Regulation of the Cardiac Ryanodine Receptor Channel by Luminal Ca^{2+} Involves Luminal Ca^{2+} Sensing Sites

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ABSTRACT The mechanism of activation of the cardiac calcium release channel/ryanodine receptor (RyR) by luminal Ca^{2+} was investigated in native canine cardiac RyRs incorporated into lipid bilayers in the presence of 0.01 μM to 2 mM Ca^{2+} (free) and 3 mM ATP (total) on the cytosolic (*cis*) side and 20 μM to 20 mM Ca^{2+} on the luminal (*trans*) side of the channel and with Cs^+ as the charge carrier. Under conditions of low *trans* Ca^{2+} (20 μM), increasing *cis* Ca^{2+} from 0.1 to 10 μM caused a gradual increase in channel open probability (P_o). Elevating *cis* Ca^{2+} above 100 μM resulted in a gradual decrease in P_o. Elevating *trans* [Ca^{2+}] enhanced channel activity ($EC_{50} \approx 2.5$ mM at 1 μM *cis* Ca^{2+}) primarily by increasing the frequency of channel openings. The dependency of P_o on *trans* [Ca^{2+}] was similar at negative and positive holding potentials and was not influenced by high cytosolic concentrations of the fast Ca^{2+} chelator, 1,2-bis(2-aminophenoxy)ethane-*N,N,N,N*-tetraacetic acid. Elevated luminal Ca^{2+} enhanced the sensitivity of the channel to activating cytosolic Ca^{2+}, and it essentially reversed the inhibition of the channel by high cytosolic Ca^{2+}. Potentiation of P_o by increased luminal Ca^{2+} occurred irrespective of whether the electrochemical gradient for Ca^{2+} supported a cytosolic-to-luminal or a luminal-to-cytosolic flow of Ca^{2+} through the channel. These results rule out the possibility that under our experimental conditions, luminal Ca^{2+} acts by interacting with the cytosolic activation site of the channel and suggest that the effects of luminal Ca^{2+} are mediated by distinct Ca^{2+}-sensitive site(s) at the luminal face of the channel or associated protein.

INTRODUCTION

In mammalian cardiac myocytes contractile activation occurs in response to Ca^{2+} influx from the extracellular space, triggering Ca^{2+}-induced Ca^{2+} release (CICR) via Ca^{2+} release channels located in the membrane of the sarcoplasmic reticulum (SR) (Bers, 1991; Stern and Lakatta, 1992). The Ca^{2+}-sensitive Ca^{2+} release channel of the SR has been identified as the ryanodine receptor (RyR), a homotetrameric protein with a total molecular mass of ~2.3 MDa. The function of the cardiac RyR has been studied extensively by measuring SR Ca^{2+} fluxes and by recording single-channel currents with the planar lipid bilayer technique (Coronado et al., 1994; Meissner, 1994). These studies have shown that the activity of the RyR is controlled by a number of cytoplasmic ligands besides Ca^{2+}, including ATP, Mg^{2+}, and calmodulin. Consequently, the cytoplasmic side of the RyR is thought to carry the corresponding binding sites.

Recently it has been demonstrated that the activity of the RyR also can be influenced by Ca^{2+} at the luminal side of the channel. It has been reported that the open probability (P_o) of single cardiac RyR channels incorporated into lipid bilayers increases if Ca^{2+} on the luminal side of the channel is elevated (Sitsapesan and Williams, 1994; Lukyanenko et al., 1996; Sitsapesan and Williams, 1997). The effects of luminal Ca^{2+} are particularly important for understanding alterations in Ca^{2+} release that occur under pathological conditions, when the Ca^{2+} content of the cell becomes exceedingly high (Ca^{2+} overload). The exact mechanism of potentiation of channel activity by luminal Ca^{2+} is not known. One possibility is that the effects of luminal Ca^{2+} are due to Ca^{2+} passing through the open channel and acting at the cytoplasmic activation site, as has been suggested for the skeletal RyR channels (Tripathy and Meissner, 1996; Herrmann-Frank and Lehmann-Horn, 1996). Alternatively, the effects of luminal Ca^{2+} could involve a distinct Ca^{2+} binding site(s) on the luminal side of the channel. This possibility would be consistent with the observations that luminal Ca^{2+} activated only channels affected by allosteric modulators such as sulmazole and ATP (Sitsapesan and Williams, 1994; Lukyanenko et al., 1996). In the present study we investigated the mechanism of action of luminal Ca^{2+} in cardiac Ca^{2+} release channels/RyRs. Crude microsomes obtained from dog heart were fused with planar lipid bilayer membranes, and the activity of single Ca^{2+} release channels was recorded in symmetrical $CsCH_3SO_3$ media. The effects of various luminal [Ca^{2+}] (0.1–20 mM) on channel activity were studied at different cytoplasmic Ca^{2+} concentrations (0.01 μM to 2 mM) and membrane potentials. Our results suggested that the effects of luminal Ca^{2+} were not mediated by the cytoplasmic Ca^{2+} activation mechanism, but rather involved a different Ca^{2+} regulatory site on the luminal side of the channel or associated protein.

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MATERIALS AND METHODS

Heavy SR microsomes were isolated by differential centrifugation from the ventricles of dog heart as described previously (Dettbarn et al., 1994). Dogs were killed by injection of Nembutal. Membrane vesicles were frozen

rapidly and stored in liquid nitrogen. SR microsomes were fused into planar lipid bilayers, and single-channel currents were monitored as described previously (Lukyanenko et al., 1996). Bilayers were composed of 80% phosphatidylethanolamine and 20% phosphatidylcholine dissolved in decane at a final concentration of 50 mg/ml. SR vesicles were added to one side of the bilayer (defined as *cis*), and the other side was defined as *trans* (virtual ground). Channel sidedness was determined by the channel's response to ATP. Reconstituted channels were consistently oriented with the cytoplasmic channel face on the *cis* side of the bilayer (Györke et al., 1994). Standard solutions contained 350 mM *cis* $CsCH_3SO_3$, 20 mM *trans* $CsCH_3SO_3$, 20 μM $CaCl_2$, 20 mM HEPES (pH 7.4). After channel incorporation, the *trans* $CsCH_3SO_3$ was adjusted to 350 mM. Single-channel recordings were performed with an Axopatch 200A (Axon Instruments, Foster City, CA) patch-clamp amplifier. Data were filtered at 2 kHz and digitized at 5–10 kHz. Acquisition and analysis of data were performed with Pcalmp 6.01 software (Axon Instruments). The free Ca^{2+} concentrations at given total Ca^{2+}, Mg^{2+}, ATP, and EGTA concentrations were determined using a computer program (Bers et al., 1994) and verified by measurements with a Ca^{2+}-selective electrode (World Precision Instruments, Sarasota, FL).

RESULTS

SR microsomes were fused into planar lipid bilayers, and single-channel currents were recorded with Cs^+ as the charge carrier. It has been shown previously that the activation of channels by luminal Ca^{2+} requires the presence of ATP (or another allosteric agonist, i.e., sulmazole) on the cytoplasmic side of the channel (Sitsapesan and Williams, 1994; Lukyanenko et al., 1996). Because the focus of this study was the action of luminal Ca^{2+}, all of our measurements were performed in the presence of 3 mM cytoplasmic ATP. First we defined the sensitivity of the channel to cytosolic (*cis*) Ca^{2+} at constant (20 μM) luminal (*trans*) Ca^{2+}. In general agreement with the results of previous single-channel studies obtained in the absence of ATP (Laver et al., 1995; Xu et al., 1996; but see Chu et al., 1993), the activity of most of the channels was regulated by cytosolic [Ca^{2+}] in a bimodal fashion. At low concentrations ($<$10 μM) *cis* Ca^{2+} activated the channel; at high concentrations ($>$ 100 μM), *cis* Ca^{2+} inhibited the channel. A significant fraction of channels (4 of 17) exhibited no inhibition in the high *cis* [Ca^{2+}] range (not shown). This is consistent with the fragility of Ca^{2+} inhibition of cardiac RyRs reported previously (Laver et al., 1995; Copello et al., 1997). Records from two typical channels, measured with and without Mg^{2+} (free *cis* [Mg^{2+}] $\approx$ 1 mM) at different free *cis* Ca^{2+} concentrations are shown in Fig. 1. Fig. 2 summarizes these series of experiments by plotting separately the relationships between P_o and free *cis* [Ca^{2+}] with and without Mg^{2+}. It is apparent that in the presence of Mg^{2+}, the concentration of Ca^{2+} needed to achieve half-maximum activation increased from $\sim$1 to $\sim$30 μM, whereas inhibition of the channel by high Ca^{2+} was not altered. Channel properties at near-threshold and maximally activating and inactivating cytosolic Ca^{2+} concentrations with and without Mg^{2+} are summarized in Table 1. Increasing *cis* Ca^{2+} resulted in a gradual prolongation of the average open time. Inhibition by Ca^{2+} was caused by an increase in the duration of closures.

To investigate the effects of luminal Ca^{2+}, we varied [Ca^{2+}] in the *trans* chamber over a range from 20 μM to 20 mM and recorded channel activity at submaximum activating concentrations of *cis* Ca^{2+} (1 μM). Fig. 3 shows a representative experiment in which channel activity was measured at $\pm$40 mV holding potentials before and after elevation of luminal Ca^{2+} from 20 μM to 5 mM. At both potentials, elevation of *trans* Ca^{2+} resulted in a dramatic increase in channel activity. In the presence of high luminal Ca^{2+}, unitary current is reduced as Ca^{2+} competes with the primary charge carrier, Cs^+, in the pore (Tu et al., 1994). Because of symmetrical distribution of Cs^+ on both sides of the lipid bilayer, the currents through the channel at +40 and $-$40 mV had opposite directions. However, at both potentials, the electrochemical gradient for Ca^{2+} favored a luminal-to-cytosolic (*trans*-to-*cis*) Ca^{2+} flux through the channel, owing to the large excess of Ca^{2+} on the luminal side of the channel ($E_{Ca} \approx$ 198 mV). It has been suggested that luminal Ca^{2+} flowing through the channel might regulate channel activity by having access to the cytoplasmic activation site of the channel (Tripathy and Meissner, 1996). Alternatively, the effects of luminal Ca^{2+} could be mediated by Ca^{2+} acting at distinct binding sites on the luminal side of the channel. The primary goal of the present study was to discriminate between these possibilities.

Fig. 4 describes the relationship between channel P_o and luminal [Ca^{2+}] at +40 and $-$40 mV determined at a constant *cis* [Ca^{2+}] of 1 μM in experiments similar to that illustrated in Fig. 3. The two P_o/*trans* [Ca^{2+}] curves virtually superimpose. The EC_{50} values, obtained by fitting the data by a logistic function, were 2.2 $\pm$ 0.9 mM and 2.8 $\pm$ 1.1 (n = 5) mM for +40 mV and $-$40 mV holding potential, respectively. Hill slopes at +40 and $-$40 mV were 1.9 and 1.6, respectively, implying that more than one Ca^{2+} ion must bind for maximum activation of the channel. These results indicate that the effect of luminal Ca^{2+} on P_o was not influenced by the membrane potential. The effects of luminal Ca^{2+} on channel properties at +40 and $-$40 and at a *cis* [Ca^{2+}] of 1 μM are summarized in Table 2. On average, P_o tended to be higher at positive than at negative membrane potentials, but the degree of potentiation by luminal Ca^{2+} was similar in the two cases. The increase in P_o was due primarily to an increase in the frequency of openings. A relatively small increase (less than twofold) in the mean duration of open events was also observed in some experiments (Table 2). The marked increase in the number of openings as well as a lack of voltage dependence of the effects of luminal Ca^{2+} are not consistent with the hypothesis that the effects of luminal Ca^{2+} are due to Ca^{2+} interacting with the cytoplasmic activation side of the channel (see Discussion).

To further assess the potential role of luminal-to-cytosolic Ca^{2+} fluxes in RyR activation, we investigated the impact of the fast Ca^{2+} buffer 1,2-bis(2-aminophenoxy)ethane-*N,N,N,N*-tetraacetic acid (BAPTA) on the activity of the channel exposed to high luminal Ca^{2+}. Channel activity was recorded in the presence of 50 nM free *cis* Ca^{2+}

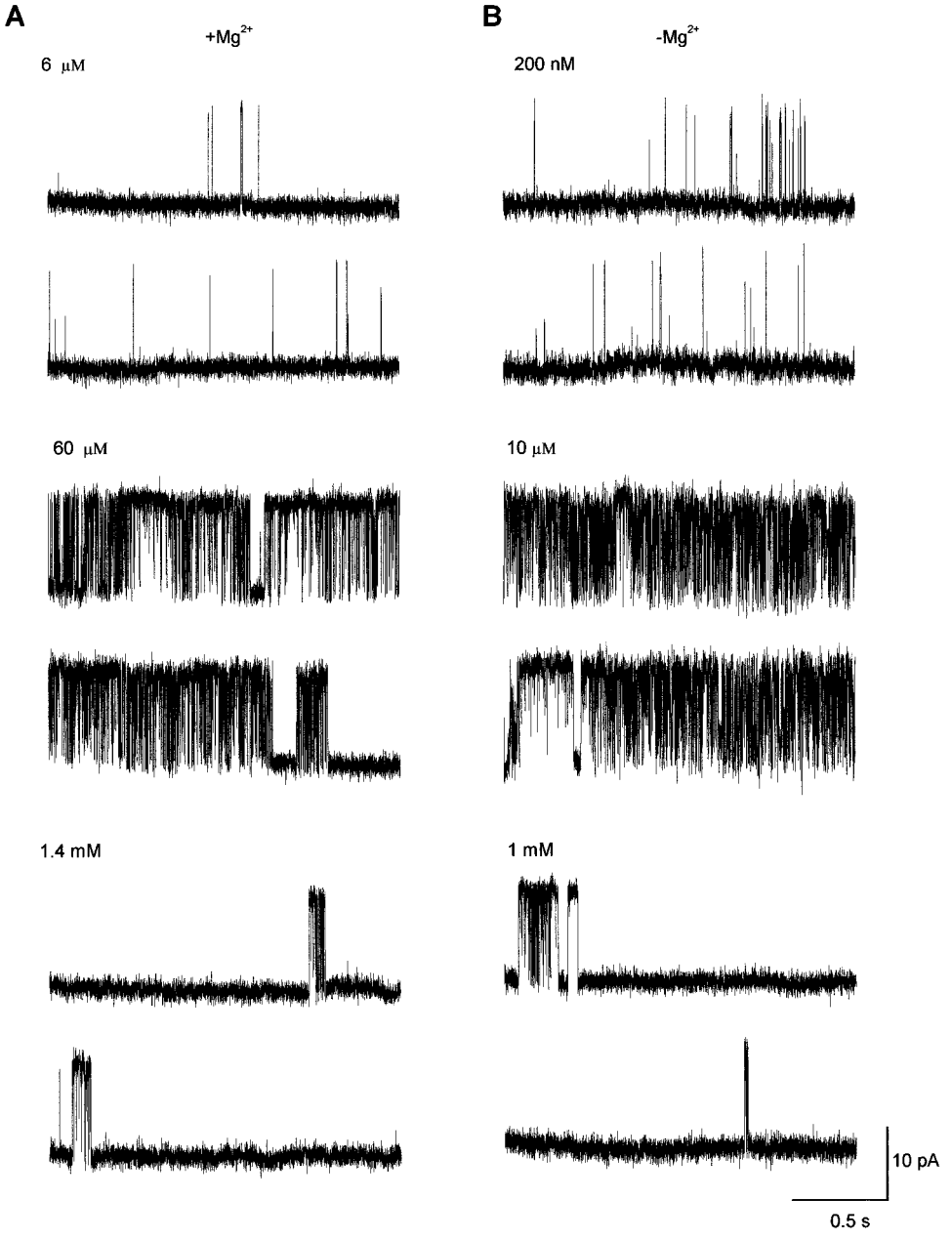

FIGURE 1 Effect of varying cytosolic [Ca^{2+}] on the activity of cardiac RyRs. Representative traces of current recordings from channels activated by Ca^{2+} and ATP with (*A*) and without (*B*) Mg^{2+}. Single-channel currents shown as upward deflections. Current recordings were obtained at +40 mV holding potential in symmetrical 350 mM $CsCH_3SO_3$, pH 7.4. The *cis* chamber contained 3 mM Na_2ATP or MgATP and the indicated free [Ca^{2+}]. The *trans* chamber contained 20 μM Ca^{2+}.

and 20 mM BAPTA with either 20 μM or 5 mM Ca^{2+} on the luminal side of the channel (Fig. 5). The addition of 5 mM Ca^{2+} to the luminal side of the channel resulted in potentiation of channel activity to an extent similar to that observed without BAPTA ($P_o = 0.018 \pm 0.001$ versus 0.137 ± 0.024; $n = 4$, $p < 0.05$). These results are consistent with the possibility that luminal Ca^{2+} acts at the luminal side of the membrane. However, they do not necessarily rule out the possibility that luminal Ca^{2+} binds to the cytoplasmic activation site, because if the site is located close enough to the channel mouth ("BAPTA-inaccessible" space; Stern, 1992a), it could be reached by Ca^{2+} before the Ca^{2+} ions are chelated by BAPTA.

To test further the possibility that luminal Ca^{2+} activates the channel by having access to the cytoplasmic activation site, we investigated the effects of luminal Ca^{2+} at increasing cytosolic [Ca^{2+}]. Our intention was to determine whether luminal Ca^{2+} would still be able to activate the channel if the cytosolic activation sites were saturated by Ca^{2+} and/or if the luminal-to-cytosolic Ca^{2+} flow were no longer supported by the electrochemical gradient for Ca^{2+}. In the series of experiments exemplified in Fig. 6, free [Ca^{2+}] on the cytosolic side of the channel was increased stepwise first from 200 nM to 20 μM and then from 20 μM to 1 mM at constant (20 μM) *trans* [Ca^{2+}], and channel activity was recorded at +40 mV. In accordance with the cytosolic Ca^{2+} sensitivity of the channel defined earlier (Fig. 2), channel activity increased upon elevating *cis* Ca^{2+} from 200 nM to 20 μM and then decreased after a further elevation of Ca^{2+} to 1 mM. Elevation of luminal Ca^{2+} from

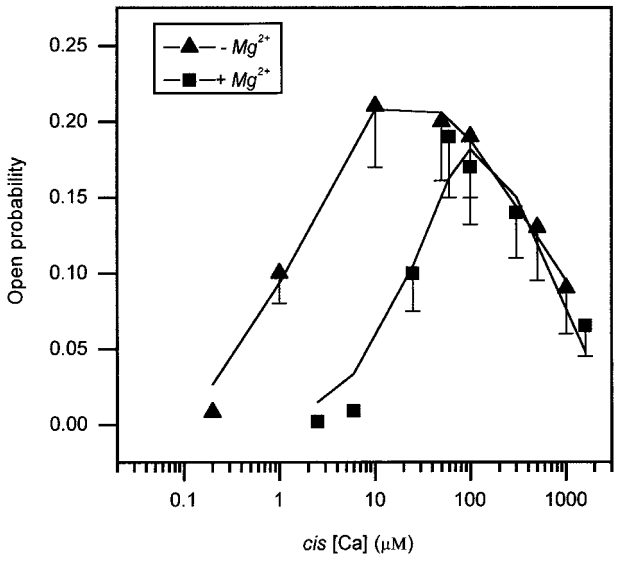

FIGURE 2 Dependence of cardiac RyR P_o on cytosolic [Ca^{2+}] in the presence of 3 mM ATP without (■) or with (total [Mg^{2+}] 3 mM, ▲) Mg^{2+}. Single-channel activities were determined at +40 mV potential in symmetrical 350 mM $CsCH_3SO_3$, pH 7.4, at indicated concentrations of free cytosolic [Ca^{2+}]. Luminal [Ca^{2+}] was 20 μM Ca^{2+}. Data are presented as means ± SEM of 5–16 determinations from diffferent experiments. Continuous curves were obtained by fitting the data according to the equation $P_o = P_o^{max}\{[Ca^{2+}]^{na}/([Ca^{2+}]^{na} + K_a^{na})\} * \{1 - [Ca^{2+}]^{ni}/([Ca^{2+}]^{ni} + K_i^{ni})\}$, where P_o^{max} = 0.25 and 0.27, K_a = 25.8 and 1.8 μM, K_i = 596 and 608 μM, na = 3.7 and 1, ni = 0.70 and 0.66 for data obtained with and without Mg^{2+}, respectively.

20 μM to 10 mM caused a dramatic increase in the activity of the channel inhibited by cytosolic Ca^{2+}. At these Ca^{2+} concentrations on the two sides of the channel and with the holding potential employed (+40 mV), the electrochemical gradient for Ca^{2+} still slightly favored a luminal-to-cytosolic Ca^{2+} flow through the channel ($E_{Ca} \approx 60$ mV). This Ca^{2+} flow, however, could not have contributed to the observed potentiation of the channel, because the activation binding sites must have been saturated by high [Ca^{2+}] already present on the cytosolic side of the channel (Fig. 2). Any surplus Ca^{2+} on the cytoplasmic side could only contribute to inhibition of the channel. Similar results were obtained in 16 other experiments, in which luminal Ca^{2+} was increased from 20 μM to 5 or 10 mM (Table 3).

In another series of experiments, the conditions were designed to prevent any Ca^{2+} flow from the luminal to the cytosolic sides of the channel. Channel activity was recorded at +40 mV in the presence of either 20 μM or 2 mM Ca^{2+} on the cytosolic and luminal sides of the channel (Fig. 7). The experiments were started at 20 μM *cis* Ca^{2+}, then *cis* Ca^{2+} was increased to 2 mM at constant *trans* Ca^{2+} (Fig. 7, *A* and *B*, respectively). As in the experiments described above, P_o decreased because of inhibition of the channel by *cis* Ca^{2+}. Under these conditions, the net Ca^{2+} flow should have a *cis*-to-*trans* direction because of the large excess of Ca^{2+} on the cytosolic side of the channel ($E_{Ca} \approx -117$ mV). Increasing luminal Ca^{2+} to 2 mM (Fig. 7 *C*) did not change the direction of the net Ca^{2+} flux through the channel because, although the concentration gradient had been abolished, the positive holding potential still favored the movement of Ca^{2+} from the cytosolic to the luminal side of the channel ($E_{Ca} \approx 0$ mV). Nevertheless, elevated luminal Ca^{2+} increased channel P_o in a manner similar to that observed in the previous experiments. Similar results were obtained in seven other experiments (Table 3). These experiments clearly show that luminal Ca^{2+} acts at the luminal but not the cytosolic side of the channel.

Fig. 8 summarizes the effects of elevating luminal Ca^{2+} from 20 μM to 5 mM on channel P_o in the range of cytosolic Ca^{2+} from 0.01 μM to 1 mM. Elevated luminal Ca^{2+} enhanced P_o at all *cis* [Ca^{2+}] investigated. The values of EC_{50} for cytosolic Ca^{2+} activation, obtained by fitting a logistic function to the raising phase of the P_o/*cis* [Ca^{2+}] curves, decreased from 0.86 ± 0.04 to 0.23 ± 0.03 μM (n = 7). The impact of luminal Ca^{2+} on P_o was most

TABLE 1 The effects of cytosolic Ca^{2+} on RyR gating

		cis [Ca^{2+}] (μM)		
	Channel properties	0.2	10	1000
$-Mg^{2+}$	Open probability (P_o)	0.009 ± 0.002	0.21 ± 0.04*	0.09 ± 0.03**
	No. of events	2150 ± 461	31560 ± 7083*	4307 ± 3860**
	Mean open time (ms)	0.59 ± 0.06	1.12 ± 0.21*	3.03 ± 0.09
	Mean closed time (ms)	55.8 ± 9.6	3.1 ± 1.6*	27.3 ± 5.8**
		6	60	1400
$+Mg^{2+}$	Open probability (P_o)	0.008 ± 0.002	0.19 ± 0.04*	0.09 ± 0.02**
	No. of events	1875 ± 325	12504 ± 2883*	4493 ± 1253**
	Mean open time (ms)	0.51 ± 0.05	2.14 ± 0.69*	2.72 ± 1.67
	Mean closed time (ms)	68.7 ± 21.63	8.6 ± 2.9*	23.3 ± 5.1**

Channel properties were obtained from 2-min continuous recordings carried out in symmetrical 350 mM $CsCH_3SO_3$ medium in the presence of 3 mM Na_2ATP or 3 mM MgATP (rows labeled $-Mg^{2+}$ and $+Mg^{2+}$, respectively) at the indicated concentrations of free *cis* [Ca^{2+}]. The *trans* chamber contained 20 μM Ca^{2+}. The holding potential was 40 mV. Data are presented as means ± SEM of 5–16 determinations from different experiments.
*$p < 0.05$ versus values at 0.2 or 6 μM Ca, respectively.
**$p < 0.05$ versus 10 or 60 μM Ca, respectively.

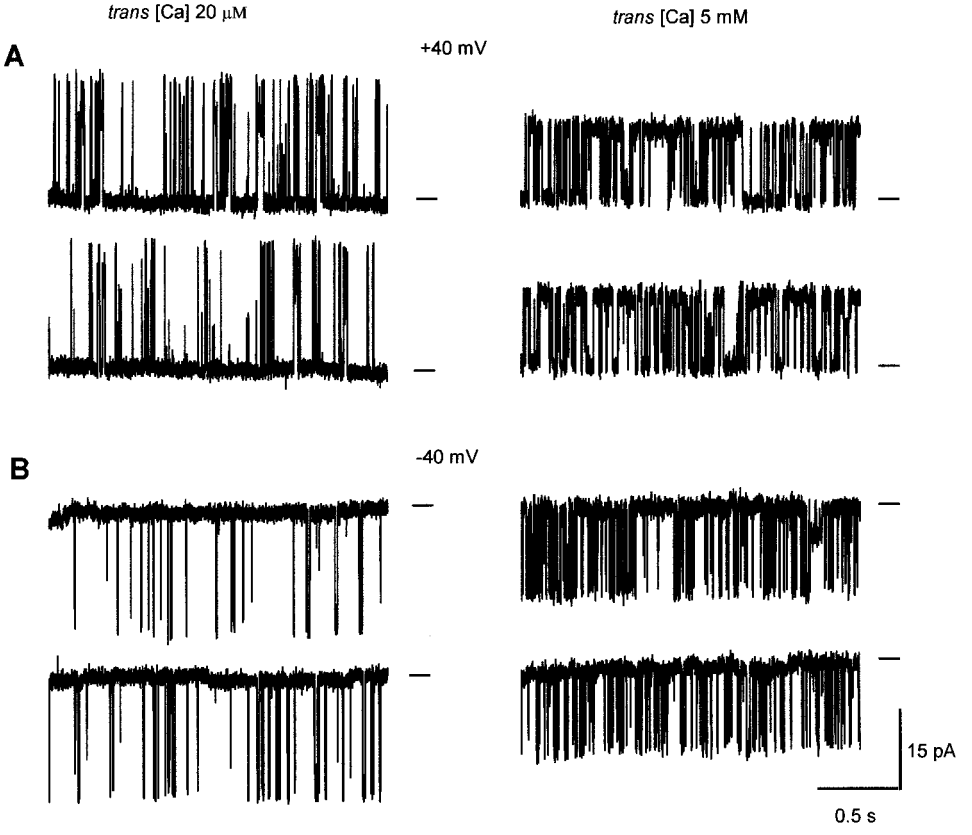

FIGURE 3 Effect on channel activity of increasing luminal [Ca^{2+}]. (*A* and *B*) Representative traces of channel recordings at +40 mV (*A*) and −40 mV (*B*) holding potential before and after increasing luminal Ca^{2+} from 20 μM Ca^{2+} to 5 mM. Current recordings were obtained in symmetrical 350 mM $CsCH_3SO_3$, pH 7.4. Channel openings are shown as downward or upward deflections from closed levels (*marked with dashes*). The *cis* chamber contained 3 mM Na_2ATP and 1 μM free [Ca^{2+}].

dramatic in the inhibitory concentration range of *cis* Ca^{2+} (>100 μM), where *trans* Ca^{2+} essentially reversed the inhibition by *cis* Ca^{2+}. These results suggest that luminal Ca^{2+} acts by enhancing the activation and by reducing the inhibition of the channel by cytosolic Ca^{2+}.

DISCUSSION

It is known that the activity of the RyR is controlled by high-affinity Ca^{2+} activation and low-affinity Ca^{2+} inactivation sites accessible from the cytosolic side of the channel (Meissner and Henderson, 1987; Chu et al., 1993; Laver et al., 1995; Xu et al., 1996, this study). In the present study we established the existence of distinct Ca^{2+} regulatory sites residing on the luminal side of the channel (or associated protein). These sites can sense changes in luminal Ca^{2+} concentration in a range of 0.2–20 mM, rendering increased activation and reduced inactivation of the channel by cytosolic Ca^{2+} upon interaction with luminal Ca^{2+}. This mechanism provides means by which the activity of the RyR can be coupled to the Ca^{2+} loading state of the SR, and it may be important for regulation of Ca^{2+} release under normal conditions as well as pathological conditions, such as Ca^{2+} overload.

Luminal Ca^{2+} acts at the luminal side of the RyR

Our conclusion that the effects of luminal Ca^{2+} do not involve the cytosolic Ca^{2+} activation site of the RyR is based on the following evidence:

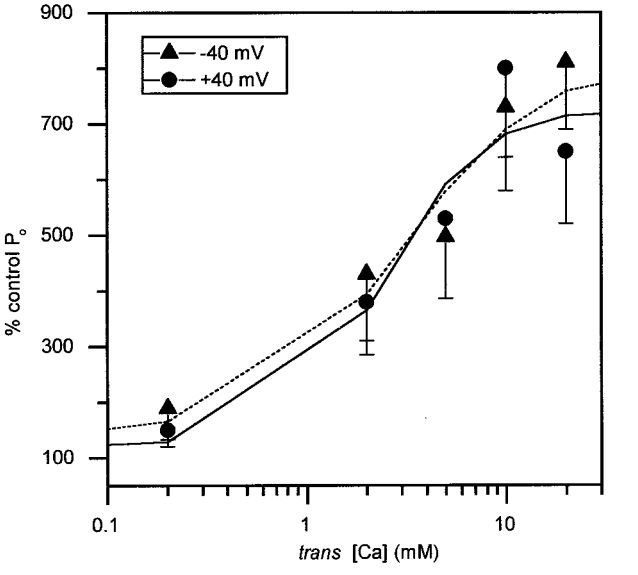

FIGURE 4 Relative open probability (P_o) as a function of luminal [Ca^{2+}]. Single-channel activities were determined at +40 mV (●) or −40 mV (▲) holding potential at indicated concentrations of luminal Ca^{2+}. The *cis* chamber contained 3 mM Na_2ATP and 1 μM free [Ca^{2+}]. Data are presented as means ± SEM of 5–9 determinations from different experiments. Continuous lines were obtained by fitting the data according to the equasion $P_o = P_o^{max}\{[Ca^{2+}]^{na}/([Ca^{2+}]^{na} + K_a^{na})\}$, where P_o^{max} = 705 and 740; K_a = 2.2 and 2.8; na = 1.9 and 1.6, for data obtained at + 40 and −40 mV, respectively.

TABLE 2 The effects of luminal Ca on RyR gating at holding potentials of +40 and −40 mV

		trans [Ca^{2+}]		
Holding potential	Channel properties	20 μM	2 mM	10 mM
+40 mV	Open probability (P_o)	0.08 ± 0.02	0.30 ± 0.08*	0.64 ± 0.14*
	No. of events	10560 ± 2950	15856 ± 2249*	32426 ± 3020*
	Mean open time (ms)	1.15 ± 0.19	2.28 ± 0.46*	1.52 ± 0.42
	Mean closed time (ms)	13.21 ± 1.9	7.12 ± 1.07*	2.81 ± 0.95*
−40 mV	Open probability (P_o)	0.06 ± 0.02	0.26 ± 0.07*	0.44 ± 0.09*
	No. of events	12750 ± 4750	31295 ± 8091*	50140 ± 8460*
	Mean open time (ms)	0.66 ± 0.05	1.05 ± 0.09*	0.84 ± 0.16
	Mean closed time (ms)	10.62 ± 2.34	3.83 ± 0.8*	2.23 ± 0.97*

Channel properties were obtained from 2-min continuous recordings carried out in symmetrical 350 mM $CsCH_3SO_3$ medium at +40 or −40 mV holding potential and the indicated [Ca^{2+}]'s in the *trans* chamber. The *cis* chamber contained 3 mM ATP (total) and 1 μM Ca^{2+} (free). Data are presented as means ± SEM of 5–9 determinations from different experiments.
*$p < 0.05$ versus values at 20 μM *trans* [Ca].

1. Luminal Ca^{2+} increased channel activity primarily by increasing the number of openings rather then by increasing their duration (Tables 2 and 3). Luminal Ca^{2+} could have access to the cytosolic activation site only when the channel opens and Ca^{2+} can flow through the pore. When the channel closes, the local Ca^{2+} gradient in the vicinity of the channel dissipates very rapidly (microseconds; Stern, 1992a), making rebinding of Ca^{2+} to the channel unlikely. Therefore, the increase in frequency of openings observed in the present study cannot easily be accounted for by Ca^{2+} flowing through the channel and binding to the activation site.

2. The relationship between P_o and luminal [Ca^{2+}] was similar at positive and negative holding potentials (+40 mV versus −40; Fig. 4). With elevated luminal Ca^{2+}, negative holding potentials should result in larger luminal-to-cytosolic Ca^{2+} fluxes than positive holding potentials. Therefore, it would be expected that if Ca^{2+} flowing from the luminal to the cytoplasmic side of the channel had access to the cytosolic Ca^{2+} activating site, P_o would be higher at negative potentials.

3. Introducing high concentrations of the fast Ca^{2+} chelator BAPTA to the cytosolic side of the channel had no significant impact on the potentiation of channel activity by increased luminal Ca^{2+} (Fig. 5).

4. Increased luminal Ca^{2+} caused a potentiation of channel activity in a [Ca^{2+}] range in which the cytosolic activation sites should be saturated by Ca^{2+} and any additional increase in [Ca^{2+}] in the vicinity of the channel could have only contributed to inhibition of channel activity (Fig. 6).

5. Luminal Ca^{2+} exerted its potentiating effects regardless of whether Ca^{2+} flowed from the luminal to the cytosolic or from the cytosolic to the luminal side of the channel. These results clearly show that the site of action of luminal

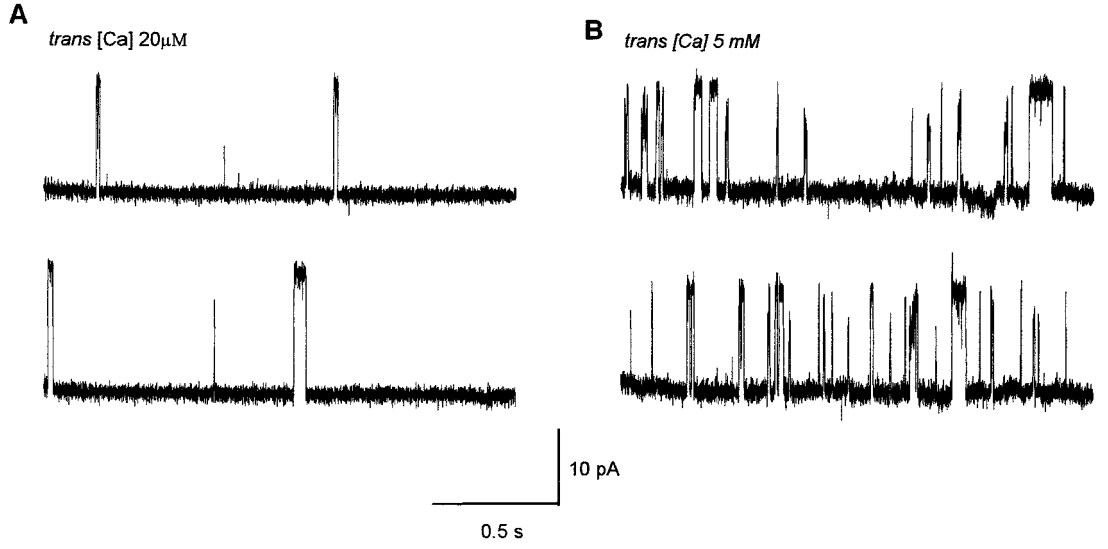

FIGURE 5 Effects of increasing luminal Ca^{2+} on channel activity in the presence of 20 mM cytosolic BAPTA. (*A* and *B*) Representative segments of single-channel traces recorded before (*A*) and after (*B*) *trans* [Ca^{2+}] was increased from 20 μM to 5 mM. The *cis* chamber contained 3 mM Mg^{2+}, 20 mM BAPTA, and 14 mM Ca^{2+} (free [Ca^{2+}] ≈ 50 nM). The *trans* chamber contained 20 μM or 5 mM Ca^{2+}, as indicated. The records were obtained in symmetrical 350 mM $CsCH_3SO_3$, pH 7.4, at +40 mV holding potential.

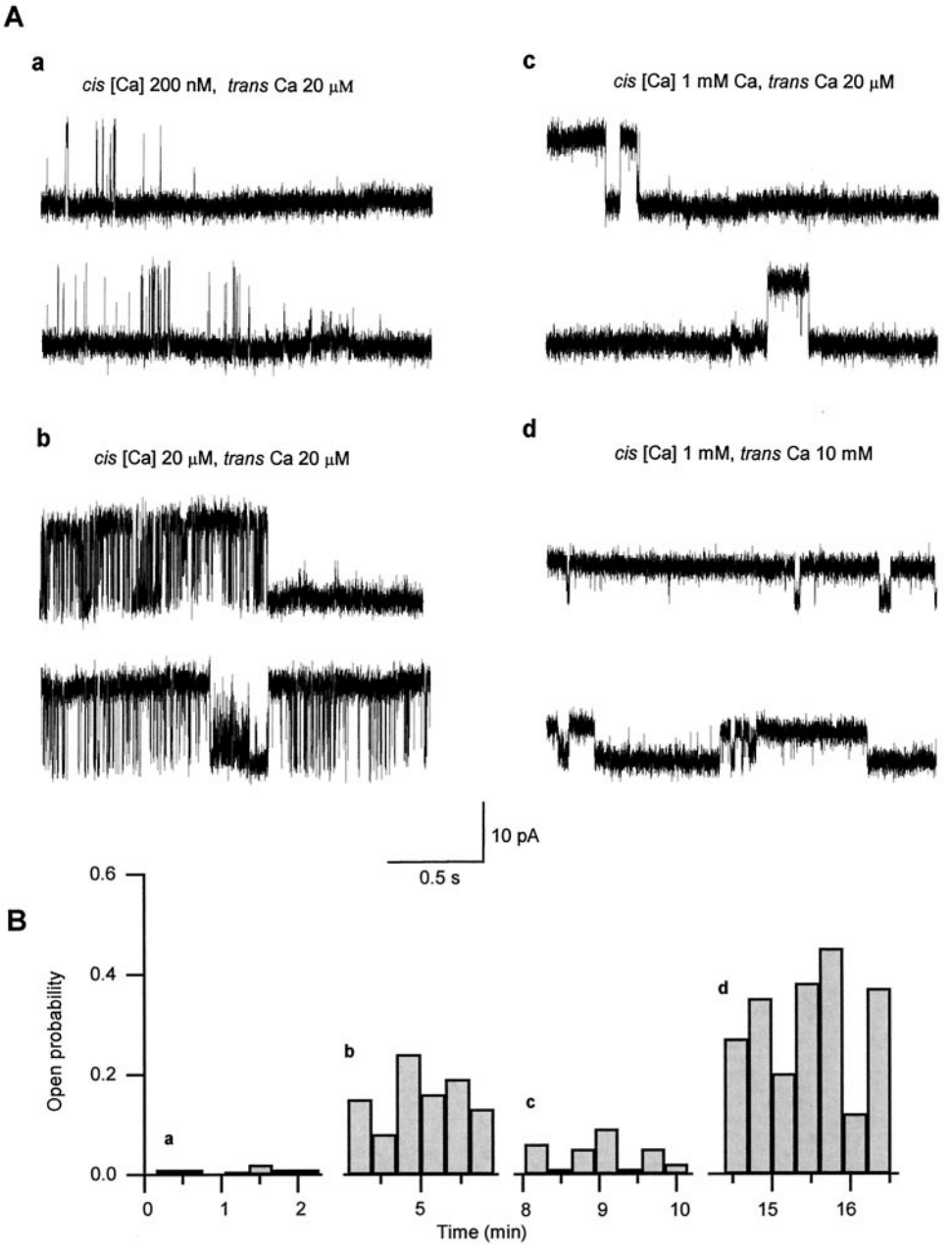

FIGURE 6 Effects of increasing luminal Ca^{2+} on activity of a channel suppressed by high cytosolic Ca^{2+}. (*A, a, b, c,* and *d*) Representative segments of single-channel traces recorded sequentially in the course of a single experiment at various cytosolic and luminal [Ca^{2+}]. The *cis* chamber contained 3 mM Na_2ATP, 100 μM EGTA, and the indicated concentration of free [Ca^{2+}]. The *trans* chamber contained 20 μM or 10 mM Ca^{2+}, as indicated. The records were obtained in symmetrical 350 mM $CsCH_3SO_3$, pH 7.4, at +40 mV holding potential. (*B*) Diary of P_o of the experiment illustrated in *A, a, b, c,* and *d,* respectively. Continuous records were divided into intervals of 20 s; P_o in each segment was plotted as a bar of length 0–1.

Ca^{2+} is at the luminal side of the channel. Because our experiments have been performed with native RyRs, they do not provide evidence regarding whether the effects of luminal Ca^{2+} are caused by interaction of Ca^{2+} with the channel protein itself or are mediated by an auxiliary regulatory protein. Growing evidence suggests that regulation

TABLE 3 The effects of luminal Ca^{2+} on RyR gating at high cytosolic [Ca^{2+}]

cis [Ca^{2+}]	1 mM			1 mM (3 mM MgATP)		2 mM	
trans [Ca^{2+}]	20 μM	5 mM	10 mM	20 μM	5 mM	20 μM	2 mM
Open probability (P_o)	0.05 ± 0.01	0.26 ± 0.04*	0.49 ± 0.09*	0.03 ± 0.01	0.21 ± 0.04*	0.03 ± 0.02	0.22 ± 0.07*
No. of events	2700 ± 450	5430 ± 985*	7925 ± 1214*	1790 ± 605	6876 ± 1730*	927 ± 2430	6105 ± 2280*
Mean open time (ms)	3.0 ± 0.9	7.8 ± 1.9	8.2 ± 1.8	2.7 ± 0.8	4.4 ± 1.3	4.7 ± 1.5	4.8 ± 0.7
Mean closed time (ms)	49.2 ± 6.3	12.3 ± 3.4*	6.4 ± 1.5*	58.2 ± 10.4	5.2 ± 1.6*	98.2 ± 21.3	17.1 ± 4.2*

Channel properties were obtained from 2-min continuous recordings carried out in symmetrical 350 mM $CsCH_3SO_3$ medium at the indicated concentrations of Ca in the *cis* and *trans* chambers. The *cis* chamber contained 3 mM Na_2ATP or 3 mM MgATP. The holding potential was +40 mV. Data are presented as means ± SEM of 6–16 determinations from different experiments.
*$p < 0.05$ versus values at 20 μM *trans* [Ca].

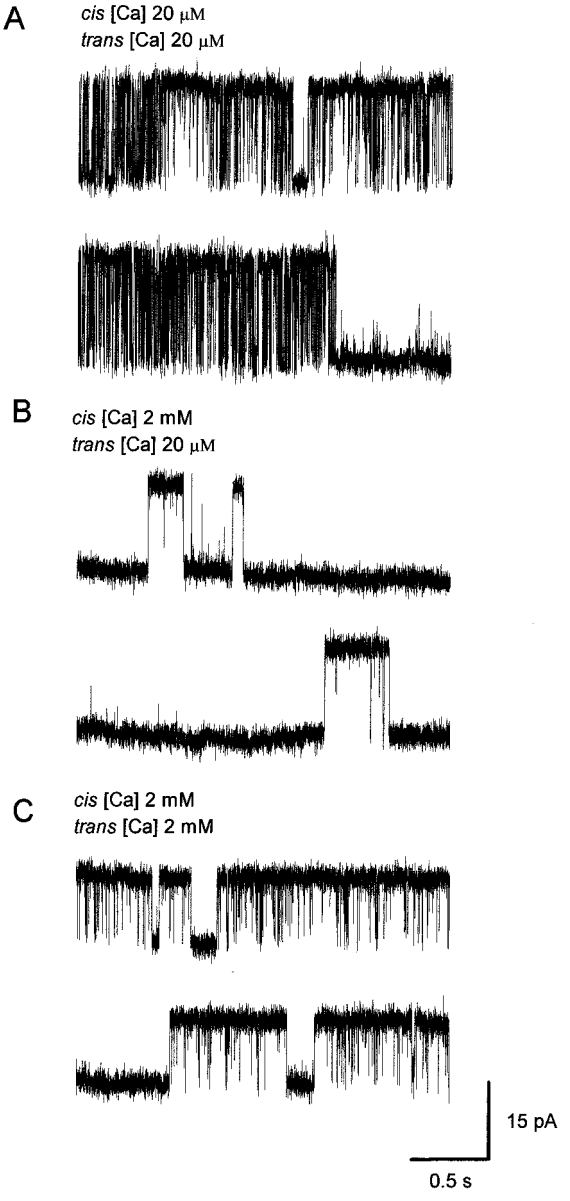

FIGURE 7 Effects of increasing luminal Ca^{2+} on channel activity under conditions when a luminal-to-cytosolic flow of Ca^{2+} through the channel is not supported by the electrochemical gradient for Ca^{2+}. Representative segments of channel recordings under control conditions (*A*), after the addition of 2 mM Ca^{2+} to the *cis* chamber (*B*), and after subsequent addition of 2 mM Ca^{2+} to the *trans* chamber (*C*). Single-channel currents are shown as upward deflections. The records were obtained in symmetrical 350 mM $CsCH_3SO_3$, pH 7.4, at +40 mV holding potential.

of Ca^{2+} release by luminal Ca^{2+} may involve a complex molecular machinery composed of multiple proteins, including RyR, clasequestrin, triadin, and junctin (Ikemoto et al., 1989; Kawasaki and Kasai, 1994; Guo and Campbell, 1995; Zhang et al., 1997). On the other hand, potentiation of P_o by increased luminal Ca^{2+} was observed in purified sheep cardiac RyRs (Sitsapesan and Williams, 1994); thus it appears luminal Ca^{2+} can also regulate the channel directly, at least in some preparations.

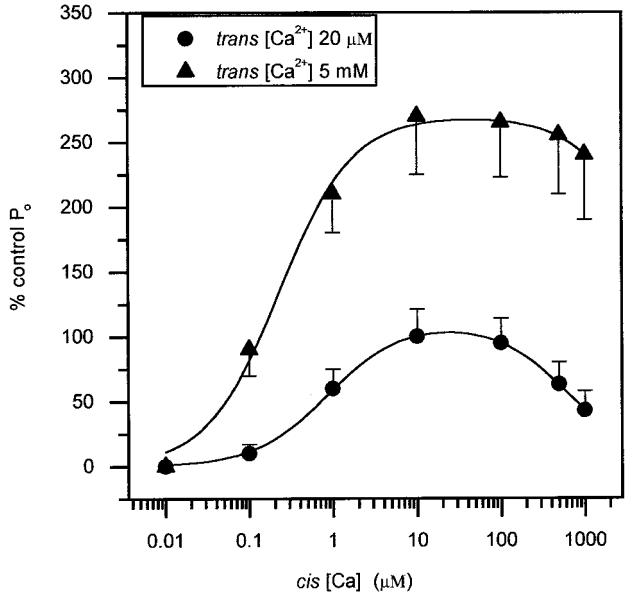

FIGURE 8 Effect of increasing luminal Ca^{2+} on the relationship between P_o and cytosolic $[Ca^{2+}]$. Single-channel activities were determined before (●) and after (▲) Ca^{2+} is elevated in the *trans* chamber from 20 μM to 5 mM at indicated concentrations of free *cis* Ca^{2+}. Data are presented as means ± SEM of 6–16 determinations from different experiments. Continuous lines were obtained by fitting the data according to the equation $P_o = P_o^{max}\{[Ca^{2+}]^{na}/([Ca^{2+}]^{na} + K_a^{na})\} * \{1 - [Ca^{2+}]^{ni}/([Ca^{2+}]^{ni} + K_i^{ni})\}$, where $P_o^{max} = 110$ and 270, $K_a = 0.86$ and 0.23, $K_i = 650$ and 8136, na = 1 and 1, ni = 1 and 1, for data obtained with 20 μM and 5 mM *trans* $[Ca^{2+}]$.

Relation to previous studies

The Ca^{2+} sensitivity of RyR activation with and without cytosolic Mg^{2+} (half-maximum activation near 1 and 30 μM, respectively) is consistent with those found previously (i.e., Coronado et al., 1994). Regarding inactivation at high *cis* $[Ca^{2+}]$, whereas some studies showed no signs of this mechanism (Rousseau et al., 1986; Rousseau and Meissner, 1989; Chu et al., 1993), others showed variable inhibition at 0.5–10 mM $[Ca^{2+}]$ (Laver et al., 1995; Copello et al., 1997; Marengo et al., 1998). Our results are in agreement with these last reports. Inactivation by 10 μM to 1 mM Ca^{2+} was consistently observed in Ca^{2+} release studies with SR vesicles (Chamberlain et al., 1984; Zimanyi and Pessah, 1991; Chu et al., 1993) and permeabilized cells (Fabiato, 1985), which more closely mimic the membrane environment of the channel in situ than do the conditions of the lipid bilayer experiments, suggesting that Ca^{2+}-dependent inactivation of the RyRs is operational in vivo. The variability in the extent of inhibition at high cytosolic $[Ca^{2+}]$ among various studies has been attributed to the fragility of the inactivation mechanism, which can be disrupted by high Cs^+ concentrations and solubilization by 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (Laver et al., 1995). Furthermore, inactivation at high Ca^{2+} can be modified by sulfhydryl oxidation (Marengo et al., 1998) and through modulation of the RyR channel by ATP (Györke and Gy-

örke, unpublished observation). In the present study we show that the extent of inactivation is also highly sensitive to luminal Ca^{2+} (Fig. 8). The reduced ability of cytosolic Ca^{2+} to promote inactivation at high luminal Ca^{2+} could also explain the results of Fabiato (1992), who showed that in skinned cardiac cells, spontaneous Ca^{2+} release can occur at high basal Ca^{2+} concentrations, at which CICR must be inactivated.

Our conclusion that the effects of luminal Ca^{2+} are not mediated by the cytosolic activation site is consistent with the results of previous studies demonstrating that the effect of luminal Ca^{2+} depends on the mode of activation of the RyR. It has been shown in both native and purified sheep (Sitsapesan and Williams, 1994) and native canine cardiac RyRs (Lukyanenko et al., 1996) that luminal Ca^{2+} has no effect on channels activated by cytosolic Ca^{2+} alone, but it potentiates channels activated by Ca^{2+} and ATP or sulmazole. Similar results were obtained in native sheep skeletal RyRs in which potentiation by luminal Ca^{2+} was observed only in the presence of ATP or ATP and Ca^{2+} but not Ca^{2+} alone on the cytosolic side of the channel (Sitsapesan and Williams, 1995). In addition, similar to the results reported here (Fig. 4), the effect of luminal Ca^{2+} on sheep skeletal RyRs activated by ATP and Ca^{2+} exhibited no voltage dependence (Sitsapesan and Williams, 1995). Recently it has been reported that luminal to cytosolic Ca^{2+} fluxes can increase the P_o in purified skeletal RyRs activated by ATP or Ca^{2+} (Tripathy and Meissner 1996; Herrmann-Frank and Lehmann-Horn, 1996). At the present time, the reasons for these different results are not clear. They may reflect species/isoform differences or differences in the degree of isolation of the RyRs in different laboratories. It is possible that luminal Ca^{2+} can influence the RyR through both (i.e., cytosolic and luminal) Ca^{2+} activation mechanisms, and the prominence of one or the other mechanism depends on the experimental conditions.

Luminal Ca^{2+} is an allosteric modulator of the RyR

It has been suggested that RyR is an allosteric enzyme, the activity of which is controlled by a number of cooperatively and noncooperatively interacting endogenous (Ca^{2+}, Mg^{2+}, ATP, calmodulin) and exogenous (caffeine, ryanodine) ligands acting at the cytosolic side of the channel (Meissner, 1992, 1994). Potentiation of RyR activity by luminal Ca^{2+} appears to be a case of allosteric modulation involving a luminal regulatory site. Luminal Ca^{2+} enhanced the sensitivity of the cytosolic activation mechanism to Ca^{2+}, and it essentially reversed inhibition of the channel induced by high cytosolic Ca^{2+}. The relief of the cytosolic Ca^{2+}-induced inhibition by luminal Ca^{2+} is consistent with our previous finding that luminal Ca^{2+} reduces the level of inhibition of the channel by the allosteric inhibitor tetracaine (Györke et al., 1994). It has been demonstrated that local anesthetics (i.e., procaine) reduce the activity of the RyR by promoting transitions to a long inactivated state in a manner similar to that of inhibition caused by high cytosolic Ca^{2+} (Zahradnikova and Palade, 1993). Thus it is conceivable that high cytosolic Ca^{2+} and tetracaine inhibit the channel through the same mechanism, which can be reversed by luminal Ca^{2+}.

Physiological implications

Nuclear magnetic resonance studies suggest that free intra-SR [Ca^{2+}] in heart varies from 1 to 5 mM (Chen et al., 1996). Thus the effects of luminal Ca^{2+} on channel activity occur within the range of free [Ca^{2+}] in the SR and may represent a physiological mechanism whereby luminal Ca^{2+} regulates Ca^{2+} release. In the CICR process, where Ca^{2+} is both the triggering signal and the output signal, the danger of spontaneous oscillations is always present. Theoretical studies suggest that to maintain a stable CICR, the cells may have to self-regulate their release gain by adjusting the activity of the RyRs to the SR Ca^{2+} load (Stern, 1992b). We have shown that the activity of the RyR is under continuous control of luminal [Ca^{2+}]. Lowering Ca^{2+} in the lumen of the SR would decrease channel activity, thereby stabilizing CICR during release. Interestingly, the effect of luminal Ca^{2+} was coupled to inhibition of RyR by high cytosolic Ca^{2+} (Fig. 8). Thus these two stabilizing mechanisms could reinforce each other during the release process when a decrease in [Ca^{2+}] in the SR would be accompanied by an increase in cytosolic [Ca^{2+}]. Alternatively, luminal Ca^{2+}-sensitive release channels could provide an adjustable leak pathway through which the cells could self-regulate their SR Ca^{2+} load to maintain a stable CICR. Consistent with this possibility, cardiac cells exhibit a considerable diastolic Ca^{2+} release, primaraly through RyRs, which increases upon elevation of the SR Ca^{2+} load (Bassani et al., 1997). Under pathological conditions of Ca^{2+} overload, the destabilizing effects of increased SR Ca^{2+} content would be exacerbated by increased activity of the channel caused by luminal Ca^{2+}, resulting in increased spontaneous Ca^{2+} release activity.

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