep cardiac CRC (Sitsapesan and Williams, 1994b). This increase was explained by assuming a Ca\(^{2+}\)-dependent pathway between two open states. High 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 Ca\(^{2+}\) decreased \(P_\text{o}\) 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 Ca\(^{2+}\) binding to the 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, lumenal Ca\(^{2+}\) is only available to cytosolic Ca\(^{2+}\) regulatory sites when a channel opens.

Ca\(^{2+}\) gradients formed by Ca\(^{2+}\) fluxes build up and dissipate in ~50 μs as channels open and close (Simon and Llinas, 1985). Accordingly, cytosolic Ca\(^{2+}\) gradients formed by lumenal Ca\(^{2+}\) fluxes likely had a lifetime that was less than that of the shortest channel events seen in the bilayers (~0.2 ms). One would then expect that the frequency of channel openings is set mainly by the spatial gradients formed by Ca\(^{2+}\) and MgATP concentrations, while lumenal Ca\(^{2+}\) does not noticeably affect the number of events and duration of mean closed events. In agreement with this prediction, lumenal Ca\(^{2+}\) did not significantly affect the number of channel events and duration of mean closed events. Much of the increase in \(P_\text{o}\) observed for lumenal 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 Ca\(^{2+}\) gradient because a similar prolongation in mean open times was observed with increasing cytosolic [Ca\(^{2+}\)] (Figs. 3 A and 7 A, third panels). We conclude that lumenal Ca\(^{2+}\) ions flowing through open channels may increase the duration of channel open events by elevating cytosolic [Ca\(^{2+}\)] at 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 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 × 29 ×
12 nm cytosolic “foot” region and a smaller transmembrane region that extends ~7 nm toward the SR lumen and likely contains a centrally located 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 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 Ca^{2+} regulatory sites on the large cardiac CRC complex, our data can provide tentative information with respect to their distance from the cytosolic Ca^{2+} release site. The cytosolic Ca^{2+} concentration profiles that were obtained at luminal Ca^{2+} fluxes of 0.1 pA and 3 pA are included in Fig. 8. Also indicated in Fig. 8 is the cytosolic Ca^{2+} concentration (1 μM, dotted line) that resulted in half-maximum activation of CRCs in the presence of 10 mM caffeine. By comparison, 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 Ca^{2+} concentration profiles that were obtained at luminal Ca^{2+} fluxes of 0.1 pA and 3 pA are included in Fig. 8. Also indicated in Fig. 8 is the cytosolic Ca^{2+} concentration (1 μM, 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 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 ≥20 nm between the 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 Ca^{2+}-activating concentrations and calculated effects of luminal Ca^{2+} fluxes was obtained for the skeletal muscle CRC (Tripathy and Meissner, 1996). To explain the paradox, skeletal muscle cytosolic 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 Ca^{2+} inactivation sites see a major portion of luminal Ca^{2+}. We propose a similar model for the cardiac CRC. The model suggests that luminal Ca^{2+} fluxes increase Ca^{2+} concentrations to a lesser extent at the Ca^{2+} activation than Ca^{2+} inactivation sites, thus explaining that, as observed in the present study, Ca^{2+} inactivation sites in the cardiac CRC can be fully activated by luminal Ca^{2+}.

The distance between the Ca^{2+} release and Ca^{2+} inactivation sites of the cardiac CRC was estimated as follows. Fig. 2 C shows that channels activated by 4 μM cytosolic Ca^{2+} in the presence of 10 mM caffeine were half-maximally inactivated at a luminal Ca^{2+} flux of ~10 pA. This flux resulted in a half-maximally inactivating cytosolic Ca^{2+} concentration of 10 mM (Fig. 1 B) at a distance of ~3 nm from the release site (Fig. 8). Single channel measurements with the fast Ca^{2+}-complexing buffer BAPTA suggest that a distance of 3 nm between the release and Ca^{2+} inactivation sites may be an upper limit. BAPTA increased channel activities at a luminal Ca^{2+} flux of 3 pA to close a maximum value (Fig. 3 B). At a distance of 3 nm, a cytosolic [Ca^{2+}] of ~3 mM is calculated (Fig. 8), which appears to be too low to cause substantial Ca^{2+} inactivation (Fig. 1 B). Higher cytosolic [Ca^{2+}] exists closer to the release site (Fig. 8). However, placement of Ca^{2+} inactivation sites too close to the release site is problematic because it renders BAPTA ineffective in lowering [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 Ca^{2+} flux of 3 pA, a cytosolic [Ca^{2+}] of ~9 mM is calculated, which is lowered by 20 mM BAPTA to ~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 Ca^{2+} inactivation site(s) lie(s) at a distance of ≤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 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 (Ca^{2+} channels, L-type), which provides a morphological basis for the Ca^{2+}-induced Ca^{2+} release (CICR) mechanism (Carl et al., 1995). Recent studies suggest that the opening of a single L-type Ca^{2+} channel may be sufficient to evoke a localized Ca^{2+} release event (“Ca^{2+} spark”) by activating one or more CRCs (Santana et al., 1996). During L-type Ca^{2+} channel opening the Ca^{2+} concentration can reach millimolar values (Langer and Peskoff, 1996), which are more than enough to activate closely apposed Ca^{2+} release channels. The present study shows that SR luminal Ca^{2+} can contribute to the regulation of cardiac SR Ca^{2+} release via direct feedback by binding to channels that release Ca^{2+}. In myocardium these events may involve more than one channel because, in addition to its own channel, luminal Ca^{2+} fluxes may activate and inactivate closely located Ca^{2+} release channels.

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