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Comparison of the Effects Exerted by Luminal Ca^{2+} on the Sensitivity of the Cardiac Ryanodine Receptor to Caffeine and Cytosolic Ca^{2+}

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Abstract. Ca^{2+} released from the sarcoplasmic reticulum (SR) via ryanodine receptor type 2 (RYR2) is the key determinant of cardiac contractility. Although activity of RYR2 channels is primary controlled by Ca^{2+} entry through the plasma membrane, there is growing evidence that Ca^{2+} in the lumen of the SR can also be effectively involved in the regulation of RYR2 channel function. In the present study, we investigated the effect of luminal Ca^{2+} on the response of RYR2 channels reconstituted into a planar lipid membrane to caffeine and Ca^{2+} added to the cytosolic side of the channel. We performed two sets of experiments when the channel was exposed to either luminal Ba^{2+} or Ca^{2+}. The given ion served also as a charge carrier. Luminal Ca^{2+} effectively shifted the EC_{50} for caffeine sensitivity to a lower concentration but did not modify the response of RYR2 channels to cytosolic Ca^{2+}. Importantly, luminal Ca^{2+} exerted an effect on channel gating kinetics. Both the open and closed dwell times were considerably prolonged over the whole range (response to caffeine) or the partial range (response to cytosolic Ca^{2+}) of open probability. Our results provide strong evidence that an alteration of the gating kinetics is the result of the interaction of luminal Ca^{2+} with the luminally located Ca^{2+} regulatory sites on the RYR2 channel complex.

Key words: Ryanodine receptor — Luminal Ca^{2+} — Caffeine — Ca^{2+} sensitivity — Planar lipid bilayer — Channel gating kinetics

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Introduction

In cardiac muscle, the Ca^{2+} required for contractile activation is rapidly released from the sarcoplasmic reticulum (SR) in response to entry of small amounts of Ca^{2+} from the extracellular space to the cytosol. The key role in this process, termed *Ca^{2+}-induced Ca^{2+} release* (CICR), is played by the cardiac ryanodine receptor (RYR2)/Ca^{2+} release channel clustered in the SR membrane (Fabiato, 1985). Eighty percent of the molecular mass of the RYR2 channel is exposed to the cytosol and only 20% is embedded in membrane, indicating that some regions are accessible from the lumen. The big cytoplasmic domain is the main regulatory domain of the channel, where the binding sites for ligands such as Ca^{2+}, caffeine, adenosine triphosphate (ATP), and Mg^{2+} are localized (Meissner, 1994). However, there is growing evidence that luminal regions of RYR2 channels may also contribute to the regulation of channel function. Fabiato & Fabiato (1979) demonstrated that the magnitude of CICR in skinned cardiac cells was enhanced as the Ca^{2+} loading of the SR was elevated. Fabiato (1992) later proposed that there are regulatory binding sites for Ca^{2+} in the lumen of the SR. This hypothesis focused more attention on luminal Ca^{2+} as a regulator, and experiments on different levels were performed to clarify the role of luminal Ca^{2+} in the regulation of CICR. Evidence from single-channel studies of RYR2 channels also points to a regulatory role for luminal Ca^{2+}. It has been consistently shown under different experimental conditions that elevating luminal Ca^{2+} leads to an increase in RYR2 channel activity (Sitsapesan & Williams, 1994a, 1997; Lukyanenko, Györke & Györke, 1996; Györke & Györke, 1998; Xu & Meissner, 1998; Ching, Williams & Sitsapesan, 2000). The results can be interpreted on the basis

of two proposed mechanisms of luminal Ca^{2+} regulation. One model suggests that Ca^{2+} flowing via the RYR2 channel activates the channel by having access to cytosolic Ca^{2+} regulatory sites (''feed-through'' model). The alternative suggestion is that luminal Ca^{2+} acts at distinct sites on the luminal side of the Ca^{2+} release complex (''true luminal regulation'' model). The feed-through model is favored by Xu & Meissner (1998). In support of this concept, they revealed a close correlation between the effects of luminal Ca^{2+} on RYR2 activity and the magnitude of Ca^{2+} flux from lumen to cytosol. However, the aforementioned findings do not rule out the possibility that luminal Ca^{2+} acts on the luminal side of the RYR2 channel. This issue was addressed directly by Györke & Györke (1998), who performed measurements at high membrane potentials, when the electrochemical gradient did not support lumen-to-cytosol Ca^{2+} fluxes, and at high cytosolic Ca^{2+} concentrations in order to saturate cytosolic Ca^{2+} activating sites. In their experiments, luminal Ca^{2+} still potentiated native canine RYR2 channels, thus supporting the existence of luminally located Ca^{2+} regulatory sites. The subsequent work of Ching et al. (2000) provided further strong evidence in favor of the true luminal regulation model. The ability of native RYR2 channels to respond to luminal Ca^{2+} was altered after the channels were exposed to luminal trypsin. Apparently, some of the luminal Ca^{2+} regulatory sites were damaged. The source of the aforementioned discrepancies is unclear, but they might reflect differences in the degree of protein purification. It has been pointed out that harsher purification procedures make the channel more prone to the feed-through action of luminal Ca^{2+} (Györke et al., 2004). Purification can result in a loss of some regulatory proteins involved in luminal Ca^{2+} sensing or damage of regulatory Ca^{2+} binding sites located directly on the luminal side of the RYR2 channel. Consistent with this hypothesis, a complex of calsequestrin, triadin 1 and junctin was found to communicate changes in luminal Ca^{2+} to the RYR2 channel (Györke et al., 2004). Triadin 1 and junctin anchor calsequestrin, which serves as the actual luminal Ca^{2+} sensor to the RYR2 channel. At low luminal Ca^{2+} concentrations, calsequestrin inhibited activity of the RYR2 channel, whereas elevating the luminal Ca^{2+} led to a dissociation of calsequestrin from the triadin 1- junction complex and thus a gradual activation of the channel. However, this finding did not exclude the possibility that luminal Ca^{2+} exerts activation effects by binding directly to the luminal aspect of the RYR2 channel. Indeed, a detailed study on the skeletal isoform of RYR channels (RYR1) showed that an increase in the luminal Ca^{2+} concentration caused a biphasic increase in channel activity. An initial, rapid phase was fully reversible and therefore attributed to a direct effect of luminal Ca^{2+}. A second, slow phase was interpreted as the result of calsequestrin dissociation from the channel because full recovery required the addition of excess calsequestrin (Beard et al., 2002; Beard, Laver & Dulhunty, 2004). A similar study should be conducted for the RYR2 channel to test whether the reported effect of luminal Ca^{2+} is partially or fully mediated by the action of calsequestrin.

Most reports on the effect of luminal Ca^{2+} on RYR2 channels have focused on determination of the differences in channel activity induced by elevating luminal Ca^{2+}, whereas a detailed examination of channel gating kinetics has not been of particular interest. Györke & Györke (1998) performed a partial analysis showing that luminal Ca^{2+} enhanced channel activity, primarily by increasing the number of openings. In contrast, Xu & Meissner (1998) found that the effect of luminal Ca^{2+} was manifested predominantly by increased open dwell times, while the number of events remained unchanged. This inconsistency is likely to be attributable to the application of different types of cytosolic activators (caffeine, ATP) that exert their own effects on gating kinetics. To contribute to a deeper understanding of the mechanism by which luminal Ca^{2+} regulates the RYR2 channel, we investigated its effects on the caffeine and cytosolic Ca^{2+} sensitivity of a single RYR2 channel, using the planar lipid bilayer method. Two sets of experiments were performed under asymmetric conditions using either 53 mM luminal Ba^{2+} or 53 mM luminal Ca^{2+} as a charge carrier. Furthermore, we examined the effects of luminal Ca^{2+} on the gating kinetics of the RYR2 channel over the whole range of channel activity. Our results suggest that luminal Ca^{2+} exerts its effect on the RYR2 channel by enhancing its sensitivity to cytosolic activators such as caffeine. Moreover, the open and closed dwell times were considerably prolonged over the whole range (response to caffeine) or the partial range (response to cytosolic Ca^{2+}) of open probability. Our results indicate that the effect on gating kinetics is likely attributed to the action of luminal Ca^{2+} on the luminal face of the channel, providing further evidence in support of the existence of distinct intraluminal Ca^{2+} sensing sites which regulate the behavior of the RYR2 channel.

Materials and Methods

Preparation of SR Membrane Vesicles

Cardiac SR microsomes were isolated from Wistar rat heart according to Buck, Lachnit & Pessah (1999) with a few modifications. The isolated left ventricles from four hearts (4 g) were homogenized with a Tissue Tearor (Biospec Products, Inc.,

Bartlesville, OK, USA) in 5 volumes of homogenization buffer (1 M KCl, 10 mM Tris-maleate) and a cocktail of protease inhibitors (Roche Applied Science, Mannheim, Germany; 1 mM benzamidine, 5 μg/ml pepstatin, 5 μg/ml leupeptin, 1 μM calpain inhibitor I, 1 μg/ml aprotinin, 1 mM 4-(2-Aminoethyl)-benzenesolfonyl . The homogenate was centrifuged for 20 min at $10,000 \times g_{max}$ at 4°C. The supernatant was discarded, and the remaining pellet was homogenized in an ice-cold homogenization buffer and centrifuged for 20 min at $6,000 \times g_{max}$ at 4°C. The supernatant was centrifuged for 25 min at $24,000 \times g_{max}$ at 4°C, and the resulting supernatant was further centrifuged for 120 min at $41,000 \times g_{max}$ at 4°C. The final pellet was resuspended in resuspension buffer: 10% sucrose, 10 mM Tris-maleate, 0.9% NaCl (pH 6.8). Aliquots were snap-frozen in liquid N_2 and stored at −70°C until used.

Single-Channel Recordings

RYR2 channels were incorporated into a planar lipid bilayer and single-channel currents recorded under voltage-clamp conditions. Cardiac SR microsomes were added to the *cis* chamber near the planar lipid bilayer formed from a 3:1 mixture of DOPE/DOPS (Avanti Polar Lipids, Alabaster, AL) across a 150 μm aperture in the wall of a polystyrene cup. Fusion of microsomes was promoted by KCl added to the *cis* chamber (corresponding to cytosol). After incorporation of a Ca^{2+} channel, the KCl gradient was eliminated by perfusion of the *cis* chamber with *cis* solution (10 ml). The *cis* chamber was filled with 1 ml of 250 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 125 mM Tris, 50 mM KCl, 1 mM ethyleneglycoltetraacetic acid (EGTA) and 0.5 or 0.36 mM $CaCl_2$ (pH 7.35); and the *trans* chamber (corresponding to lumen) was filled with 1 ml of 53 mM $Ca(OH)_2$, 50 mM KCl, 250 mM HEPES or 53 mM $Ba(OH)_2$, 50 mM KCl and 250 mM HEPES (pH 7.35). The free Ca^{2+} concentration was calculated by WinMaxc32 version 2.50 (http://www.stanford.edu/~cpatton/maxc.html). In cytosolic Ca^{2+}/Ba^{2+} competition experiments, various mixtures of 1 mM EGTA, 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM N-Carboxylmethyl-N-(2-hydroxyethyl)-N,N′-ethylenediglycine (HEDTA) were used to buffer both cytosolic Ca^{2+} and Ba^{2+}. To investigate the possibility that luminal Ba^{2+} modulates RYR2 channel activity by direct binding to the luminal face of the channel, experiments were conducted in asymmetric Cs^+ solutions (*cis* solution = 400 mM $CsCH_3O_3S$, 10 mM HEPES, 1 mM EGTA, 0.5 mM $CaCl_2$, 8 mM caffeine [pH 7.35]; *trans* solution = 20 mM $CsCH_3O_3S$, 10 mM HEPES [pH 7.35]). Cs^+ was used as a charge carrier. The *trans* chamber was connected to the head-stage input of a Warner BC-525D amplifier (Warner Instruments, Hamden, CT), and the *cis* chamber was held at ground. The holding potential was 0 m*V* in all experiments. Electrical signals were filtered at 1 kHz, digitized at 4 kHz and analyzed. Data acquisition and analysis were performed with a commercially available software package (pCLAMP 5.5; Axon Instruments, Burlingame, CA) using an IBM-compatible Pentium computer and an A/D-D/A converter (Digidata 1322A, Axon Instruments). The open probability (P_o) was calculated from continuous records of >2-min duration. For the purpose of gating kinetics analysis, the records were divided into 30-s intervals for luminal Ba^{2+} and 60-s intervals for luminal Ca^{2+}. The analyzed intervals were prolonged for luminal Ca^{2+} due to an insufficient number of events collected on shorter intervals. The average open and closed times were calculated on these intervals as a standard arithmetic average. The resulting values for luminal Ca^{2+} and Ba^{2+} were further averaged on the defined intervals of P_o and statistically compared. At $P_o \sim 0.5$, the open and closed time histograms were constructed and fit by a sum of exponential curves, yielding values of the mean open and closed times. The results are reported as average ± standard deviation (SD). The significance of differences was analyzed by the Student *t*-test with Welch's approximation and statistical significance was accepted at $P < 0.05$.

Results

SR microsomes were fused into a planar lipid membrane, and single-channel currents through RYR2 channels were recorded under asymmetric conditions with either luminal Ca^{2+} or luminal Ba^{2+} as a charge carrier. The net current at 0 m*V* membrane potential was in the lumen-to-cytosol (*trans* to *cis*) direction, and the magnitude was constant. Experiments with luminal Ba^{2+} were considered to serve as a control. To exclude the potential masking effects of contaminating Ca^{2+}, 1 mM EGTA was added to the *trans* solution containing $Ba(OH)_2$. To establish that experiments with luminal Ba^{2+} can indeed be used as a control, we tested whether gradual elevation of luminal Ba^{2+} concentration up to 53 mM results in changes in channel activity. Experiments were performed in the absence of luminal Ca^{2+}, and current recordings were obtained in asymmetric 400/20 mM (*cis*/*trans*) Cs^+ solutions at 0 m*V* holding potential. When luminal Ba^{2+} concentration was <40 mM, the primary charge carrier was Cs^+ and a cytosol-to-lumen ion flux through the channel pore was favored. However, elevation of luminal Ba^{2+} concentration from 40 to 53 mM led to a change in the current direction. Ba^{2+} became the primary charge carrier defining the current direction from lumen to cytosol. To clearly see a potential decrease or increase in channel activity, RYR2 channels were activated by ~8 mM caffeine to $P_o \sim 0.5$. Free cytosolic Ca^{2+} concentration was kept constant (100 nM). Under these experimental conditions, we did not observe any significant activation or inhibition of the RYR2 channel when Ba^{2+} concentration on the luminal side was varied over a range from 0 to 53 mM (*data not shown*).

Effects of Luminal CA^{2+} on Caffeine-Activated RYR2 Channels

Caffeine has been widely used as a pharmacological tool for releasing Ca^{2+} from intracellular stores in the study of excitation-contraction coupling (Fabiato & Fabiato, 1976; Endo & Kitazawa, 1978; Song et al., 2002; Yoshihara et al., 2005). In our work, we used caffeine as a probe to clarify the way in which luminal Ca^{2+} affects the function of RYR2 channels. All of the experiments were conducted at ~90 nM cytosolic Ca^{2+} when RYR2 channels exhibited low activity regardless of whether Ca^{2+} or Ba^{2+} was present on the luminal side of the channel. In Figure 1A, a single RYR2 channel was recorded in the presence of various concentrations of caffeine using either luminal

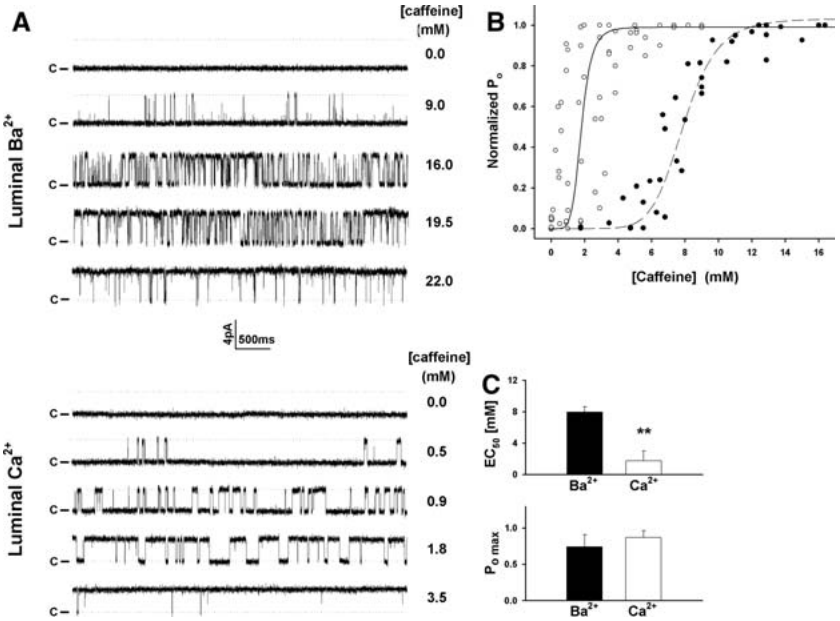

Fig. 1. Effects of luminal Ca^{2+} on the response of the RYR2 channel to caffeine. (*A*) Representative current traces of single RYR2 channel at varying concentrations of caffeine applied from the cytosolic side of the channel in the presence of either luminal Ba^{2+} (*upper traces*) or luminal Ca^{2+} (*lower traces*). Channel openings are in the upward direction. *Dashes* at the left of the tracings indicate closed state of the channel (C). (*B*) The relationship between normalized P_o and caffeine concentration is displayed for each analyzed experiment. Single-channel activities were determined at 0 m*V* potential and under asymmetric conditions using either luminal Ba^{2+} (●) or luminal Ca^{2+} (○) as a charge carrier. (*C*) Statistical comparison of average EC_{50} and $P_{o\ max}$ for caffeine response of RYR2 channel recorded at either luminal Ba^{2+} or Ca^{2+}. The graphs show that luminal Ca^{2+} shifted significantly the EC_{50} to lower values (1.76 ± 1.25 mM for luminal Ca^{2+} *vs.* 7.94 ± 0.69 mM for luminal Ba^{2+}, $^{**}P < 0.01$) but did not exert any effect on the maximal extent of channel activation (0.87 ± 0.10 for luminal Ca^{2+} *vs.* 0.74 ± 0.17 for luminal Ba^{2+}). Data are presented as average ± SD. More than four experiments were used to calculate average values.

Ba^{2+} (upper traces) or luminal Ca^{2+} (lower traces) as a charge carrier. An elevation of the caffeine concentration resulted in the sequential activation of the channel approximately to its maximum extent under both conditions. Figure 1B summarizes these experiments by plotting P_o against caffeine concentration for each experiment. Individual curves were fit with the Hill equation, and the values of EC_{50} (concentration of caffeine needed to achieve half-maximal activation) were further averaged. It is apparent that in the presence of luminal Ca^{2+} the EC_{50} was significantly reduced from 7.94 ± 0.69 mM ($n = 5$) to 1.76 ± 1.25 mM ($n = 8$) ($P < 0.01$) (Fig. 1C). The maximal extent of channel activation by caffeine was not affected by luminal Ca^{2+}. $P_{o\ max}$ was 0.74 ± 0.17 ($n = 5$) for luminal Ba^{2+} and 0.87 ± 0.10 ($n = 8$) for luminal Ca^{2+} (Fig. 1C). Our results support and complement the finding of Xu & Meissner (1998), who kept the concentration of caffeine constant and increased the luminal Ca^{2+} concentration.

An additional source that can contribute to elucidating the molecular mechanism of luminal Ca^{2+} action is the analysis of channel gating kinetics. In the presence of low caffeine and cytosolic Ca^{2+} concentrations, an insufficient number of open and closed events for constructing and fitting dwell time histograms was collected. Therefore, as a first step, we determined the average dwell times calculated as a standard arithmetic average (Fig. 2).

It is apparent from the representative current traces (Figure. 1A) that luminal Ca^{2+} influenced not only the EC_{50} for caffeine activation but also the duration of open and closed events. Figure 2 summarizes the results of gating kinetics analysis. The average open and closed times and the frequency of opening as an additional parameter describing channel behavior were plotted against P_o. In both cases, for luminal Ba^{2+} and luminal Ca^{2+}, the activation of the channel by caffeine was manifested by a prolongation of the average open time, a shortening of the average closed time and a bell-shaped dependence of the frequency of opening on P_o. However, in the presence of luminal Ca^{2+}, the RYR2 channel exhibited a much lower frequency of opening over the whole range of P_o. The maximum value reached for luminal Ba^{2+} was 65 ± 16 Hz ($n = 4$) and that for luminal Ca^{2+} was 22 ± 7 Hz ($n = 5$). Luminal Ca^{2+} effectively slowed down the channel gating kinetics, inevitably resulting in significant prolongation of average open and closed times in order to reach a similar degree of activation as for luminal Ba^{2+}. This conclusion was confirmed by statistical comparison of gating kinetics parameters over the whole range of channel activity (Fig. 2).

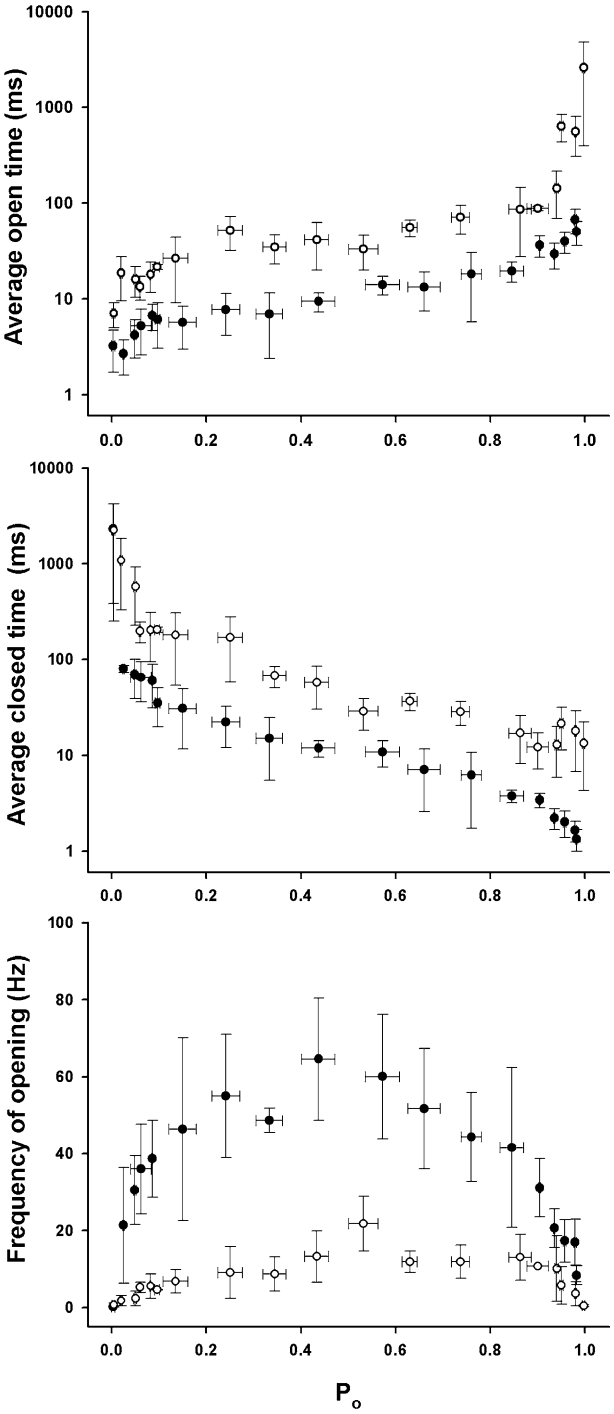

Fig. 2. Gating kinetics of caffeine-activated RYR2 channel is modified by luminal Ca^{2+}. Average open time, closed time and frequency of opening accumulated from 30-s recordings for luminal Ba^{2+} (●) and 60-s recordings for luminal Ca^{2+} (○) were further averaged on defined intervals of P_o and compared. Statistically significant differences between luminal Ba^{2+} and Ca^{2+} ($P < 0.05$) are shown for all tested intervals of P_o except the first interval for the average closed time *vs.* P_o dependence and the first and the last intervals for the frequency of opening *vs.* P_o dependence. Error bars represent SD.

At $P_o \sim 0.5$, the frequency of opening reached a maximum; therefore, a sufficient number of events for the construction and fitting of dwell time distributions was collected. For luminal Ba^{2+}, the open and closed time distributions were well fit by the sum of three exponential curves, demonstrating the occupancy of at least three different open and three different closed states. The mean dwell time and corresponding occupancy ($n = 4$) for each open/closed component are listed in Table 1. Similarly, luminal Ca^{2+} produced a pattern of gating which was also characterized with at least three open and three closed states (Table 1). Each of the three open and two of three closed times (τ_{C2} and τ_{C3}) determined for luminal Ca^{2+} were significantly longer than for luminal Ba^{2+}. Furthermore, the channel exposed to luminal Ca^{2+} preferentially occupied the states with the two longest open times (τ_{O2} and τ_{O3}) in contrast to luminal Ba^{2+}, where the states with the two shortest open times were preferred (τ_{O1} and τ_{O2}). A similar tendency as in the case of the open states was found for the occupancy of closed states in the presence of luminal Ba^{2+}, and some minor differences were revealed for luminal Ca^{2+} when the channel preferentially occupied the state with the longest and the shortest closed times (τ_{C1} and τ_{C3}). Taken together, luminal Ca^{2+} significantly prolonged the mean open and closed times and, in general, shifted the occupancy in favor of states with longer dwell times.

The enhanced sensitivity of the RYR2 channel to caffeine might be explained by both of the suggested mechanisms of luminal Ca^{2+} regulation. To separate them, we performed an additional set of experiments in the absence of luminal Ca^{2+} to eliminate its direct effects on the luminal face of the channel. Furthermore, an accumulation of Ca^{2+} ions near the cytosolic domain of the channel was mimicked by elevating the cytosolic Ca^{2+} concentration above basal level (~90 nM). Under these conditions, we tested the sensitivity of the RYR2 channel to caffeine. EC_{50} values were plotted against the corresponding cytosolic Ca^{2+} concentrations (Fig. 3A). Cytosolic Ca^{2+} (173 nM) was able to shift the EC_{50} for caffeine sensitivity to a similar concentration as was found for luminal Ca^{2+} ($EC_{50} = 1.57 \pm 1.00$ mM, $n = 4$; $EC_{50} = 1.76 \pm 1.25$ mM, $n = 8$, respectively). Furthermore, we compared the gating kinetics obtained from this set of experiments with those determined for the caffeine-activated channel exposed to luminal Ca^{2+} and 90 nM cytosolic Ca^{2+}. The average open and closed times were significantly shorter, and the frequency of opening was significantly higher over the whole range of P_o (Fig. 3B). Again, at $P_o \sim 0.5$, we performed a detailed analysis of gating kinetics by fitting open and closed time histograms by the sum of three exponential curves. The resulting parameters are listed in Table 2. All of

Table 1. Effects of luminal Ca^{2+} on mean dwell times of caffeine-activated RYR2 channel determined at $Po \sim 0.5$

	Mean open time (ms)			Occupancy (%)		
Luminal	τ_{O1}	τ_{O2}	τ_{O3}	W_{O1}	W_{O2}	W_{O3}
Ba^{2+}	0.86 ± 0.10	3.56 ± 0.50	16.78 ± 2.56	47.70 ± 2.20	29.20 ± 1.60	23.00 ± 3.70
Ca^{2+}	2.44 ± 0.26**	17.64 ± 0.91**	77.24 ± 8.95**	17.70 ± 2.60**	51.20 ± 5.40**	31.00 ± 7.40
	Mean closed time (ms)			Occupancy (%)		
Luminal	τ_{C1}	τ_{C2}	τ_{C3}	W_{C1}	W_{C2}	W_{C3}
Ba^{2+}	0.83 ± 0.47	3.86 ± 1.46	17.49 ± 1.40	41.00 ± 4.00	32.90 ± 6.42	25.93 ± 7.26
Ca^{2+}	0.73 ± 0.21	9.92 ± 3.40*	51.73 ± 5.60**	38.00 ± 16.00	16.20 ± 7.60*	45.70 ± 9.10*

Data are presented as average ± SD of four different experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* values determined for luminal Ba^{2+}.

the three open times and two of the three closed times were significantly shorter. Furthermore, the occupancy of open and closed states characterized by the two shortest times (τ_{O1}, τ_{O2} and τ_{C1}, τ_{C2}) was preferred. Whereas the shift in EC_{50} of caffeine sensitivity might be interpreted in terms of both proposed mechanisms of luminal Ca^{2+} regulation, the outcomes of a gating kinetics analysis are difficult to reconcile with the feed-through model.

Luminal CA^{2+} and its Impact on Cytosolic CA^{2+}-activated RYR2 channels

In most studies, the activation effect of luminal Ca^{2+} was not seen when solely Ca^{2+} was used as a cytosolic activator of RYR2 channels (Sitsapesan & Williams, 1994a, 1997; Györke & Györke, 1998). We reexamined this conclusion under our experimental conditions and performed a detailed gating kinetics analysis. This set of experiments was conducted in the absence of caffeine, and RYR2 channels were activated solely by cytosolic Ca^{2+}. Recordings at five different cytosolic steady-state Ca^{2+} concentrations are shown for both luminal Ba^{2+} (Fig. 4A, upper traces) and luminal Ca^{2+} (Fig. 4A, lower traces). All channels exhibited very low activity at <0.1 μM Ca^{2+} and reached maximal activation at 0.5 μM Ca^{2+}. The relationships between P_o and the cytosolic Ca^{2+} concentration (Fig. 4B) were analyzed using the Hill equation. These analyses yielded similar EC_{50} values of 0.18 ± 0.04 μM ($n = 10$) for luminal Ba^{2+} and 0.19 ± 0.04 μM ($n = 11$) for luminal Ca^{2+}. Likewise, the extent of maximal activation was not altered (0.94 ± 0.08 [$n = 10$] for luminal Ba^{2+} and 0.97 ± 0.07 [$n = 11$] for luminal Ca^{2+}, Fig. 4C). A detailed analysis of the gating kinetics profile of Ca^{2+}-activated RYR2 channels was one of the main aims of our study. When no flux of Ca^{2+} via channel in the lumen-to-cytosol direction is allowed (luminal Ba^{2+}), an increase in P_o to ~0.5 was due to the remarkable increase in the frequency of opening (Fig. 5). A reduction in the average closed time was found mainly to account for this observed increase in P_o during activation. A further decrease in the frequency of opening for $P_o > 0.5$ was mediated by a considerable increase in the average open time. An interesting behavior was revealed for the channel exposed to luminal Ca^{2+}. For $P_o < 0.5$, the frequency of opening was very low in comparison with luminal Ba^{2+}, thus resembling the gating kinetics of caffeine-activated RYR2 channels. Surprisingly, at $P_o \sim 0.5$, the channel switched to a different mode of gating and started to behave as in the presence of luminal Ba^{2+}. This change is documented by a considerable increase in the frequency of opening and a decrease in the average open and closed times. At $P_o > 0.5$, no significant differences in the examined parameters for luminal Ca^{2+} and Ba^{2+} were detected. This observation was further supported with the fitting of dwell time distributions by the sum of three exponential curves. At $P_o \sim 0.5$, no changes were found between luminal Ca^{2+} and Ba^{2+} in either magnitude of dwell time or occupancy of the corresponding open and closed states (Table 3).

Competition Between CA^{2+} and BA^{2+} for Cytosolic Regulatory Sites of RYR2 Channels

Knowledge about the interaction of Ba^{2+} and RYR2 channels is limited only to the findings that Ba^{2+} is not able to activate the channel from the cytosolic side (Liu, Pasek & Meissner, 1998). There is missing information about the ability of Ba^{2+} to compete with Ca^{2+} for cytosolic activation sites. However, this information is crucial for the correct interpretation of our results. To address this issue, we examined the sensitivity of RYR2 channels to cytosolic Ca^{2+} at various concentrations of cytosolic Ba^{2+}. This set of experiments was performed in the presence of luminal Ba^{2+} to avoid the direct effects of luminal Ca^{2+} on the channel properties. The determined values of EC_{50} were plotted against

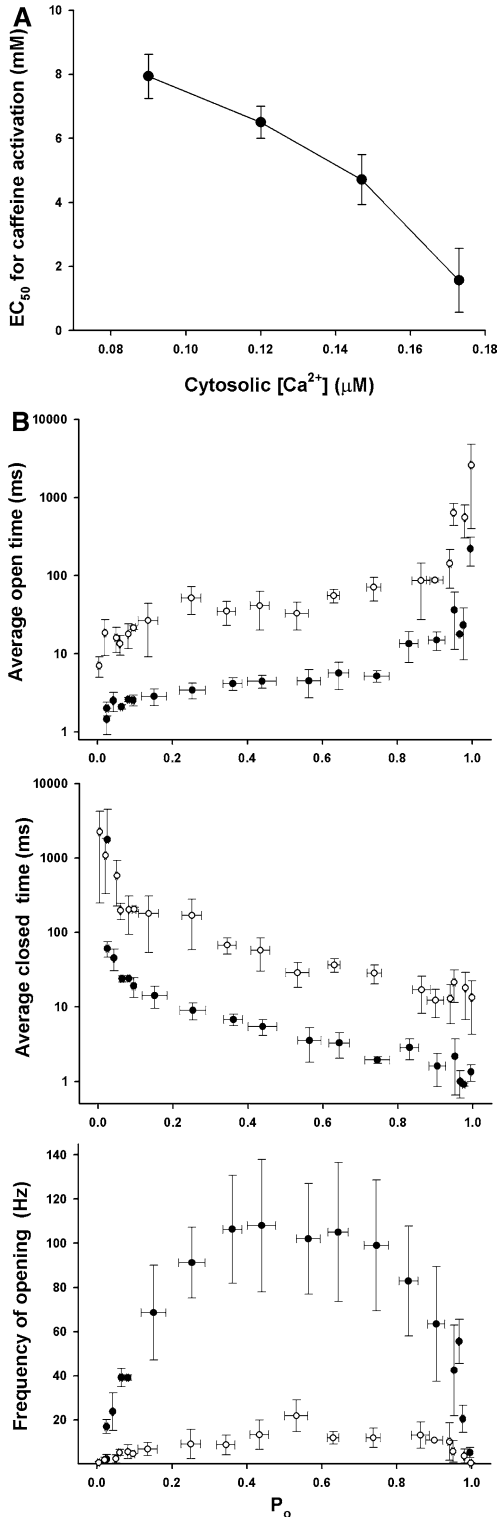

Fig. 3. Effects of cytosolic Ca^{2+} on caffeine response of RYR2 channel exposed to luminal Ba^{2+}. (*A*) Relationship between EC_{50} for caffeine activation and cytosolic Ca^{2+} concentration for RYR2 channel. Data points are displayed as average ± SD from three or four experiments. (*B*) Average open time, closed time and frequency of opening accumulated from 30-s recordings for luminal Ba^{2+} (●) and 60-s recordings for luminal Ca^{2+} (○) were further averaged on defined intervals of P_o and compared. Statistically significant differences between luminal Ba^{2+} and Ca^{2+} ($P < 0.05$) are shown for all tested intervals of P_o except the first interval for the average closed time *vs.* P_o dependence and the first and last intervals for the frequency of opening *vs.* P_o dependence. Error bars represent SD.

the concentration of cytosolic Ba^{2+} (Fig. 6). Surprisingly, we found that Ba^{2+} is an effective competitor, with the onset of competition at ~30 μM (Fig. 6). Due to the fact that there was not a difference in EC_{50} for the sensitivity of RYR2 channels to cytosolic Ca^{2+} examined for luminal Ba^{2+} and luminal Ca^{2+}, we assumed that the Ba^{2+} passing the channel could temporarily increase the Ba^{2+} concentration near the cytosolic face of the channel up to 30 μM. Considering this finding, we examined whether 30 μM cytosolic Ba^{2+} is able to shift the EC_{50} of caffeine activation. The experiments were done in the presence of luminal Ca^{2+} to eliminate the flux of Ba^{2+} to cytosol and, thus, to keep the concentration of cytosolic Ba^{2+} constant. The RYR2 channel exposed to cytosolic Ba^{2+} was activated by caffeine with an EC_{50} of 1.85 ± 1.50 mM ($n = 6$), which is comparable to the EC_{50} determined in the absence of cytosolic Ba^{2+} (1.76 ± 1.25 mM, $n = 8$). Thus, this indicates that it is not essential to consider the competition effect between Ca^{2+} and Ba^{2+} to cytosolic regulatory sites in the interpretation of observed differences in the values of EC_{50} for caffeine activation of the RYR2 channel exposed to either luminal Ca^{2+} or luminal Ba^{2+}.

Discussion

In the present study, the effects of luminal Ca^{2+} on caffeine and cytosolic Ca^{2+} activation of RYR2 channels were investigated. We already know that the RYR2 channel is activated by increasing the concentration of luminal Ca^{2+}. What is clear from the present study is that luminal Ca^{2+} exerts noticeable effects also on channel gating kinetics. We were inspired by the work of Sitsapesan & Williams (1994a), Györke & Györke (1998) and Xu & Meissner (1998). All three groups performed the single-channel measurements and systematically manipulated the concentration of luminal Ca^{2+} while the concentration of cytosolic activators was maintained constant (sulmazol, ATP, caffeine). We decided to conduct complementary experiments, keeping luminal Ca^{2+} concentration constant and changing the concentration of caffeine and Ca^{2+} applied to the cytosolic face of the RYR2 channel. We conducted experiments at either 53 mM Ca^{2+} or 53 mM Ba^{2+} on the luminal face of the channel to strengthen the proposed effects of luminal Ca^{2+}. At the same time, chosen ions served as charge carriers.

Table 2. Mean dwell times of the RYR2 channel exposed to luminal Ba^{2+} and coactivated by cytosolic Ca^{2+} and caffeine determined at *Po* ~ 0.5

	Mean open time (ms)			Occupancy (%)		
Luminal	τ_{O1}	τ_{O2}	τ_{O3}	W_{O1}	W_{O2}	W_{O3}
Ca^{2+}	2.44 ± 0.26	17.64 ± 0.91	77.24 ± 8.95	17.70 ± 2.60	51.20 ± 5.40	31.00 ± 7.40
Ba^{2+}	0.94 ± 0.26**	3.65 ± 0.83**	18.06 ± 3.01**	45.30 ± 9.00**	33.30 ± 9.40*	20.15 ± 11.00
	Mean closed time (ms)			Occupancy (%)		
Luminal	τ_{C1}	τ_{C2}	τ_{C3}	W_{C1}	W_{C2}	W_{C3}
Ca^{2+}	0.73 ± 0.21	9.92 ± 3.40	51.73 ± 5.60	38.00 ± 16.00	16.20 ± 7.60	45.70 ± 9.10
Ba^{2+}	1.03 ± 0.38	3.29 ± 0.96*	14.37 ± 2.60**	36.70 ± 22.40	43.40 ± 9.62*	20.00 ± 5.80*

A gating kinetics analysis was performed for the caffeine response of RYR2 channel exposed to luminal Ba^{2+} and 173 nM cytosolic Ca^{2+} and compared with the parameters obtained for the channel exposed to luminal Ca^{2+} and 90 nM cytosolic Ca^{2+}. Data are presented as average ± SD of four different experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* values determined for luminal Ca^{2+}.

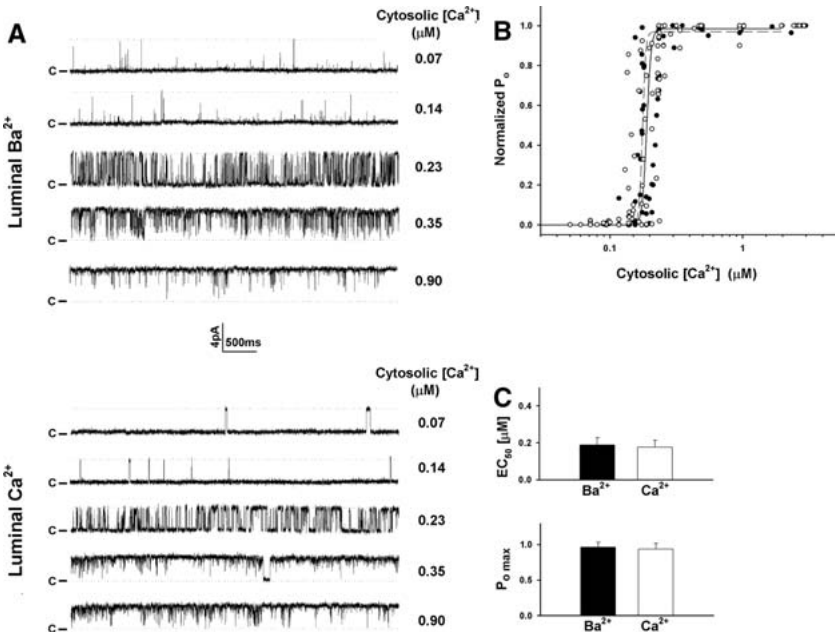

Fig. 4. Luminal Ca^{2+} does not alter response of RYR2 channel to cytosolic Ca^{2+}. (*A*) Representative current traces for RYR2 channel activated by cytosolic Ca^{2+} recorded under experimental conditions when either luminal Ca^{2+} (*upper traces*) or luminal Ba^{2+} (*lower traces*) was present. Channel openings are in the upward direction. *Dashes* at the left of the tracings indicate closed state of the channel (C). The relationship between P_o and cytosolic Ca^{2+} concentration is shown in (*B*). Data points displayed are individual P_o measurements from more than nine experiments. Single-channel activities were determined at 0 m*V* membrane potential and under asymmetric conditions using either luminal Ba^{2+} (●) or luminal Ca^{2+} (○) as a charge carrier. (*C*) Statistical analysis revealed that neither the EC_{50} (0.19 ± 0.04 μM for luminal Ca^{2+} *vs.* 0.18 ± 0.04 μM for luminal Ba^{2+}) nor the $P_{o\ max}$ was modified by luminal Ca^{2+} (0.97 ± 0.07 for luminal Ca^{2+} *vs* .0.94 ± 0.08 for luminal Ba^{2+}). Data are presented as average ± SD.

Experiments with luminal Ba^{2+} mimicked the situation when no Ca^{2+} is present on the luminal face of the channel. They were considered to be a control on the following evidence. Firstly, we found that luminal Ba^{2+} as high as 53 mM was not able to significantly activate or inhibit caffeine-activated RYR2 channels when asymmetric Cs^{+} solutions were used. Our experiments were performed in the absence of luminal Ca^{2+} to avoid any competition between luminal Ca^{2+} and Ba^{2+} for both luminal and cytosolic regulatory sites on the RYR2 channel. However, the competition between Ca^{2+} and Ba^{2+} was studied by Tripathy & Meissner (1996). They reported that the addition of 1–5 mM Ba^{2+} to the luminal side of the RYR1 channel (skeletal muscle isoform) caused a significant decrease in P_o due to competition between Ba^{2+} and Ca^{2+} for binding sites in the channel pore and on the cytosolic face. When Ca^{2+} flux from the

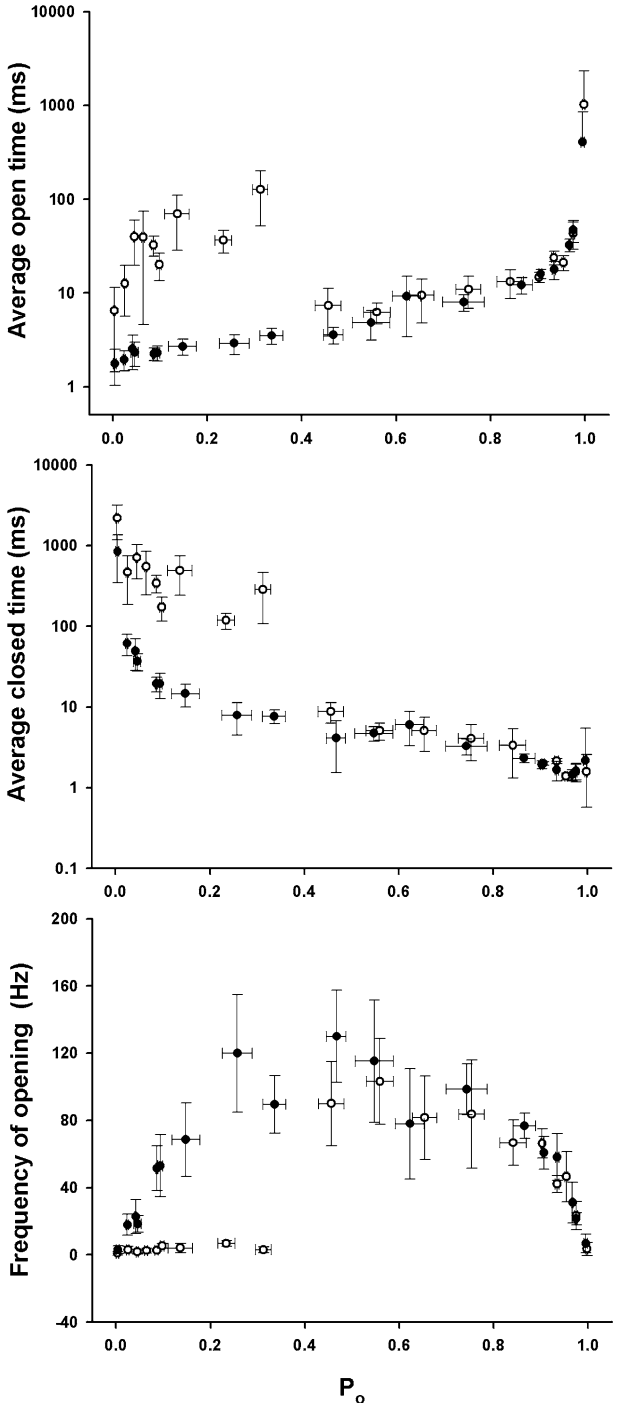

Fig. 5. Luminal Ca^{2+} exerts effect on gating kinetics of cytosolic Ca^{2+}-activated RYR2 channel with $P_o < 0.5$. Average values for open time, closed time and frequency of opening accumulated from 30-s recordings for luminal Ba^{2+} (●) and 60-s recordings for luminal Ca^{2+} (○) were further averaged on defined intervals of P_o and compared. Statistically significant differences between luminal Ba^{2+} and Ca^{2+} ($P < 0.05$) are shown for $P_o < 0.5$ except the first P_o interval for the frequency of opening *vs.* P_o dependence. For $P_o > 0.5$, differences were not statistically significant. Error bars represent SD.

lumen was eliminated by appropriate holding potential, no activation or inhibition was observed when luminal Ba^{2+} was added. Importantly, this finding supports our result. It seems that under these condition, Ba^{2+} did not compete with Ca^{2+} for luminally located regulatory sites. The possible explanation could be that 50 μM luminal Ca^{2+} is much lower than the K_D for Ca^{2+} binding to the luminal face of the RYR1 channel. Thus, Ba^{2+} had access to unoccupied binding sites, and this situation was similar to our finding. Secondly, Ba^{2+} is not able to activate RYR2 channels from the cytosolic side (Liu et al., 1998). Thirdly, we suggest that, although Ba^{2+} competes with Ca^{2+} for cytosolic regulatory sites, the observed difference in the EC_{50} for caffeine activation of RYR2 channels exposed to either luminal Ba^{2+} or luminal Ca^{2+} is presumably not attributed to this competition effect. Luminal Ba^{2+} did not alter sensitivity of the RYR2 channel to cytosolic Ca^{2+}, indicating that accumulation of Ba^{2+} near the cytosolic face due to flux of Ba^{2+} in the lumen-to-cytosol direction did not exceed 30 μM concentration when the onset of competition between Ca^{2+} and Ba^{2+} for cytosolic regulatory sites appeared. Importantly, the EC_{50} for caffeine activation was not affected by 30 μM cytosolic Ba^{2+}. Considering the fact that the sensitivity of the RYR2 channel to caffeine is regulated by subactivating concentrations of cytosolic Ca^{2+} (Sitsapesan & Williams, 1990), we can conclude that luminal Ba^{2+} permeating the channel does not cause the shift in the EC_{50} for caffeine activation to a higher concentration due to the competition with Ca^{2+} for cytosolic regulatory sites but, rather, that luminal Ca^{2+} sensitized the RYR2 channel to caffeine in respect to luminal Ba^{2+}. Fourthly, the value of EC_{50} for caffeine activation of RYR2 channels exposed to luminal Ba^{2+} obtained in our study is similar to values observed in the absence of luminal Ba^{2+} and Ca^{2+} (Xu & Meissner, 1998; Lokuta et al., 1997). At present, it is impossible to do precise quantitative comparisons of our and already published parameters of the gating kinetics for RYR2 channels due to missing detailed information about the gating kinetics profile in the absence of luminal Ba^{2+} and Ca^{2+}. Despite this, a simple coarse comparison showed us that there is some trend of a slight prolongation of the mean and average open and closed times by luminal Ba^{2+} (Sitsapesan & Williams, 1994b; Györke & Györke, 1998). Thus, it is unlikely that luminal Ba^{2+} exerts a significant effect on the channel gating kinetics and the remarkable prolongation of average and mean dwell times by luminal Ca^{2+} is obviously related to the presence of Ca^{2+} on the luminal face of the channel.

Our study led to new observations that may have important implications for understanding the principles of the mechanisms underlying the regulation of RYR2 channels by luminal Ca^{2+}.

Table 3. Mean dwell times of cytosolic Ca^{2+}-activated RYR2 channel determined at $Po \sim 0.5$ are not altered by luminal Ca^{2+}

	Mean open time (ms)			Occupancy (%)		
Luminal	τ_{O1}	τ_{O2}	τ_{O3}	W_{O1}	W_{O2}	W_{O3}
Ba^{2+}	0.95 ± 0.08	3.92 ± 0.54	52.28 ± 4.98	47.36 ± 4.50	36.13 ± 4.25	16.50 ± 3.46
Ca^{2+}	1.18 ± 0.69	4.56 ± 1.44	64.57 ± 10.94	41.46 ± 7.17	35.13 ± 5.48	23.33 ± 2.56
	Mean closed time (ms)			Occupancy (%)		
Luminal	τ_{C1}	τ_{C2}	τ_{C3}	W_{C1}	W_{C2}	W_{C3}
Ba^{2+}	0.68 ± 0.10	2.56 ± 0.79	32.54 ± 5.04	59.10 ± 8.08	29.20 ± 7.80	11.70 ± 4.32
Ca^{2+}	0.91 ± 0.11	3.97 ± 0.21	32.53 ± 4.80	50.95 ± 2.33	27.20 ± 6.80	21.80 ± 4.45

Data are presented as average ± SD of four different experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* values determined for luminal Ba^{2+}.

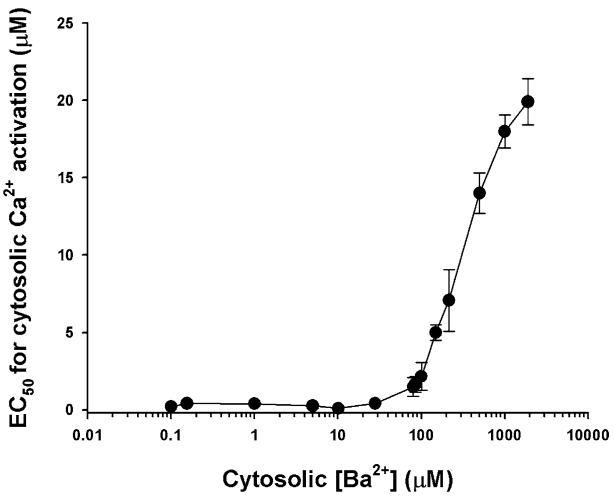

Fig. 6. Competition effect between Ba^{2+} and Ca^{2+} for cytosolic regulatory sites on the RYR2 channel. Relationship between the EC_{50} for cytosolic Ca^{2+} activation of RYR2 channel and cytosolic Ba^{2+} concentration. Various mixtures of EDTA, EGTA and HEDTA were used to chelate both cytosolic Ca^{2+} and Ba^{2+}. The onset of competition appeared at ~30 μM of cytosolic Ba^{2+}. Data points are displayed as average ± SD from five to seven experiments.

First, the sensitivity of RYR2 channels to caffeine was enhanced when luminal Ca^{2+} was present. Moreover, the gating kinetics of caffeine-activated RYR2 channels exposed to luminal Ca^{2+} was remarkably slowed down. Although the shift in EC_{50} to a lower concentration can also be induced in the absence of luminal Ca^{2+} by direct addition of Ca^{2+} to *cis* solution (cytosolic face of the channel), remarkable prolongation in dwell times typical for luminal Ca^{2+} was not induced by this maneuver. We found that even a small increase in cytosolic Ca^{2+} from 90 to 173 nM was enough to shift the EC_{50} of caffeine activation. Importantly, 173 nM cytosolic Ca^{2+} alone did not induce considerable activation of the channel but, rather, sensitized the channel to caffeine (Sitsapesan & Williams, 1990).

We simulated the potential accumulation of luminal Ca^{2+} near the cytosolic face of the channel due to Ca^{2+} flow from the lumen by simple addition of cytosolic Ca^{2+} to the *cis* solution. We are aware that our steady-state experimental model only provides a course approximation to what actually occurs in the close vicinity of the cytosolic face. According to Xu & Meissner (1998), luminal Ca^{2+} has access to cytosolic regulatory sites only during channel opening. When the channel closes, Ca^{2+} accumulated near the cytosolic face rapidly dissipates and the channel feels only the equilibrated concentration of cytosolic Ca^{2+} in the bulk. This hypothesis was further supported by findings of Laver, O'Neill & Lamb (2004). They used the competition between Mg^{2+} and Ca^{2+} for cytosolic regulatory sites of the RYR1 channel (skeletal muscle isoform) as an elegant probe for the ionic environment near the cytosolic mouth of the channel. The lack of expected competition between cytosolic Mg^{2+} and luminal Ca^{2+} for a single channel was suggested to result from limited temporal access between cytosolic and luminal solutions. Interestingly, the presence of competition for raft of RYR1 channels demonstrated that the cytosolic regulatory sites of the closed channel can still have access to luminal Ca^{2+} via the adjacent open channel.

In our experimental model, cytosolic Ca^{2+} concentration mimicking the accumulation of luminal Ca^{2+} on the cytosolic face is constant regardless of the channel state. However, according to mathematical models, it could be a relevant situation (Mazzag, Tignanelli & Smith, 2005). It was predicted that Ca^{2+} released by the open channel (residual Ca^{2+}) may influence subsequent channel gating. This effect depends on the time scale for Ca^{2+} domain formation and collapse near the cytosolic face compared to the characteristic time scale for channel gating. When the localized Ca^{2+} domain forms and collapses

slowly, the cytosolic regulatory binding sites for Ca^{2+} experience essentially the same concentration of cytosolic Ca^{2+} regardless of channel state (Mazzag et al., 2005). Our experimental conditions (~4 pA Ca^{2+} current in the lumen-to-cytosol direction, slow cytosolic Ca^{2+} chelating by 1 mM EGTA) favor slow generation and dissipation of Ca^{2+} gradients, supporting the validity of our experimental model.

In summary, we can conclude that at least alteration of the gating kinetics of the caffeine-activated RYR2 channel is accounted for by binding of Ca^{2+} to distinct luminally located regulatory sites.

Secondly, consistent with published findings (Sitsapesan & Williams, 1994a; Györke & Györke, 1998), the values of EC_{50} for cytosolic Ca^{2+} activation were not significantly different when the RYR2 channel was exposed to either luminal Ba^{2+} or Ca^{2+}. We can state that if there was a global rise in cytosolic Ca^{2+} to 173 nM due to accumulation of ions passing the channel, we would be able to see the shift in EC_{50} to a lower concentration under our experimental conditions. For this purpose, we extended the tested interval of cytosolic Ca^{2+} concentration down to 50 nM to get a definable baseline for dose response. The lack of any effect on the EC_{50} has been previously interpreted in terms of both the true luminal regulation (Sitsapesan & Williams, 1997) and feedthrough (Laver et al., 2004) models. Sitsapesan & Williams (1997), preferring the true luminal regulation model, hypothesized that the conformation of the channel activated solely by cytosolic Ca^{2+} is such that the regulatory sites for Ca^{2+} on the luminal face of the RYR2 channel to which luminal Ca^{2+} binds in the presence of a second cytosolic ligand are inaccessible. However, we revealed that luminal Ca^{2+} modified the gating kinetics of cytosolic Ca^{2+}-activated RYR2 channel, thus providing evidence that luminally located binding sites for Ca^{2+} are also accessible when cytosolic Ca^{2+} is used as a sole activator. Experiments with luminal Ba^{2+} defined the activation effect of Ca^{2+} acting solely on the cytosolic side of the channel; therefore, they were crucial for appropriate interpretation of our results. When the channel exposed to luminal Ca^{2+} exhibited low activity ($P_o < 0.5$), the gating kinetics was very slow and resembled the behavior of the caffeine-activated channel exposed to luminal Ca^{2+}. When the activity was enhanced ($P_o > 0.5$) by increasing the cytosolic Ca^{2+} concentration, the channel started to open and close more frequently, resembling the behavior of the RYR2 channel exposed to luminal Ba^{2+}. If the lumen-to-cytosol Ca^{2+} flux was required for this effect, we would expect to see a faster gating kinetics profile also for $P_o < 0.5$, inasmuch as such gating kinetics is typical for the channel exposed to luminal Ba^{2+} and activated solely by cytosolic Ca^{2+}. Furthermore, the remarkable prolongation of average dwell times for $P_o < 0.5$ is more likely accounted for by binding of Ca^{2+} to distinct luminally located regulatory sites. Considering this conclusion, we can suggest that cytosolic Ca^{2+} is a specific activator of the RYR2 channel due to its ability to take control of the channel gating, at least for $P_o > 0.5$. In contrast, caffeine does not exhibit a similar potency as cytosolic Ca^{2+} to compete with luminal Ca^{2+} for regulation of the gating behavior.

In contrast, Laver et al. (2004) favored the feedthrough model to interpret the findings that the activation of RYR2 channels by luminal Ca^{2+} required the presence of cytosolic activators other than Ca^{2+}. Considering the fact that binding of Ca^{2+} to cytosolic regulatory sites must take place for transition of the resting RYR2 channel to an open state, it has been hypothesized that luminal Ca^{2+} emanating from the channel pore does not augment channel opening because the cytosolic regulatory sites are already occupied. According to our analysis, the action of luminal Ca^{2+} should not be hampered by cytosolic Ca^{2+}. It is reasonable to assume that the channel does not recognize whether Ca^{2+} was added directly to the cytosolic side or flowed from the luminal side. When the channel opens, Ca^{2+} flux in the lumen-to-cytosol direction will cause a temporal increase in the local concentration of Ca^{2+} near the already occupied cytosolic regulatory sites. Under these conditions, dissociation/association of Ca^{2+} from or to the channel will be more frequent, leading to gradual channel activation as observed for the RYR2 channel activated solely by cytosolic Ca^{2+}.

Obviously, in order to clearly distinguish the effects attributed to the action of luminal Ca^{2+} on either the luminal or cytosolic face of the RYR2 channel, it is necessary to find an experimental approach when only one mechanism will operate. Testing the mutated RYR2 channels resistant to cytosolic Ca^{2+} activation could be the next step (Li & Chen, 2001).

In summary, we demonstrate for the first time that luminal Ca^{2+} modifies the gating kinetics of RYR2 channels manifested by a significant prolongation of the open and closed event durations over the whole range of P_o. Importantly, this effect is presumably related to the action of luminal Ca^{2+} on the luminal side of the channel. Whether the alteration of gating kinetics induced by luminal Ca^{2+} is the result of calsequestrin dissociation from the triadin 1–junctin complex or if it is attributed to the binding of Ca^{2+} to regulatory sites localized directly on the luminal part of the RYR2 channel remains to be clarified. In general, our results favor the true luminal regulation model and provide further evidence of the existence of functional Ca^{2+} regulatory sites on the luminal side of the RYR2 channel.

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