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Coupled gating modifies the regulation of cardiac ryanodine receptors by luminal Ca^{2+}

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ABSTRACT

Cardiac ryanodine receptors (RYR2s) infrequently exhibit coupled gating that is manifested by synchronous opening and closing. To better characterize this phenomenon, we investigated the regulation of coupled RYR2 channels by luminal Ca^{2+} focusing on effects that are likely mediated by the true luminal activation mechanism. By reconstituting an ion channel into a planar lipid bilayer and using substantially lower concentration of luminal Ba^{2+} (8 mM, the virtual absence of Ca^{2+}) and luminal Ca^{2+} (8 mM), we show that response of coupled RYR2 channels to caffeine at a diastolic cytosolic Ca^{2+} (90 nM) was affected by luminal Ca^{2+} in a similar manner as for the single RYR2 channel except the gating behavior. Whereas, the single RYR2 channel responded to luminal Ca^{2+} by prolongation in open and closed times, coupled RYR2 channels seemed to be resistant in this respect. In summary, we conclude that the class of Ca^{2+} sites located on the luminal face of coupled RYR2 channels that is responsible for the channel potentiation by luminal Ca^{2+} is functional and not structurally hindered by the channel coupling. Thus, the idea about non-functional luminal Ca^{2+} sites as a source of the apparent gating resistance of coupled RYR2 channels to luminal Ca^{2+} appears to be ruled out.

1. Introduction

In the heart, Ca^{2+} release from the intracellular Ca^{2+} store (sarcoplasmic reticulum, SR) through the ryanodine receptor (RYR2) channel plays an essential role in mediating the cardiac myocyte contraction. RYR2 channels are located on the SR membrane and packed into elongated clusters that exhibit unique "checkerboard-like" organization [1–3]. The striking feature of this arrangement is that the individual square-shaped RYR2 channels are in close proximity at each corner. More precisely, *in vitro* studies on reconstituted "checkerboard-like" arrays showed that channels are physically interlocked *via* specific protein–protein interactions at the corners [4]. This finding strongly supports the concept of allosteric mechanism in the channel regulation based on a direct communication among individual RYR2 channels within the array.

On the functional level, the first cooperative interactions among skeletal as well as cardiac RYR channels were reported by Marx et al. [5,6]. In both cases, they observed that multiple RYR channels opened and closed in a concerted fashion and referred to this behavior as "coupled gating". Subsequent studies of our group and others brought the further evidence about coupled RYR channels [7–12]; and thus, proving a strong platform for studying this phenomenon primarily from the biophysical point of view.

Luminal Ca^{2+} has been shown to play an important but not fully understood role in the regulation of the single RYR2 channel [10,13–21]. Although, the precise roles of Ca^{2+} feedthrough (Ca^{2+} flux *via* the RYR2 pore) and direct luminal activation mechanisms are not yet determined, the existence of luminally located Ca^{2+} sites is strongly evidenced in several functional studies [13,14,16,17,21].

For coupled RYR2 channels, a regulatory effect of luminal Ca^{2+} has not been examined extensively. Most of the studies published yet attempted to answer the question about the molecular nature of the channel coupling and the role of luminal Ca^{2+} as the source of Ca^{2+} flux in this process. Recently, Porta et al. [12] have revised the concept of Ca^{2+} flux requirement so far for coupled skeletal RYR (RYR1) channels and highlighted a potential role of direct effect of luminal Ca^{2+} on the luminal channel face in the stabilization of the coupled gating phenomenon.

In the present paper, we were interested in the luminal Ca^{2+} regulation of coupled RYR2 channels isolated from the rat heart that is mediated by a class of luminally located Ca^{2+} sites. Our main aim was to investigate the function of a single coupled unit excluding the function of individual channels in a unit that has been examined in more detail in our previous works [9,11]. Using a method of reconstitution of an ion channel into a planar lipid bilayer (BLM), we systematically examined the activation of coupled RYR2 channels by caffeine at a diastolic cytosolic Ca^{2+} (90 nM) in the presence of either luminal Ba^{2+} (8 mM, the virtual absence of luminal Ca^{2+}) or luminal Ca^{2+} (8 mM). The obtained dose responses were fitted by Hill equation and gating behavior parameters were determined for both conditions. For comparison, similar datasets were obtained and a similar analysis was

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performed for the single RYR2 channel. Although, the gating of coupled RYR2 channels was not affected by luminal Ca^{2+} in contrast to the single one, luminal Ca^{2+} substantially enhanced the sensitivity to caffeine regardless of whether RYR2 channels were single or coupled. Importantly, from all studied luminal Ca^{2+} effects, which have been shown to be mediated by the true luminal regulation mechanism, at least the modulation of caffeine sensitivity was retained and not abolished by coupled gating. Thus, we conclude that the class of Ca^{2+} sites located on the luminal face of coupled RYR2 channels that is responsible for the channel potentiation by luminal Ca^{2+} is functional and not structurally hindered by the channel coupling.

2. Material and methods

2.1. Preparation of membrane vesicles

All procedures with animals were approved by the State veterinary and food administration of the Slovak Republic (Ro-1730/11-221 and Ro-1522/10-221). Sarcoplasmic reticulum (SR) microsomes enriched in RYR2 channels were isolated from the ventricles of adult rat hearts using homogenization and ultracentrifugation steps that follow the procedure published previously [17].

2.2. Drugs and chemicals

Phospholipids were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). All other chemicals were from Sigma-Aldrich (St. Louis, MO), if not stated otherwise.

2.3. Single-channel recordings

RYR2 channels were incorporated into a planar lipid bilayer (BLM) and single-channel currents were recorded under voltage-clamp conditions. The BLMs of a 3:1 mixture of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and 1,2-dioleoyl-*sn*-glycero-3-[phospho-L-serine] (DOPS) were painted on 50–70 μm diameter circular apertures in the wall of a polystyrene cup. The *cis* chamber (corresponding to cytosol) was filled with 1 ml of 250 mM HEPES, 125 mM Tris, 50 mM KCl, 1 mM EGTA, and 0.5 mM $CaCl_2$ (pH = 7.35), and the *trans* chamber (corresponding to lumen) was filled with 1 ml of 8 mM $Ca(OH)_2$ or $Ba(OH)_2$, 50 mM KCl, and 22 mM HEPES (pH = 7.35). In the *cis* solution, Ca^{2+} was buffered using ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) and free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_C$) was calculated by WinMaxc32 version 2.50 (http://www.stanford.edu/~cpatton/maxc.html). To make free $[Ca^{2+}]_C$ calculation more accurate the purity of EGTA was determined by potentiometric titration. Experiments with 8 mM $[Ba^{2+}]_L$ as the sole permeant ion mimicked the situation when no Ca^{2+} is present at the luminal face of the RYR2 channel. The level of contaminating Ca^{2+} in the *trans* solution with 8 mM $[Ba^{2+}]_L$ was 5 μM, as determined by Ca^{2+}-selective electrode (Type 25-20+, Elektrochemické detektory, Ltd., Turnov, Czech Republic) [21]. In all datasets, caffeine was used to gradually activate RYR2 channels up to the maximal level. Cardiac SR microsomes were added to the *cis* solution and their fusion with the BLM was promoted by KCl added to the *cis* chamber. After Cl^- or K^+ currents were observed, the KCl gradient was eliminated by perfusion of the *cis* chamber with *cis* solution (10 ml). The *trans* chamber was connected to the head-stage input of a Warner BC-535D amplifier (Warner Instruments, Inc., Hamden, CT) and the *cis* chamber was held at ground. The holding potential was 0 mV in all experiments. Electrical signals were filtered through the Warner BC-535D low-pass Bessel filter at 1 kHz and digitized at 4 kHz with an A/D-D/A converter (Digidata 1322A, Molecular Devices, Sunnyvale, CA).

2.4. Single-channel analysis

Data acquisition and analysis were performed with a commercially available software package (pCLAMP 5.5, Molecular Devices, Sunnyvale, CA). The open probability (P_O) was calculated from continuous records of >2 min duration using the 50%-amplitude threshold method. To analyze coupled RYR2 channels as a single functional unit, the threshold was set between the baseline and the amplitude of the single RYR2 channel ($P_O^{coupled}$). This strategy enabled us to ignore the flicker gating manifested by brief transitions of individual RYR2 channels in the unit between open and closed states. For each dataset, the dependence of P_O on caffeine concentration was globally fitted by the Hill function with no shared parameters. For the purpose of gating behavior analysis, the records were divided into 30 s intervals. The average open and closed times and the frequency of opening were calculated on these intervals as a standard arithmetic average. The resulting values for luminal Ba^{2+} and luminal Ca^{2+} were further averaged on the defined intervals of P_O and statistically compared. The results are reported as average ± SEM. The significance of differences was analyzed by the Student t-test with Welch approximation and is regarded as statistically significant at $P < 0.05$.

3. Results

Cardiac SR microsomes were fused into the BLM, and single-channel currents were recorded under asymmetric conditions with either luminal Ba^{2+} or luminal Ca^{2+} used as the only charge carrier. We decided to use Ba^{2+} ions as a replacement for Ca^{2+} ions in order to withdraw the regulation effects of luminal Ca^{2+} predominantly on the luminal channel face. Ba^{2+} is divalent ion as Ca^{2+} and despite it competes with Ca^{2+} on both cytoplasmic as well as luminal channel faces [17,20], alone or in a robust predominance did not have the significant effect on the single RYR2 regulation by caffeine when present on the luminal face [17]. In addition, cytosolic Ba^{2+} did not activate RYR2 channels in contrary to cytosolic Ca^{2+} [20,22]. Considering the fact that used chemicals did not reach 100% purity, we assessed in our previous study [21] that the level of contaminating Ca^{2+} in the *trans* solution with Ba^{2+} was ~5 μM; thus, we can declare that our present experiments were performed in the virtual absence of luminal Ca^{2+}.

Almost all the papers published yet reported the coupled RYR activity at the considerably high luminal Ca^{2+} concentration ($[Ca^{2+}]_L$ ~ 50 mM) that is outside the physiologically relevant range. Thus, we attempted to examine coupled RYR2 channels at 8 mM $[Ca^{2+}]_L$ that is in a physiological context much closer to "cell-like" conditions. The feasibility of these conditions was validated in our previous work [9] where we studied the effect of various $[Ca^{2+}]_L$ (8–53 mM) on the coupling stability and as a control we were able to reconstitute coupled RYR2 channels also in the virtual absence of luminal Ca^{2+} using 8 mM $[Ba^{2+}]_L$.

3.1. RYR2 channel coupling alters the regulation of gating behavior by luminal Ca^{2+}

The gating behavior is of particular interest to us, because, we have previously reported that luminal Ca^{2+} (53 mM) substantially affected the single RYR2 gating and most importantly our results strongly indicated that this effect was likely attributed to the action of luminal Ca^{2+} on the luminal channel face [17]. Thus, we considered the channel gating behavior as a useful tool for the exploration of a luminal component of the coupled RYR2 regulation by luminal Ca^{2+}. Here, we determined gating parameters over the whole range of the coupled RYR2 activity that was increased by a caffeine addition from the cytosolic channel face. Fig. 1A shows representative recordings of coupled RYR2 channels obtained at 8 mM $[Ba^{2+}]_L$ (left panel) or 8 mM $[Ca^{2+}]_L$ (right panel) at caffeine concentrations spanning the whole activation range (0–15 mM, 0–5 mM, respectively). $[Ca^{2+}]_C$ was kept constant at

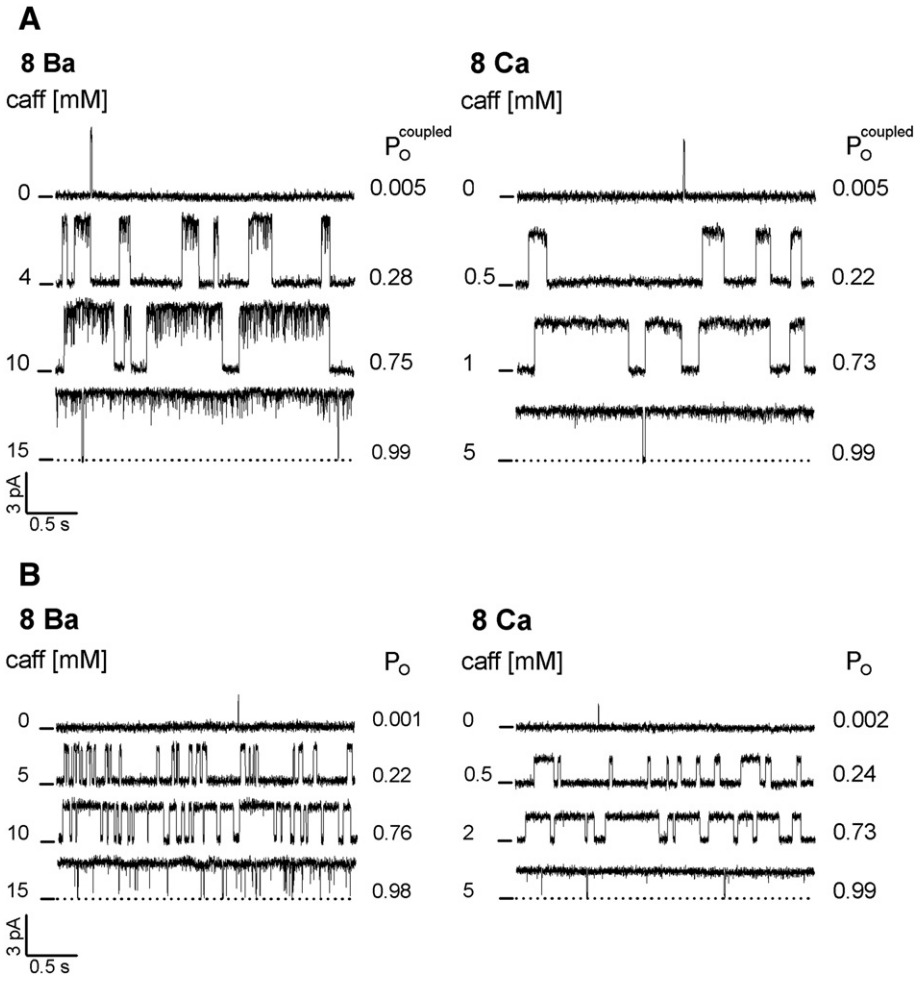

Fig. 1. Activation of RYR2 channels by caffeine. (A) Representative current traces of coupled RYR2 channels at various caffeine concentrations in the presence of 8 mM $[Ba^{2+}]_L$ (left) or 8 mM $[Ca^{2+}]_L$ (right). Increasing caffeine concentration from 0 mM to 15 mM for luminal Ba^{2+} and from 0 mM to 5 mM for luminal Ca^{2+} resulted in a gradual activation of coupled RYR2 channels up to the maximal level. Recordings were selected to illustrate the channel behavior at $P_O^{coupled}$ ~ 0, 0.25, 0.75 and 1.0. For comparison, representative current traces of the single RYR2 channel under the same experimental conditions are also shown (B left — 8 mM $[Ba^{2+}]_L$, B right — 8 mM $[Ca^{2+}]_L$). In all four datasets, $[Ca^{2+}]_C$ was 90 nM and recordings were conducted under steady-state conditions at 0 mV. Channel openings are in the upward direction. Dashes at the left of the tracings indicate the closed state of the channel.

90 nM. Under both luminal conditions, two RYR2 channels opened and closed at the same time that resulted in coupled events with double single-channel current amplitude. For comparison, Fig. 1B shows sample current traces for the single RYR2 channel under the same luminal conditions (8 mM $[Ba^{2+}]_L$ (left panel) and 8 mM $[Ca^{2+}]_L$ (right panel)). From the cytosolic side, the single RYR2 channel was activated by caffeine in the same range as for coupled channels (0–15 mM caffeine for luminal Ba^{2+} and 0–5 mM caffeine for luminal Ca^{2+}). Considering the fact that in the present study we decreased $[Ca^{2+}]_L$ to 8 mM and luminal Ca^{2+} has been shown to effectively regulate the RYR2 sensitivity to ATP in several aspects of the channel function [21], we had to carry out a new set of experiments for the single RYR2 channel using 8 mM instead of 53 mM $[Ca^{2+}]_L$ and caffeine as the cytosolic channel activator.

It is obvious directly from the raw current traces that even 8 mM $[Ca^{2+}]_L$ significantly slowed down the single RYR2 gating that is manifested by prolonged open and closed events (Fig. 1B). Surprisingly, coupled channels displayed greatly long openings and closings regardless of whether or not Ca^{2+} was present on the luminal channel face implying that luminal Ca^{2+} is not involved in the gating regulation. Here, we would like to emphasize that the focus of our present paper was narrowed only to coupled channels functioning as a single unit. Thus, we ignored remarkable brief closings from the main open state that can be easily recognized on the raw current traces when luminal Ca^{2+} was absent (Fig. 1A, left panel). We have previously suggested that intensity of such flickering likely reflects the stability of coupled gating. In addition, we reported that coupling stability was significantly enhanced by 8 mM $[Ca^{2+}]_L$ compared with 8 mM $[Ba^{2+}]_L$ [9].

To better visualize the qualitative differences in the gating regulation by luminal Ca^{2+} Fig. 2A and B shows representative recordings of caffeine activated coupled and single RYR2 channels on the expanded time scale and at the appropriate channel activity, respectively. The coupled channels were activated to $P_O^{coupled}$ ~ 0.5 by 6 mM and 0.8 mM caffeine in the presence of 90 nM $[Ca^{2+}]_C$ when exposed to luminal Ba^{2+} (Fig. 2A, top panel) or luminal Ca^{2+} (Fig. 2A, bottom panel), respectively. For comparison, Fig. 2B shows sample current traces for the single RYR2 channel under the same luminal conditions (8 mM $[Ba^{2+}]_L$ (top panel) and 8 mM $[Ca^{2+}]_L$ (bottom panel)). To increase the single RYR2 activity to P_O ~ 0.5 caffeine concentrations of 7 mM and 1 mM were used for luminal Ba^{2+} and luminal Ca^{2+}, respectively. To quantify our subjective impression from the raw current traces we determined the main gating parameters such as the average open and closed times and the frequency of opening over the whole range of a channel activity. We used the method described in our previous work [17] and Fig. 2C and D summarizes obtained results. For both coupled and single RYR2 channels, the increase in channel activity by caffeine (0–15 mM for luminal Ba^{2+} and 0–5 mM for luminal Ca^{2+}) under both tested luminal conditions arose from a prolongation of the average open time and a decrease in the average closed time. Aforementioned changes in both dwell times resulted in a biphasic dependence of the frequency of opening. While for coupled channels their gating behavior

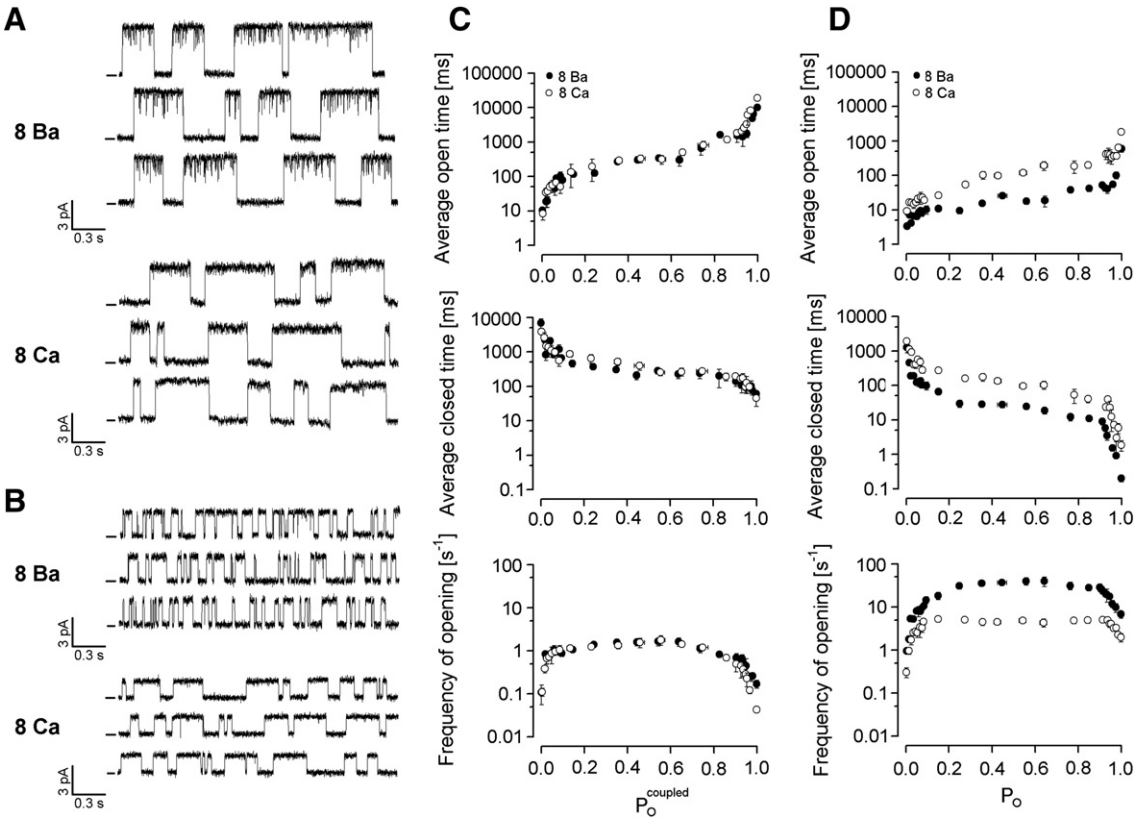

Fig. 2. Luminal Ca^{2+} regulation of the RYR2 gating behavior. (A) Representative current traces of coupled RYR2 channels activated by caffeine to $P_O^{coupled}$ ~ 0.5 in the presence of 8 mM $[Ba^{2+}]_L$ (top) or 8 mM $[Ca^{2+}]_L$ (bottom). Recordings were made at constant $[Ca^{2+}]_C$ of 90 nM and caffeine concentration of 6 mM (luminal Ba^{2+}) and 0.8 mM (luminal Ca^{2+}). (C) The gating behavior parameters for coupled RYR2 channels determined for luminal Ba^{2+} (black circles, n = 3) and luminal Ca^{2+} (open circles, n = 7). The average open time, closed time and the frequency of opening accumulated from 30 s recordings and determined for both conditions were further averaged on defined intervals of $P_O^{coupled}$ and compared. All three gating parameters of coupled RYR2 channels were similar and not statistically different when compared 8 mM $[Ba^{2+}]_L$ with 8 mM $[Ca^{2+}]_L$ on all $P_O^{coupled}$ intervals. For comparison, representative current traces (B) and the gating behavior parameters (D) of the single RYR2 channel under the same luminal experimental conditions are also shown (B top — 8 mM $[Ba^{2+}]_L$, B bottom — 8 mM $[Ca^{2+}]_L$, D (black circles) — 8 mM $[Ba^{2+}]_L$, D (open circles) — 8 mM $[Ca^{2+}]_L$). Seven single RYR2 channels were recorded for both tested conditions and channels were activated to P_O ~ 0.5 by 7 mM caffeine in the presence of luminal Ba^{2+} and 1 mM caffeine in the presence of luminal Ca^{2+}. Recordings with P_O ~ 0.5 were selected to clearly demonstrate the absent effect of luminal Ca^{2+} on the gating behavior of coupled RYR2 channels in contrast to the single RYR2 channel. All three gating parameters of the single RYR2 channel determined for luminal Ba^{2+} and luminal Ca^{2+} were shown to be significantly different on P_O ranging from 0.1 to 0.9 ($P < 0.05$). For $P_O < 0.1$ and $P_O > 0.9$, the differences were too small to achieve statistical significance for all included P_O intervals. Recordings in A and B were conducted under steady-state conditions at 0 mV. Channel openings are in the upward direction. Dashes at the left of the tracings indicate the closed state of the channel. Data in C and D are presented as average ± SEM and error bars are shown only when SEM is larger than symbol size.

was insensitive to luminal Ca^{2+}, for the single channel luminal Ca^{2+} led to the significant prolongation in both dwell times that inevitably caused a substantial reduction in the frequency of opening for P_O ranging from 0.1 to 0.9. For $P_O < 0.1$ and $P_O > 0.9$, the differences were too small to achieve statistical significance for all included P_O intervals. Note that the graph scale for a given gating parameter in Fig. 2C and D remained the same to clearly demonstrate the magnitude of differences induced by luminal Ca^{2+}. In summary, we can state that over the whole range of $P_O^{coupled}$ the gating behavior of coupled RYR2 channels activated by caffeine is not regulated by luminal Ca^{2+} that is in a big contrast to the single RYR2 channel. This finding may suggest that the class of Ca^{2+} sites on the luminal channel face involved in the channel regulation is not functional anymore. To test this hypothesis, other components from the functional profile of the single RYR2 channel that are related to the caffeine activation and apparently mediated by the true luminal regulation mechanism should be identified and explored for coupled channels.

3.2. The enhanced caffeine sensitivity caused by luminal Ca^{2+} is attributed to binding of Ca^{2+} to luminally located Ca^{2+} sites

In our previous work concentrated on the single RYR2 channel, we found that luminal Ca^{2+} greatly enhanced the channel sensitivity to caffeine [17]. Unfortunately, in 2006, we were unable to convincingly ascribe this stimulating effect of luminal Ca^{2} to either Ca^{2+} feedthrough or true luminal mechanisms, because 173 nM $[Ca^{2+}]_C$ in the virtual absence of luminal Ca^{2+} had a similar power to shift the single RYR2 sensitivity to caffeine to lower concentration as was reported for 53 mM $[Ca^{2+}]_L$ [17]. Hence, in this study, we used a completely different approach when we tested whether effects of cytosolic and luminal Ca^{2+} on caffeine action are additive or competitive. Particularly, we attempted to assess whether cytosolic Ca^{2+} competes for Ca^{2+} sites located on the cytosolic channel face with luminal Ca^{2+} permeating the RYR2 pore. We recorded the single RYR2 channel at 53 mM $[Ca^{2+}]_L$ to maximize the Ca^{2+} flux. This strategy promoted the saturation of cytosolic Ca^{2+} sites, if there are sensitive to Ca^{2+} permeating the RYR2 pore. $[Ca^{2+}]_C$ was gradually increased from 90 nM to 309 nM and the whole dose response to caffeine was examined again. Fig. 3A displays P_O plotted as a function of caffeine concentration for 132, 204 and 309 nM $[Ca^{2+}]_C$. Solid lines are the best fits by the Hill equation. In addition, the dotted curve for 90 nM $[Ca^{2+}]_C$ was replotted from our previous work [17]. Fig. 3B summarizes results and documents that the half-activating caffeine concentration, EC_{50}, is a function of cytosolic Ca^{2+} at a constant luminal Ca^{2+} and was significantly decreased from 1.76 ± 0.33 mM to 0.70 ± 0.22 mM by increasing $[Ca^{2+}]_C$ from 90 nM to 309 nM. The remaining tested $[Ca^{2+}]_C$ of 132 nM and 204 nM caused only a marginal reduction in EC_{50} (1.751 ± 0.096 mM and 1.26 ± 0.12 mM, respectively). P_{OMax} was

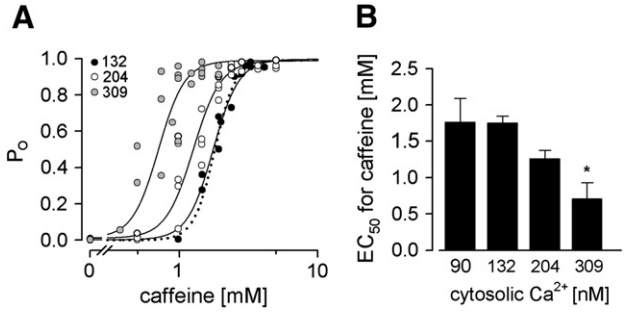

Fig. 3. Additive effect of cytosolic and luminal Ca^{2+} on the caffeine activated single RYR2 channel. (A) Caffeine dependence of P_O for 132, 204 and 309 nM $[Ca^{2+}]_C$ (black, open and gray circles, respectively). $[Ca^{2+}]_L$ was kept constant at 53 mM to ensure Ca^{2+} flux in the lumen-to-cytosol direction as big as possible in order to reliably distinguish between potential additive and competitive effects of cytosolic and luminal Ca^{2+} on the cytosolic channel face. The dotted line represents the data obtained at 90 nM $[Ca^{2+}]_C$ that are replotted from our previous work [17]. Data points displayed in each dataset are individual P_O measurements collected from more than five experiments. Solid lines are Hill curves with averaged values of parameters obtained by fitting the whole datasets for 132, 204 and 309 nM $[Ca^{2+}]_C$ simultaneously when all fitted parameters were free. (B) Cytosolic Ca^{2+} dependence of EC_{50} for caffeine. Data are represented as average ± SEM. Asterisk denotes a significant decrease of EC_{50} for caffeine ($P < 0.05$).

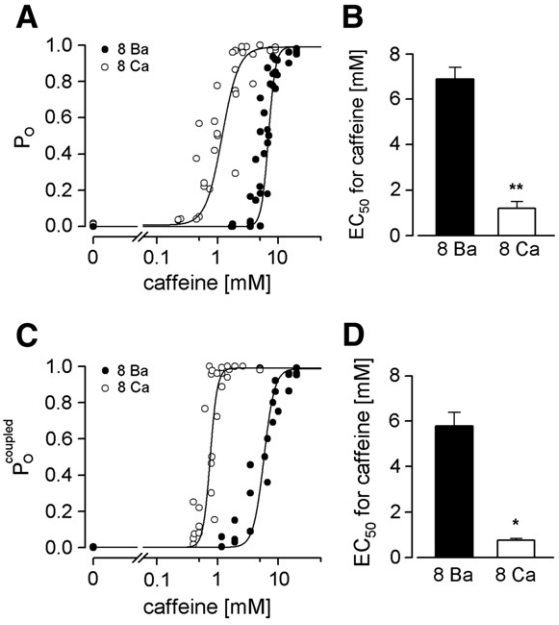

Fig. 4. Dose response of single and coupled RYR2 channels to caffeine. The relationship between P_O and caffeine concentration of single (A) and coupled (C) RYR2 channels when luminal Ba^{2+} or luminal Ca^{2+} was present (black and open circles, respectively). $[Ca^{2+}]_C$ was kept constant at 90 nM. Data points displayed in each dataset are individual P_O measurements collected from seven experiments, except for coupled channels at 8 mM $[Ba^{2+}]_L$ where we reconstituted only three coupled channel units. Lines in A and C are Hill curves with averaged values of parameters obtained by fitting the whole datasets for luminal Ba^{2+} and luminal Ca^{2+} simultaneously when all fitted parameters were free. The statistical comparison of EC_{50} for caffeine determined in the presence of either luminal Ba^{2+} or luminal Ca^{2+} for the single (B) and coupled RYR2 channels (D). Data are presented as average ± SEM. Asterisks denote a significant decrease of EC_{50} for caffeine (*$P < 0.05$, **$P < 0.01$).

not influenced by cytosolic Ca^{2+}, as we expected, because even at 90 nM $[Ca^{2+}]_C$ the channel reached almost the full activation (0.92 ± 0.05). These data indicate that under our conditions cytosolic Ca^{2+} did not compete with luminal Ca^{2+} on the cytosolic channel face because it caused an additive decrease in EC_{50} for caffeine; thus, cytosolic Ca^{2+} sites were not occupied or saturated by Ca^{2+} flux and were available for cytosolic Ca^{2+}. Importantly, this observation strongly indicates that cytosolic Ca^{2+} and luminal Ca^{2+} acted on different channel faces. Thus, it is evident that the luminal Ca^{2+} sensitivity of caffeine action for single and presumably for coupled RYR2 channels has indeed a dominant luminal component involving the binding of luminal Ca^{2+} to the luminal channel face. Taken together, the stimulating effect of luminal Ca^{2+} on the caffeine sensitivity seems to be an appropriate candidate for the additional component from the RYR2 functional profile that could be tested for coupled RYR2 channels in an attempt to resolve the issue about the functionality of luminally located Ca^{2+} sites.

3.3. Luminal Ca^{2+} affects the caffeine sensitivity of coupled RYR2 channels similarly as for the single RYR2 channel

We obtained the whole caffeine dose response curve for both single and coupled RYR2 channels when luminal Ba^{2+} or luminal Ca^{2+} were present. First, we analyzed a new dataset for the single RYR2 channel exposed to 8 mM instead of 53 mM $[Ca^{2+}]_L$ to verify the stimulation effect of luminal Ca^{2+} on the RYR2 sensitivity to caffeine. Fig. 4A illustrates the effect of luminal Ca^{2+} on the whole dose response of the single RYR2 channel to caffeine. Each dataset was globally fitted with Hill equation with EC_{50} of 6.89 ± 0.53 mM for luminal Ba^{2+} and 1.19 ± 0.31 mM for luminal Ca^{2+} (Fig. 4B). This indicates that even 8 mM $[Ca^{2+}]_L$ significantly shifted EC_{50} for caffeine to a lower concentration. Furthermore, in both cases the single RYR2 channel was substantially activated by caffeine and fitted P_{OMax} that is the maximum achievable P_O induced by caffeine was not influenced by luminal Ca^{2+} ($P_{OMax} = 0.9795 \pm 0.0066$ for luminal Ba^{2+} and $P_{OMax} = 0.9897 \pm 0.0067$ for luminal Ca^{2+}). For coupled RYR2 channels, we obtained similar results. Fig. 4C shows pooled data summarizing $P_O^{coupled}$ caused by caffeine. Each dataset was globally fitted with Hill equation with EC_{50} of 5.79 ± 0.61 mM for luminal Ba^{2+} and 0.753 ± 0.083 mM for luminal Ca^{2+} (Fig. 4D). Thus, luminal Ca^{2+} when increased to 8 mM significantly potentiated the caffeine sensitivity of coupled RYR2 channels similarly as we found for the single channel. Again, caffeine evoked the full activation of coupled RYR2 channels even in the virtual absence of luminal Ca^{2+} indicating a resistance of this parameter to luminal Ca^{2+} ($P_{OMax}^{coupled} = 0.987 \pm 0.013$ for 8 mM $[Ba^{2+}]_L$ and $P_{OMax}^{coupled} = 0.9965 \pm 0.0023$ for 8 mM $[Ca^{2+}]_L$). Taken together, our results strongly suggest that coupled gating does not make RYR2 channels insensitive to luminal Ca^{2+} and the class of luminally located Ca^{2+} sites that is responsible for the channel activation by luminal Ca^{2+} is functional and not structurally hindered.

4. Discussion

We have studied the coupled gating among RYR2 channels for more than 10 years. Although, reconstitution of this phenomenon in the BLM is an infrequent event comparing with the single RYR2 channel we collected a substantial amount of data characterizing two main aspects of this synchronous channel functioning. First, we and the others are interested in identifying determinants influencing the coupling stability [8,9,12]. Second, we attempt to understand the significance of this phenomenon currently from the biophysical point of view. In this respect, we have consistently studied the functional profile of coupled RYR2 channels considering them as a single functional unit and its regulation by known ligands of the single RYR2 channel [11]. We believe that this approach, when we compare coupled with single RYR2 channels, could provide relevant information for understanding the molecular nature of the RYR2 coupling. In support of this concept, Porta et al. [12] highlighted, in their recent study about coupled RYR1 channels, the importance of studying the regulation of coupled channels by luminal Ca^{2+} primarily under physiological conditions. In respect to coupled gating, this opens a completely new field of research because until now the role of luminal Ca^{2+} either in the RYR coupling stability or in coupled unit regulation has not been widely considered. Hence, in the present study we examined the luminal Ca^{2+} regulation of the smallest channel array composed of two RYR2 channels isolated

from the rat hearts. We used caffeine as a pharmacological probe for elucidating this regulation mechanism because the same strategy was successfully applied for the single RYR2 channel [17,20].

4.1. Luminal Ca^{2+} regulation of caffeine activated coupled RYR2 channels

For the single RYR2 channel, an alteration in the gating behavior by luminal Ca^{2+} has been clearly evidenced and we consider it to be one of the most prominent effects of luminal Ca^{2+}, although poorly understood from the physiological point of view [17,20]. Importantly, this specific action of luminal Ca^{2+} was difficult to reconcile with the Ca^{2+} feedthrough mechanism, therefore, it has been ascribed to the direct action on the luminal channel face [17]. Albeit, we previously reported that gating behavior of coupled RYR2 channels is significantly slowed down when compared with the single channel at 53 mM $[Ca^{2+}]_L$, we really did not expect that the gating of coupled channels would be insensitive to luminal Ca^{2+}. This apparent lack of luminal Ca^{2+} regulation might be seemingly explained by non-functional state of luminal Ca^{2+} sites. They could be structurally inaccessible as was originally suggested by Sitsapesan and Williams, however in the different context [23]. They proposed that conformational changes resulting from the binding of cytosolic agonist on the single RYR2 channel determine the functionality of luminal Ca^{2+} sites. However, this is not our case because we clearly showed that coupled RYR2 channels exhibited a substantially higher sensitivity to caffeine when luminal Ca^{2+} was present. This finding is in agreement with what we found for the single RYR2 channel. The EC_{50} was reduced approximately sixfold for the single channel and eightfold for coupled ones when comparing luminal Ba^{2+} with luminal Ca^{2+}. Our motivation to explore the stimulating effect of luminal Ca^{2+} on the response of coupled RYR2 channels to caffeine, in respect to EC_{50}, was our new convincing evidence about a pivotal role of the true luminal mechanism in this regulation process. We used an alternative approach to that employed in our previous study that failed to distinguish between the actions of luminal Ca^{2+} on the luminal and cytosolic faces of the caffeine activated single RYR2 channel [17]. Here, we monitored a potential competition between cytosolic Ca^{2+} and luminal Ca^{2+} (permeating the RYR2 pore) for cytosolic Ca^{2+} sites. The apparent lack of competition, demonstrated in our study, highlights the dominance of a luminal component in the luminal Ca^{2+} impact on the single RYR2 activation by caffeine. We can extend this significant finding also on coupled RYR2 channels, because it is feasible to assume that coupled gating does not switch between true luminal and Ca^{2+} feedthrough mechanisms. In other words, when the caffeine response of the single RYR2 channel is enhanced by luminal Ca^{2+} from the luminal channel face then the same regulation mechanism of luminal Ca^{2+} will be likely involved also in the regulation of coupled RYR2 channels. This idea is supported by our new finding that luminal Ca^{2+} shifted the caffeine sensitivity similarly in both cases because the values of EC_{50} for caffeine in the presence of 8 mM $[Ca^{2+}]_L$ were not significantly different when comparing single and coupled RYR2 channels. In addition, EC_{50} for caffeine determined in the virtual absence of luminal Ca^{2+} (8 mM $[Ba^{2+}]_L$) was not influenced by coupled gating. Importantly, in our previous work, we showed that the values of EC_{50} for cytosolic Ca^{2+} determined for single and coupled RYR2 channels were not significantly different when 53 mM $[Ca^{2+}]_L$ was used [11]. Notably, this collective data imply that Ca^{2+} flux through one RYR2 channel unlikely enhances the true luminal effect of luminal Ca^{2+} on the neighboring RYR2 channel by an indirect Ca^{2+} feedthrough mechanism, at least for caffeine sensitivity.

4.2. Ca^{2+} flux and the coupled gating phenomenon

Until recently, it was strongly believed that the appearance of coupled RYR channels in BLM recordings is caused by the channel-to-channel activation by Ca^{2+} flux in the lumen-to-cytosol direction [8,10,24,25]. Although, this concept was contradicted immediately from the beginning by Marx et al. [5,6] who showed that functional synchronicity among either RYR1 or RYR2 channels is not dependent on Ca^{2+} flux *via* channel pores because coupled events were observed also when luminal Ba^{2+} was used as a charge carrier instead of Ca^{2+}. In support of this finding, we also observed coupled gating when RYR2 channels were exposed to luminal Ba^{2+} [9]. In contrast, Liu et al. [10] and Porta et al. [12] revealed by their elegant studies that after abolishing Ca^{2+} flux by replacing luminal Ca^{2+} with luminal Ba^{2+} the functional synchronicity among either RYR2 or RYR1 channels was no longer evident, respectively. When the charge carrier was changed back to Ca^{2+}, coupled events reappeared suggesting the Ca^{2+} flux dependence. For a long time, this apparent controversy has not been resolved, until Porta et al. [12] debated in their recent paper the role of luminal Ca^{2+} in the channel coupling that could be mediated in addition to Ca^{2+} flux also by a direct action on the luminal channel face. Although, they were not successful in the reconstitution of coupled channels in Ba^{2+} solutions (53 mM) they admitted that there is an appreciable probability, albeit much smaller comparing with Ca^{2+} solutions (53 mM), of finding coupled gating under this Ca^{2+} free conditions. Surprisingly, we found the strong evidence for this conclusion in our present as well as previous works [9,11]. We calculated that the experiments with coupled RYR2 channels represent 25% and 28% of all the observations with multichannel incorporations at 8 mM and 53 mM $[Ca^{2+}]_L$, respectively. This probability is similar to that reported by Porta et al. for coupled RYR1 channels studied under the similar conditions [12]. Thus, in our hands, $[Ca^{2+}]_L$ does not appreciably affect the chance for coupled RYR2 reconstitution. In contrast, we obtained completely different numbers for Ba^{2+} solutions. Using SR microsomes isolated from rat hearts we have never observed coupled gating when RYR2 channels were exposed to 53 mM $[Ba^{2+}]_L$. Unexpectedly, this probability was substantially enhanced to 14% when $[Ba^{2+}]_L$ was decreased to 8 mM. Importantly, the exclusive evidence for the existence of RYR1 and RYR2 coupling at 53 mM $[Ba^{2+}]_L$ was provided only by Marx et al. [5,6]. Nevertheless, in their studies, coupled channels were evidenced only in two independent experiments for each RYR isoform. Taken together, aforementioned findings point to the solid fact that in addition to luminal Ca^{2+}, luminal Ba^{2+} could also be involved in the functional communication among RYR channels. In this case we can rule out a possibility that this positive effect of luminal Ba^{2+} is mediated by Ba^{2+} flux because it has been documented that Ba^{2+} is a competitive non-agonist and by itself does not activate RYR2 channels from the cytosolic site [17,20]. How can then one explain the existence of coupled gating in Ba^{2+} solutions? One possible, albeit still speculative, explanation is that Ba^{2+} acts as a competitive agonist on a distinctive class of luminal Ca^{2+} sites that is predominantly involved in the coupling mechanism. In such scenario, Ba^{2+} in addition to competing with Ca^{2+} for these luminally located Ca^{2+} sites is also capable by itself to accomplish the coupled gating but in a lesser degree in comparison with Ca^{2+}.

5. Conclusions

The role of luminal Ca^{2+} in the regulation of functional profile of coupled RYR2 channels has not been systematically investigated yet, albeit, this type of regulation is of significant interest for the single RYR2 channel due to its potential implication in life-threatening diseases such as cardiac arrhythmias. Our study provides the first evidence that caffeine sensitivity of coupled RYR2 channels is regulated by luminal Ca^{2+} similarly as the single RYR2 channel. This effect is likely mediated by luminally located Ca^{2+} sites suggesting that coupled gating phenomenon does not preclude luminal Ca^{2+} from binding to these sites. However, we identified one important difference in the action of luminal Ca^{2+} on the gating behavior. Whereas, the single RYR2 channel responded to luminal Ca^{2+} by prolongation in open and closed times, coupled RYR2 channels seemed to be immune in this respect. In

accordance with our previous work [11] we again revealed that the tight functional communication between RYR2 channels substantially affected the gating behavior and its regulation. This strengthens our hypothesis that the individual gates of coupled RYR2 channels may be one of the main subjects affected by a synchronous functioning.

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