# Mechanism of Calmodulin Inhibition of Cardiac Sarcoplasmic Reticulum $Ca^{2+}$ Release Channel (Ryanodine Receptor)

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ABSTRACT The functional effects of calmodulin (CaM) on single cardiac sarcoplasmic reticulum $Ca^{2+}$ release channels (ryanodine receptors) (RyR2s) were determined in the presence of two endogenous channel effectors, MgATP and reduced glutathione, using the planar lipid bilayer method. Single-channel activities, number of events, and open and close times were determined at varying cytosolic $Ca^{2+}$ concentrations. CaM reduced channel open probability at $<10$ $\mu$M $Ca^{2+}$ by decreasing channel events and mean open times and increasing mean close times. At $>10$ $\mu$M $Ca^{2+}$, CaM was less effective in inhibiting RyR2. CaM decreased mean open times but increased channel events, without significantly affecting mean close times. A series of voltage pulses was applied to the bilayer from +50 to –50 mV and from –50 mV to +50 mV to rapidly increase and decrease open channel-mediated sarcoplasmic reticulum lumenal to cytosolic $Ca^{2+}$ fluxes. CaM decreased the duration of the open events after the voltage switch from –50 mV to +50 mV. In parallel experiments, a $Ca^{2+}$-insensitive calmodulin mutant was without effect on RyR2 activity. The results are discussed in terms of a possible role of CaM in the termination of cardiac sarcoplasmic reticulum $Ca^{2+}$ release.

## INTRODUCTION

Cardiac muscle contracts during an action potential when the influx of extracellular $Ca^{2+}$ triggers the release of $Ca^{2+}$ from an intracellular $Ca^{2+}$-storing organelle, the sarcoplasmic reticulum (SR). $Ca^{2+}$ entry through voltage- and dihydropyridine-sensitive L-type $Ca^{2+}$ channels (DHPRs) in the surface membrane and transverse tubule opens closely apposed SR $Ca^{2+}$ release channels (ryanodine receptors) (RyR2s), a process termed calcium-induced calcium release (Fabiato, 1985). $Ca^{2+}$-gated $Ca^{2+}$ release through the RyR2 ion channel is modulated by allosteric effectors such as MgATP, inhibitors such as $Mg^{2+}$, redox active molecules, protein kinases and phosphatases, and calmodulin (CaM) (Franzini-Armstrong and Protasi, 1997; Fill and Copello, 2002; Meissner, 2002).

CaM is a ubiquitous cytosolic $Ca^{2+}$-binding protein that modulates cellular events through calmodulin-dependent protein kinases or by direct binding of CaM (Rhoads and Friedberg, 1997). Direct binding of CaM inhibits the ryanodine receptors (Balshaw et al., 2002). RyR2 has one high-affinity binding domain that is shared by the $Ca^{2+}$-free and $Ca^{2+}$-bound forms of CaM (Yamaguchi et al., 2003). Binding and release of the $Ca^{2+}$-free and $Ca^{2+}$-bound forms of CaM occur within seconds to minutes (Balshaw et al., 2001). It is thus unlikely that CaM regulates cardiac SR $Ca^{2+}$ release by binding to or dissociating from the RyR2 during a cardiac action potential.

An unresolved question is how $Ca^{2+}$ induced $Ca^{2+}$ release from the SR is terminated during an action potential. During a cardiac action potential, $Ca^{2+}$ ions entering the cell via the L-type $Ca^{2+}$ channel trigger the release of massive amounts of $Ca^{2+}$ from the SR via the RyR2 and the release of $Ca^{2+}$ triggers further $Ca^{2+}$ release. Such a high-gain, positive feedback system is potentially unstable, resulting in a none-or-all response, in disagreement with experimental evidence of graded $Ca^{2+}$ release (Bassani et al., 1995; Negretti et al., 1995; Chen et al., 1998). Proposed mechanisms for terminating SR $Ca^{2+}$ release include $Ca^{2+}$-induced inactivation of RyR2 (Fabiato, 1985), depletion of SR $Ca^{2+}$ (Luo and Rudy, 1994), and the simultaneous closing of all active RyRs in a release unit to reduce the $Ca^{2+}$ concentration to a subthreshold activation level (Stern, 1992; Sobie et al., 2002).

In this study we tested the hypothesis that CaM facilitates the termination of cardiac SR $Ca^{2+}$ release by altering the kinetics of $Ca^{2+}$-gated RyR2 activity. The functional effects of CaM on the RyR2 ion channel were examined using the planar lipid bilayer technique. Single-channel activities and the kinetics of channel opening and closing were determined at varying cytosolic $Ca^{2+}$ concentrations in the absence and presence of CaM. A voltage-pulse protocol was used to determine the effects of CaM on RyR2s that were activated by open channel-mediated SR lumenal to cytosolic $Ca^{2+}$ fluxes. The results show that CaM inhibits RyR2 ion channel activity in a $Ca^{2+}$-dependent manner by modifying the transition rates of the open-to-close and close-to-open channel states.

## MATERIALS AND METHODS

### Materials

Phospholipids were obtained from Avanti Polar Lipids (Alabaster, AL). The cDNA encoding CaM was kindly provided by Dr. Claude Klee at the National Institutes of Health, Bethesda, MD. The $CaM_{D1234A}$ cDNA was a generous gift of Dr. John Adelman at Oregon Health Sciences University, Portland, OR. CaM and the non-$Ca^{2+}$ binding CaM mutant were expressed in *Escherichia coli* and purified as described (Balshaw et al., 2001). All other chemicals were of analytical grade.

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## Preparation of SR vesicles and purification of the RyR2

Canine cardiac SR vesicle fractions enriched in RyR2 were prepared in the presence of protease inhibitors (Balshaw et al., 2001). Endogenous CaM was removed by incubating SR vesicles for 30 min at 24°C with 1 $\mu$M myosin light chain kinase-derived calmodulin binding peptide in 100 $\mu$M $Ca^{2+}$ followed by centrifugation through 15% sucrose to remove complexed and free peptide (Balshaw et al., 2001). The 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)-solubilized 30S RyR2 complexes were 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 cardiac SR vesicles or proteoliposomes containing the purified RyR2 with Mueller-Rudin type bilayers containing phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine in the ratio 5:3:2 (25 mg of phospholipid per ml *n*-decane) (Xu and Meissner, 1998). The side of the bilayer to which the SR vesicles or proteoliposomes were added was defined as the *cis* side. The *trans* side was defined as ground. Single channels were recorded in solutions containing 0.25 M $CsCH_3SO_3$, 10 mM Cs-Hepes, pH 7.3 (SR vesicles), or 0.25 M KCl, 20 mM KHepes, pH 7.4 (purified RyR2) on both sides of the bilayer and additions as indicated. Electrical signals were filtered at 2 kHz (Figs. 1, 3 and 4) or 300 Hz (Fig. 2), digitized at 10 kHz, and analyzed as described (Xu and Meissner, 1998). Free $Ca^{2+}$ concentrations were obtained by including 0.5–1 mM EGTA and levels of $Ca^{2+}$ as determined by a computer program (Schoenmakers et al., 1992). Free $Ca^{2+}$ concentrations of $\geq 1$ $\mu$M were verified with the use of a $Ca^{2+}$ selective electrode (detectION, Philadelphia, PA) and those of $<1$ $\mu$M using Fluo-3.

In voltage pulse experiments, 500 $\mu$M *trans* $Ca^{2+}$ and 0.1 $\mu$M *cis* $Ca^{2+}$ and a series of voltage pulses were used to rapidly increase or decrease open channel-mediated lumenal-to-cytosolic $Ca^{2+}$ fluxes (Xu and Meissner, 1998). Large capacitance currents occurring during voltage pulses were

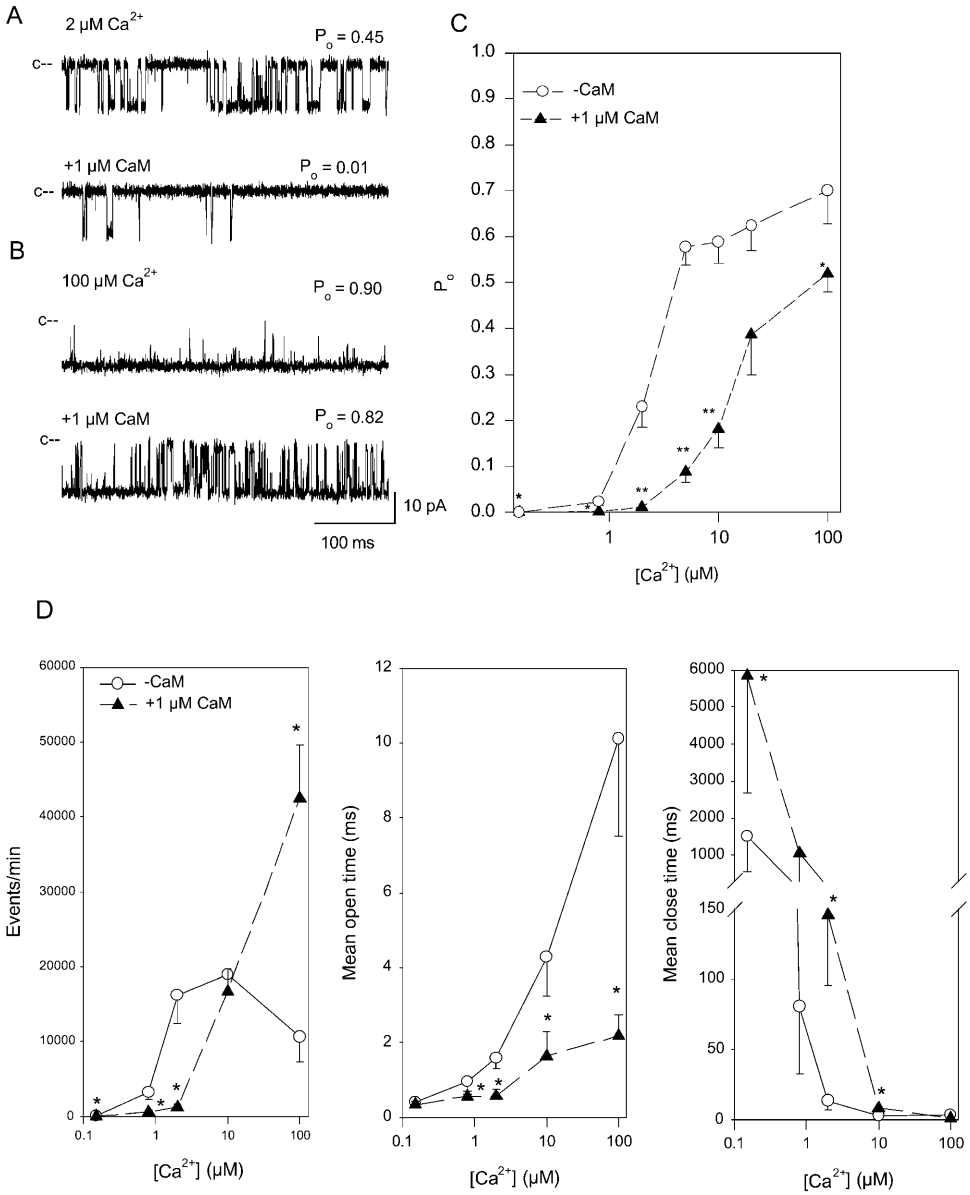


FIGURE 1 Effect of calmodulin on single cardiac SR $Ca^{2+}$ release channel with $Cs^+$ as current carrier. Cardiac SR vesicles were fused with a lipid bilayer and single-channel currents were recorded at −35 mV (downward deflection from close levels, *c*--) in symmetric 0.25 M $CsCH_3SO_3$, 10 mM Cs-Hepes, pH 7.3 media containing 2 $\mu$M (*A*) or 100 $\mu$M (*B*) free cytosolic $Ca^{2+}$, 5 mM cytosolic GSH, and 5 mM MgATP in the absence (*top traces*) and presence (*bottom traces*) of 1 $\mu$M cytosolic CaM. *Trans* (SR lumenal) $Ca^{2+}$ was 2 $\mu$M. (*C*) Effect of CaM on cardiac $Ca^{2+}$ release channel activity at the shown cytosolic $Ca^{2+}$ concentrations without (○) or with (▲) 1 $\mu$M CaM. Data are the mean ± SE of 5–16 experiments. (*D*) Effect of CaM on single-channel kinetic parameters at 0.15–100 $\mu$M cytosolic $Ca^{2+}$. Data are the mean ± SE of 3–11 experiments. * and ** significantly different from controls (−CaM) at $p < 0.05$ and $p < 0.001$, respectively.

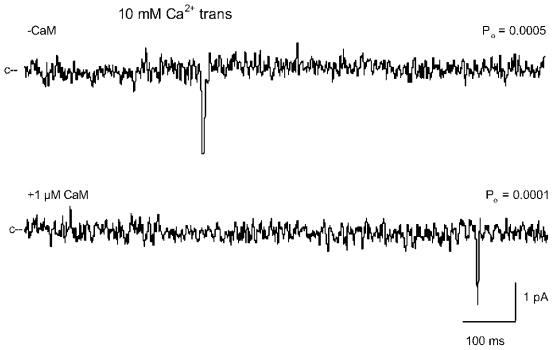


FIGURE 2 Effect of calmodulin on single cardiac SR $Ca^{2+}$ release channel with $Ca^{2+}$ as current carrier. Cardiac SR vesicles were fused with a lipid bilayer, and single-channel currents were recorded at 0 mV (downward deflection from close levels, *c--*) in symmetric 0.25 M $CsCH_3SO_3$, 10 mM Cs-Hepes, pH 7.3 media containing 5 mM cytosolic GSH, 5 mM MgATP, and 0.15 $\mu$M cytosolic $Ca^{2+}$ in the absence (*top trace*) and presence (*bottom trace*) of 1 $\mu$M CaM. *Trans* (SR lumenal) $Ca^{2+}$ was 10 mM.

corrected by subtracting those of blank (no channel openings) episodes and ''nulled out'' using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) with an integrative headstage.

### Data analysis

Results are given as mean ± SE. Significance of differences in the data was analyzed with Student's *t*-test. Differences were regarded to be statistically significant at $p < 0.05$.

## RESULTS

### CaM inhibition of single RyR2 channels activated by cytosolic $Ca^{2+}$

Functional effects of CaM on the cardiac $Ca^{2+}$ release channel (RyR2) were examined using the planar lipid bilayer method. Cardiac SR vesicles enriched in RyR2 were fused with the lipid bilayer. Single-channel activities and the kinetics of channel opening and closing were determined at varying cytosolic $Ca^{2+}$ concentrations in the absence and presence of a maximally inhibiting concentration of 1 $\mu$M CaM (Balshaw et al., 2001) under steady-state conditions.

Fig. 1 *A* shows a representative single RyR2 ion channel recording in the absence (*upper trace*) and presence (*lower trace*) of 1 $\mu$M CaM at 2 $\mu$M free $Ca^{2+}$ in the *cis* (cytosolic) bilayer chamber. The free *trans* (SR lumenal) $Ca^{2+}$ concentration was 2 $\mu$M. Channels were recorded in a cesium methanesulfonate buffer to minimize $K^+$ and $Cl^-$ channel activities also present in the SR (Meissner, 1983). Use of $Cs^+$ rather than $Ca^{2+}$ as a current carrier allowed tight control of RyR2 by cytosolic $Ca^{2+}$. Experiments were performed in the presence of two endogenous effector molecules, MgATP and reduced glutathione (GSH), that affect regulation of RyR2 by CaM (Balshaw et al., 2001). Addition of CaM to the *cis* chamber reduced single-channel open probability ($P_o$) from 0.45 to 0.01. Elevation of cytosolic $Ca^{2+}$ from 2 to 100 $\mu$M increased $P_o$ from 0.45 to 0.90 (Fig. 1 *B*, *upper trace*). Under this condition, CaM was less effective in inhibiting single RyR2 channel activity (Fig. 1 *B*, *lower trace*). Fig. 1 *C* summarizes the effects of CaM on $P_o$ at 0.15–100 $\mu$M free cytosolic $Ca^{2+}$. Under steady-state conditions in the presence of 5 mM reduced GSH and 5 mM MgATP, $P_o$ increased from 0.0007 at 0.15 $\mu$M $Ca^{2+}$ to 0.7 at 100 $\mu$M $Ca^{2+}$. CaM inhibition of RyR2 channel activity depended on $Ca^{2+}$ concentration. CaM reduced the averaged $P_o$ 25-fold from 0.25 to 0.01 at 2 $\mu$M cytosolic $Ca^{2+}$, compared to a 1.25-fold decrease from 0.7 to 0.56 at 100 $\mu$M $Ca^{2+}$.

Analysis of single-channel recordings showed that at cytosolic $[Ca^{2+}] \leq 2$ $\mu$M, CaM reduced channel open probability by decreasing the number channel events and mean open times and by increasing mean close times (Fig. 1 *D*). At 2 $\mu$M $Ca^{2+}$, CaM decreased the average mean open time 2.7-fold from 1.6 ms to 0.6 ms. The average mean close time increased more than 10-fold from 13.6 to 146 ms. At 100 $\mu$M $Ca^{2+}$, CaM had a moderate effect on $P_o$. Although the average mean open time decreased fivefold from 10 ms to 2 ms, channel events increased fourfold. Taken together, the data indicate that the effects of CaM on RyR2 ion channel opening and closing depend on the cytosolic $Ca^{2+}$ concentration.

The SR membrane is highly permeable to monovalent cations and anions, which suggests that the SR membrane potential in resting muscle is near 0 mV (Meissner, 1983). The effects of CaM on RyR2 were therefore also determined at 0 mV holding potential using 10 mM lumenal $Ca^{2+}$ as current carrier. Fig. 2 (*upper trace*) shows a representative RyR2 ion channel recording in which in the presence of 0.15 $\mu$M cytosolic $Ca^{2+}$ long channel closings were observed. Addition of 1 $\mu$M CaM decreased $P_o$ fivefold (Fig. 2, *lower trace*). Table 1 summarizes the effects of 1 $\mu$M CaM on single-channels recorded with 10 mM $Ca^{2+}$ as current carrier in the presence of 0.15 $\mu$M and 0.8 $\mu$M cytosolic $Ca^{2+}$. The data suggest that CaM inhibited the channels by decreasing the number of channel events and mean open times and increasing the mean close times; <1 channel opening per 2 s recording time was detected at 0.15 $\mu$M cytosolic $Ca^{2+}$. Addition of 1 $\mu$M cytosolic CaM decreased this number to <1 event per 5 s recording time. Thus CaM inhibited RyR2 independent of whether channels were activated by cytosolic $Ca^{2+}$ or by way of lumenal-to-cytosolic $Ca^{2+}$ fluxes.

### Effects of CaM on RyR2 activated by open channel-mediated lumenal $Ca^{2+}$ fluxes

We used a voltage-pulse protocol to study the effects of CaM on RyR2 ion channels that were activated by open channel-mediated lumenal-to-cytosolic $Ca^{2+}$ fluxes. The protocol relied on the observation that lumenal $Ca^{2+}$ flowing through the open channel regulates channel activity by binding to cytosolic $Ca^{2+}$ activating and inhibitory sites (Xu and

Meissner, 1998). In these studies we used proteoliposomes containing the purified RyR2. Fusion of proteoliposomes with the lipid bilayer yielded a stable baseline and small error when subtracting the large capacitance currents occurring during voltage pulses. Capacitance currents were obtained from blank episodes that showed no channel currents. To optimize the conditions for the measurement of RyR2 in nonsteady-state conditions, the regulation of the purified RyR2 by lumenal $Ca^{2+}$ and cytosolic CaM was first determined in steady-state conditions (Fig. 3). Channels were recorded in 250 mM KCl on both sides of the bilayer with 5 mM MgATP, 5 mM GSH, and 0.1 $\mu$M $Ca^{2+}$ in the *cis*, cytosolic bilayer chamber, and 0.1 $\mu$M or 500 $\mu$M $Ca^{2+}$ in the *trans*, SR lumenal bilayer chamber. The *cis* chamber also contained 10 mM caffeine to partially activate the channels. Holding potentials of −50 mV and +50 mV were used to yield open channel-mediated lumenal-to-cytosolic $Ca^{2+}$ fluxes of 1.7 pA and 0.02 pA, respectively, with 500 $\mu$M $Ca^{2+}$ in the lumenal bilayer chamber, as calculated according to a barrier model that describes the ionic conduction of the RyR2 (Tinker et al., 1992). Fig. 3 *A* shows that with 0.1 $\mu$M $Ca^{2+}$ in the *trans* chamber, $P_o$ was low and did not differ substantially at −50 mV and +50 mV. In contrast, single-channel activities differed greatly at the two holding potentials when the lumenal $Ca^{2+}$ concentration was increased from 0.1 to 500 $\mu$M (Fig. 3 *C*). The average single-channel activity increased by >50-fold from 0.004 ± 0.002 at +50 mV to 0.255 ± 0.085 at −50 mV ($n = 7$). The RyR2 ion channel was significantly inhibited by CaM at –50 mV and +50 mV with 0.1 $\mu$M $Ca^{2+}$ (Fig. 3, *A* and *B*) or 500 $\mu$M $Ca^{2+}$ (Fig. 3, *C* and *D*) in the *trans* chamber. CaM increased close times and decreased open times and number of channel events. Although small, a majority of the differences were statistically significant (Fig. 3, *B* and *D*).

A representative RyR2 channel recording is shown in Fig. 4 using the voltage pulse protocol, 0.1 $\mu$M or 500 $\mu$M $Ca^{2+}$ in the *trans* bilayer chamber, and otherwise the conditions of Fig. 3. A series of voltage pulses was applied to the bilayer from +50 to −50 mV and from −50 mV to +50 mV (Fig. 4 *A*) to repeatedly vary lumenal-to-cytosolic $Ca^{2+}$ fluxes and thereby local cytosolic $Ca^{2+}$ concentration and RyR2 activity (Fig. 4 *C*). In the absence of CaM and presence of 500 $\mu$M lumenal $Ca^{2+}$, after a mean lag period of 100 ± 4 ms (Fig. 4 *D*), voltage pulses from +50 mV to −50 mV caused nearly full activation of the RyR2 ion channel (Fig. 4 *C*, *upper single-channel current traces*). The mean lag period of channel opening significantly increased to 162 ± 4 ms in the presence of 1 $\mu$M cytosolic CaM. In eight recordings, the portion of episodes without channel openings significantly increased from 15.7 ± 8.5% in the absence of CaM to 28.0 ± 9.0% in the presence of CaM. Long channel openings or an increase in channel activity after a voltage switch were not observed in the presence of 0.1 $\mu$M $Ca^{2+}$ in the *trans* bilayer chamber (Fig. 4 *B*). Voltage pulses from −50 mV to +50 mV decreased the driving force of lumenal $Ca^{2+}$ and reduced lumenal-to-cytosolic $Ca^{2+}$ fluxes. In this case with 500 $\mu$M $Ca^{2+}$ in the *trans* chamber, a prolonged channel opening was observed immediately after the voltage switch (Fig. 4 *C*, *upper single-channel current traces*). Prolonged channel openings were only observed in episodes that showed a long opening immediately before the voltage switch. CaM (1 $\mu$M) significantly decreased the mean open time of these open events from 25.4 ± 1.1 ms to 12.5 ± 0.5 ms (Fig. 4 *E*). Subsequent channel events had open times that were similar to those observed under steady-state conditions ($T_o \sim$ 1–2 ms, Fig. 3 *C*). RyR2 channel activation or inhibition showed no refractoriness when the duration of the voltage pulses was varied from 150 ms to 1000 ms. Taken together, results of Fig. 4 suggest that CaM shortens the lifetimes of channel openings formed by lumenal-to-cytosolic $Ca^{2+}$ fluxes.

To further delineate the $Ca^{2+}$ dependence of CaM inhibition of RyR2, experiments were performed with a non-$Ca^{2+}$ binding mutant of CaM ($CaM_{D1234A}$, Keen et al., 1999). The binding affinity of the CaM mutant was determined at 0.1 $\mu$M $Ca^{2+}$ by measuring its ability to compete with 10–30 nM [$^{35}$S]CaM for binding to RyR2 using an equilibrium displacement binding assay. The data yielded a $K_d$ value of 820 ± 224 nM ($n = 4$). The functional effects of the CaM mutant on RyR2 were explored in single-channel recordings. We found that 2 $\mu$M $CaM_{D1234A}$ had no effect on single RyR2 channel activities using assay conditions identical to those of Figs. 1 *A* and 4.

**TABLE 1 Effect of CaM on single-channel kinetic parameters with $Ca^{2+}$ as current carrier**

| | 0.15 $\mu$M $Ca^{2+}$ | | 0.8 $\mu$M $Ca^{2+}$ | |
|---|---|---|---|---|
| | −CAM | +CaM | −CaM | +CaM |
| $P_o$ | 0.00034 ± 0.00014 | 0.00008 ± 0.00002* | 0.050 ± 0.007 | 0.0006 ± 0.0002* |
| Events/min | 29 ± 4 | 11 ± 3* | 163 ± 32 | 32 ± 12* |
| $T_o$ (ms) | 0.68 ± 0.18 | 0.68 ± 0.29 | 4.57 ± 0.29 | 3.36 ± 0.40* |
| $T_c$ (ms) | 2023 ± 273 | 9103 ± 3272 | 831 ± 195 | 5579 ± 1750* |

Single-channel activities were determined as in Fig. 2 with 0.15 $\mu$M and 0.8 $\mu$M $Ca^{2+}$ in the *cis* chamber. Data are the mean ± SE of 4–6 single-channel recordings.

*$p < 0.05$ compared to controls (−CaM).

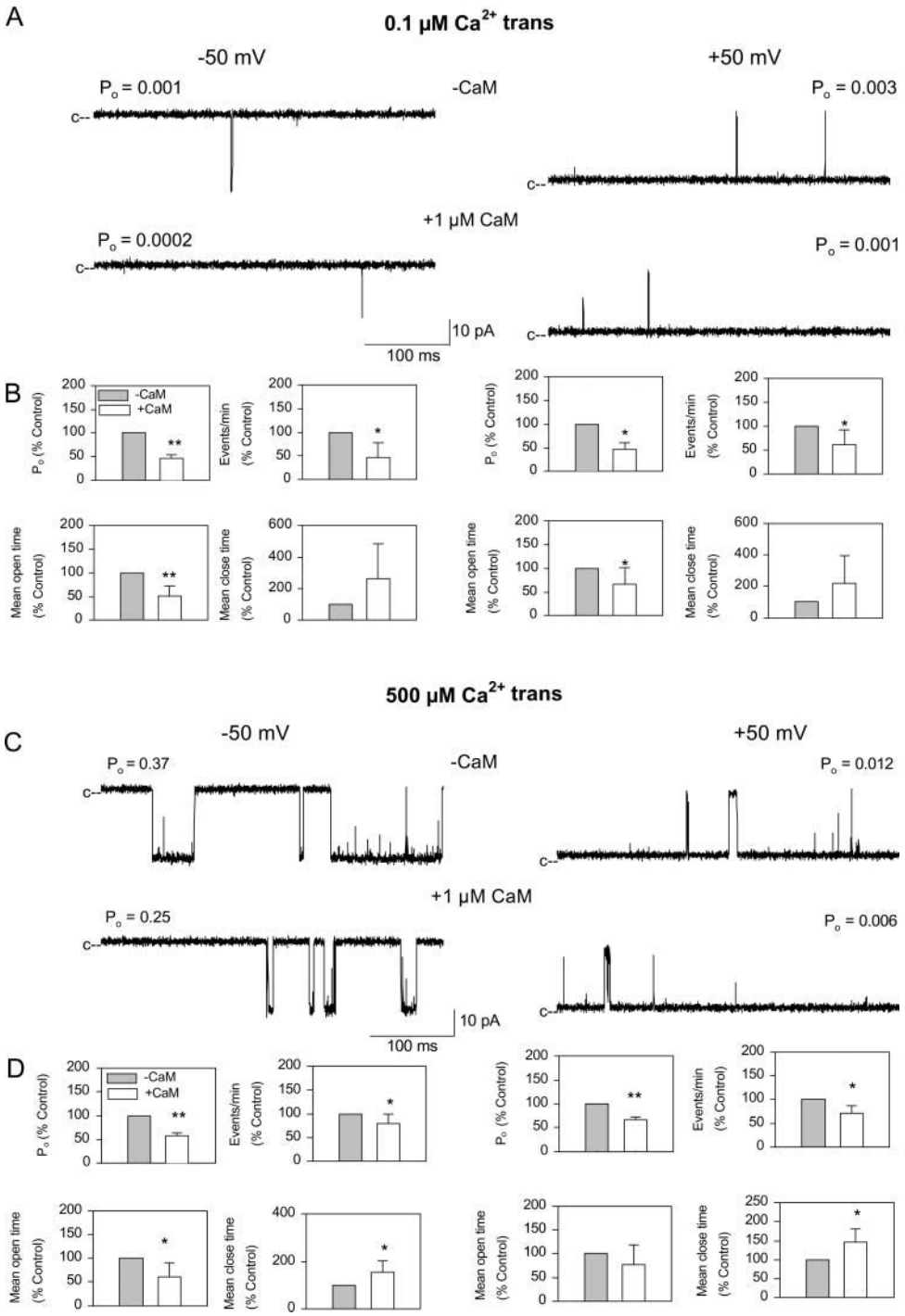


FIGURE 3 Single-channel activities and inhibition by CaM in 0.1 $\mu$M lumenal $Ca^{2+}$ (*A* and *B*) and 500 $\mu$M lumenal $Ca^{2+}$ (*C* and *D*). Proteoliposomes containing purified RyR2 were fused with a lipid bilayer. Single-channel currents were recorded at –50 mV (*left panels*) and +50 mV (*right panels*) in symmetric 0.25 M KCl media with 5 mM cytosolic GSH, 5 mM MgATP, 10 mM caffeine (an activator of the $Ca^{2+}$ release channel), and 0.1 $\mu$M $Ca^{2+}$ with either 0.1 $\mu$M lumenal $Ca^{2+}$ (*A*) or 500 $\mu$M lumenal $Ca^{2+}$ (*C*) in the absence (*top traces*) and presence (*bottom traces*) of 1 $\mu$M CaM. (*B*) Normalized $P_o$ values, number of channel events, and mean open ($T_o$) and close ($T_c$) times at −50 mV and +50 mV for *A*. Averaged control parameters (−CaM) were in (*A*) at −50 mV: $P_o = 0.002 \pm 0.001$, events/min $= 174 \pm 109$, $T_o = 1.41 \pm 0.17$ ms, $T_c = 2381 \pm 1233$ ms; at +50 mV: $P_o = 0.008 \pm 0.003$, events/min $= 435 \pm 166$, $T_o = 1.35 \pm 0.26$ ms. $T_c = 1110 \pm 756$ ms. (*D*) Normalized parameters for *C*. Averaged control parameters (−CaM) for *C* were at −50 mV: $P_o = 0.255 \pm 0.085$, events/min $= 431 \pm 233$, $T_o = 134 \pm 51$ ms, $T_c = 1167 \pm 627$ ms; at +50 mV: $P_o = 0.004 \pm 0.002$, events/min $= 179 \pm 100$, $T_o = 2.13 \pm 0.44$ ms, $T_c = 1316 \pm 325$ ms. Data are the mean ± SE of seven experiments. $*p < 0.05$ and $**p < 0.001$ compared to normalized data in the absence of CaM.

## DISCUSSION

This study shows that CaM inhibits $Ca^{2+}$ release channel activity both when the channels are activated by cytosolic $Ca^{2+}$ or by lumenal-to-cytosolic $Ca^{2+}$ fluxes. At $<10$ $\mu$M cytosolic $Ca^{2+}$, CaM decreased channel open probability by decreasing the frequency of channel events and mean open times and by increasing mean close times. At $\geq 10$ $\mu$M $Ca^{2+}$, CaM was less effective in reducing channel activity, decreasing mean open times without affecting mean close times. CaM delayed channel openings when luminal-to-cytosolic $Ca^{2+}$ fluxes were rapidly increased. In contrast, channel closure was accelerated by CaM when lumenal-to-cytosolic $Ca^{2+}$ fluxes decreased.

RyR2 is activated by micromolar $Ca^{2+}$ concentrations and inhibited by millimolar $Ca^{2+}$ concentrations, which suggests the presence of high-affinity activating and low-affinity

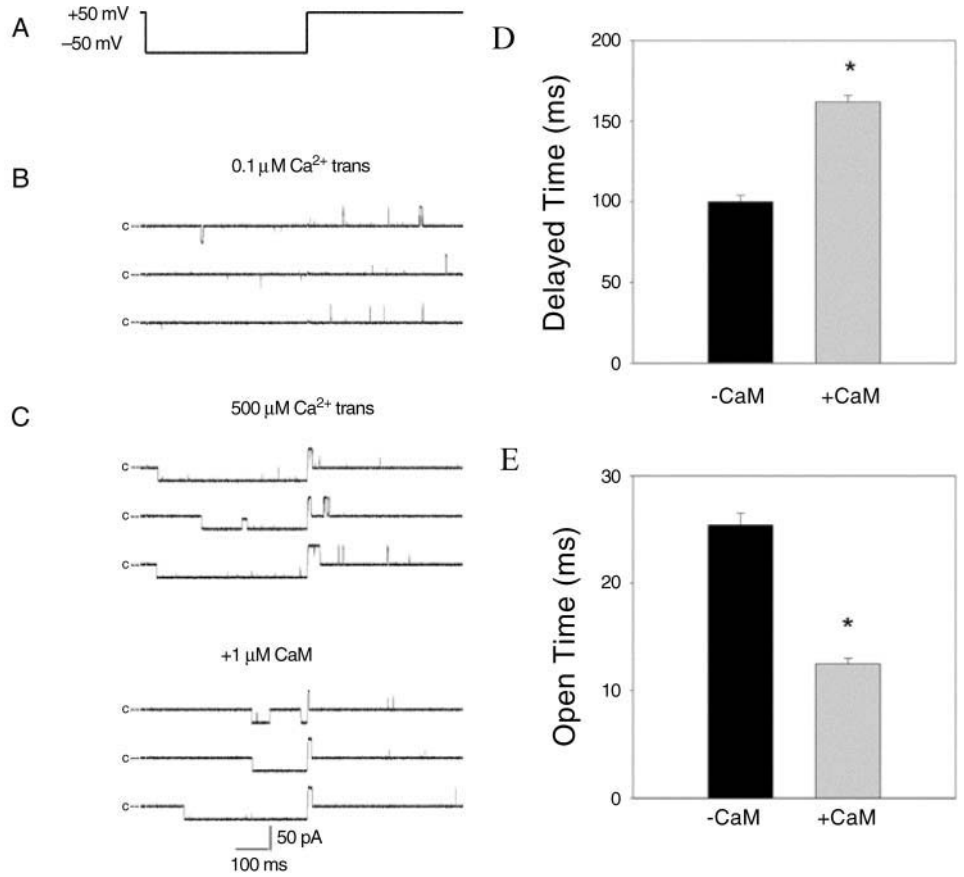


FIGURE 4 Regulation of RyR2 by CaM using a rapid voltage pulse protocol. A representative purified RyR2 ion channel recording in the absence and presence of 1 $\mu$M *cis* CaM in a 0.25 M KCl, pH 7.4 medium that contained 0.1 $\mu$M free $Ca^{2+}$, 10 mM caffeine, 5 mM GSH, and 5 mM MgATP in the cytosolic (*cis*) chamber, and 0.1 $\mu$M (*B*) or 500 $\mu$M (*C*) $Ca^{2+}$ in the SR lumenal (*trans*) chamber. (*A*) A series of 300 voltage pulses from + 50 mV to −50 mV and from −50 mV to +50 mV was applied to the bilayer to increase and reduce open channel-mediated lumenal to cytosolic $Ca^{2+}$ fluxes, respectively. (*B*) Three episodes in the presence of 0.1 $\mu$M *trans* $Ca^{2+}$ without added CaM are shown. (*C*) Three episodes each of channel currents in the presence of 500 $\mu$M *trans* $Ca^{2+}$ without (*upper current traces*) and with 1 $\mu$M CaM (*lower current traces*) are shown. Channel openings are shown as downward (−50 mV) or upward (+50 mV) deflections from close levels (marked *c*). (*D*) Mean times it took to observe the first channel opening after the voltage switch from +50 mV to −50 mV in the absence and presence of 1 $\mu$M CaM. Data are the mean ± SE of 1264 (−CaM) and 1169 (+CaM) episodes of eight recordings. (*E*) Mean open times of single-channel events immediately after the voltage switch from −50 mV to +50 mV in the absence and presence of 1 $\mu$M CaM. Data are the mean ± SE of 895 (−CaM) and 648 (+CaM) episodes of eight recordings. *$p < 0.05$ compared to controls (−CaM).

inhibitory $Ca^{2+}$ binding sites (Franzini-Armstrong and Protasi, 1997; Fill and Copello, 2002; Meissner, 2002). Both types of $Ca^{2+}$ binding sites are accessible from the cytosolic side in the large cytosolic foot region of RyRs. Single-channel measurements show that SR lumenal $Ca^{2+}$ also regulates RyR2 by binding to lumenal channel sites (Sitsapesan and Williams, 1997; Gyorke and Gyorke, 1998) or by gaining access to cytosolic $Ca^{2+}$ activation and inhibition sites after passage to the cytosolic receptor side (Xu and Meissner, 1998). $Ca^{2+}$-gated RyR2 ion channel activity is modulated by allosteric factors such as protein phosphorylation, MgATP, redox active molecules, and CaM (Franzini-Armstrong and Protasi, 1997; Fill and Copello, 2002; Meissner, 2002). In this study, single-channel recordings were performed in the presence of MgATP and reduced GSH. In the presence of the two endogenous effector molecules, CaM affected the $Ca^{2+}$ dependence of [$^3$H]ryanodine binding similarly as in this study (Balshaw et al., 2001). However, the [$^3$H]ryanodine binding studies lacked the detailed kinetic information provided by the single-channel measurements of this study.

The results of this study support a previously proposed model (Xu and Meissner, 1998) in which lumenal $Ca^{2+}$ can activate the purified RyR2 only when the channel is open. Activation requires initial channel opening so that lumenal $Ca^{2+}$ can gain access to the cytosolic regulatory $Ca^{2+}$ sites. Therefore, the delay after a change in holding potential from +50 mV to –50 mV reflected the fact that at the positive holding potential, channels opened infrequently under the conditions used in this study. CaM delayed channel activation by decreasing the frequency of channel openings, as determined in steady-state conditions, which suggests that CaM may affect the occurrence of cellular $Ca^{2+}$ release events called $Ca^{2+}$ sparks that arise from the spontaneous openings of RyR2s. However, it should be noted that the activating conditions used in this study are not the same as those during a cardiac action potential in cardiomyocytes. Rather than by SR lumenal $Ca^{2+}$, cardiac SR $Ca^{2+}$ release is triggered by $Ca^{2+}$ ions that enter the cells by way of the L-type $Ca^{2+}$ channel.

$Ca^{2+}$ gradients formed by lumenal-to-cytosolic $Ca^{2+}$ fluxes dissipate within ~100 $\mu$s as channels close (Simon and Llinas, 1985; Stern, 1992). One would have then expected that channels closed rapidly as the lumenal-to-cytosolic $Ca^{2+}$ flux was reduced by a voltage change from −50 mV to +50 mV. However, the open events immediately after the voltage switch were much longer than those at +50 mV in steady-state conditions. Thus, a prolonged lumenal to cytosolic $Ca^{2+}$ flux preceding the voltage switch appeared to affect the time course of channel closure without eliminating

CaM inhibition. CaM binds to and is released from RyR2 within seconds to minutes. Therefore, CaM affected channel activity by remaining constitutively bound to RyR2. Inhibition appeared to depend on the binding of $Ca^{2+}$-sensitive CaM because a non-$Ca^{2+}$ binding mutant of CaM was without effect.

During a cardiac action potential, $Ca^{2+}$ ions entering the cell via the L-type $Ca^{2+}$ channel trigger the release of massive amounts of $Ca^{2+}$ from the SR via the RyR2 and the release of $Ca^{2+}$ triggers further $Ca^{2+}$ release. Such a high-gain, positive feedback system is potentially unstable, resulting in a none-or-all response. Several mechanisms have been proposed for ending $Ca^{2+}$ release from SR. First, $Ca^{2+}$- and time-dependent inactivation terminates SR $Ca^{2+}$ release (Fabiato, 1985). This inactivation mechanism is challenged by single-channel and SR vesicle $Ca^{2+}$ flux measurements that show no $Ca^{2+}$- or time-dependent inhibition except a $Ca^{2+}$-dependent inhibition at nonphysiological $Ca^{2+}$ concentrations >1 mM (Rousseau et al., 1986). Second, $Ca^{2+}$ release is terminated because the supply of releasable $Ca^{2+}$ in the SR is exhausted. This mechanism requires ''local'' depletion of SR $Ca^{2+}$ because substantial amounts of $Ca^{2+}$ remain within the SR after a $Ca^{2+}$ transient (Bassani et al., 1995; Negretti et al., 1995; Chen et al., 1998). However, as recently reported, $Ca^{2+}$ ions rapidly diffuse from the free to junctional SR (Shannon et al., 2003). It is therefore unlikely that $Ca^{2+}$ release is limited by SR $Ca^{2+}$ availability. A third mechanism is that when the L-type $Ca^{2+}$ channel closes, SR $Ca^{2+}$ release is terminated locally through ''stochastic attrition'' (Stern, 1992). Modeling studies have shown that the simultaneous stochastic closing of a cluster of closely apposed release channels can reduce the local cytosolic $Ca^{2+}$ concentration to a subthreshold level, thereby ending SR $Ca^{2+}$ release (Stern, 1992; Sobie et al., 2002). RyR2s are organized in release units of ~100 release channels depending on the species (Franzini-Armstrong et al., 1999), which if all are activated would require the simultaneous closing of a large number of channels, however, as few as 4–6 ryanodine receptors may suffice to trigger a $Ca^{2+}$ release event ($Ca^{2+}$ spark) (Wang et al., 2001). In support of a simultaneous closing, Marx et al. (2001) have reported that RyR2 ion channels display synchronized openings and closings in lipid bilayers (coupled gating). The results of this study suggest that CaM may facilitate termination of SR $Ca^{2+}$ release when the local activator $Ca^{2+}$ concentration decreases to ~10 $\mu$M by a mechanism that remains to be resolved. We offer the following model. Our data suggest that during diastole at a myoplasmic $Ca^{2+}$ concentration of ~100 nM, the binding of CaM maintains the RyR2 in a close state. During an action potential, influx of $Ca^{2+}$ via the L-type $Ca^{2+}$ channel rapidly raises the $Ca^{2+}$ concentration at the $Ca^{2+}$ release sites to submillimolar values, resulting in the activation of the RyR2. During the initial release phase, SR $Ca^{2+}$ release is little affected by CaM because of a high local $Ca^{2+}$ activator concentration that is formed by $Ca^{2+}$ ions that both enter the cell and are released by the SR. On the other hand, our in vitro observations suggest that CaM may have a role in the termination of SR $Ca^{2+}$ release by reducing the release of $Ca^{2+}$ ions when the local activator $Ca^{2+}$ concentration decreases to ~10 $\mu$M, with maximal effects observed at low micromolar to submicromolar $Ca^{2+}$ concentrations. CaM increasingly prolongs the close channel times as the $Ca^{2+}$ concentration decreases, allowing $Ca^{2+}$ more time to diffuse away from the release sites and thereby reducing the probability of channel reopening.

CaM is likely only one of several factors that may have a role in the termination of SR $Ca^{2+}$ release. Other proteins reported to affect SR $Ca^{2+}$ release include calsequestrin (Szegedi et al., 1999), sorcin (Farrell et al., 2003; Seidler et al., 2003), and S100 (Most et al., 2003). Furthermore, CaM acts on other proteins that regulate SR $Ca^{2+}$ release such as the sarcolemmal voltage dependent $Ca^{2+}$ channel (DHPR), calmodulin dependent protein kinase (CaMKII), and calmodulin stimulated protein phosphatase (calcineurin) (Anderson, 2002).

In conclusion, our single-channel measurements provide the first detailed kinetic examination of the regulation of the $Ca^{2+}$-gated cardiac $Ca^{2+}$ release channel by CaM. CaM provides a complementary mechanism of regulating SR $Ca^{2+}$ release, in addition to the regulation of RyR2 by $Ca^{2+}$. Our in vitro observations support a model in which the action of CaM in ending SR $Ca^{2+}$ release is facilitatory. CaM lowers RyR2-mediated SR $Ca^{2+}$ release at low micromolar to submicromolar $Ca^{2+}$ concentrations by decreasing the number of channel events and increasing the duration of close times. Deciphering the kinetics of channel opening and closing associated with the inhibition of the cardiac $Ca^{2+}$ release channel by one of its endogenous effectors should help to elucidate the not well-understood mechanism of SR $Ca^{2+}$ release in the myocardium.

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