J Membrane Biol (2010) 234:159–169
DOI 10.1007/s00232-010-9243-8

Identification of Changes in the Functional Profile of the Cardiac Ryanodine Receptor Caused by the Coupled Gating Phenomenon

Jana Gaburjakova · Marta Gaburjakova

Received: 6 October 2009 / Accepted: 4 March 2010 / Published online: 25 March 2010

Abstract The objective of this work was to identify and further characterize potential changes in the functional profile of the cardiac ryanodine receptor (RyR2) channel caused by the coupled gating phenomenon. By reconstituting an ion channel into a planar lipid membrane, we showed that coupled RyR2 channels were activated by cytosolic Ca^{2+} with similar efficacy and potency as reported for the single RyR2 channel. In contrast, all examined parameters of gating kinetics were affected by the functional interaction between channels. Ignoring brief closings during main open events, the average open and closed times were considerably prolonged and the frequency of opening was reduced. Interestingly, when luminal Ca^{2+} was used as a charge carrier, Ca^{2+}-activated coupled RyR2 channels did not exhibit a sudden switch from slow to fast gating kinetics at an open probability of 0.5 as reported for the single RyR2 channel. Regarding flicker gating, the average closed time was significantly shorter and the frequency of closing was greatly enhanced. Furthermore, in contrast to the single RyR2 channel, both parameters for coupled channels were independent of cytosolic Ca^{2+}. Selected permeation properties of coupled RyR2 channels were comparable to those found for the single RyR2 channel. The Ca^{2+} current amplitude-luminal Ca^{2+} relationship displayed a simple saturation and the channel selectivity for Ba^{2+} and Ca^{2+} ions was similar. Our results suggest that the major targets influenced by coupled gating are likely the gates of individual RyR2 channels recruited into a functional complex, thus ensuring the correlation of Ca^{2+} fluxes.

Keywords Ryanodine receptor · Coupled gating · Planar lipid membrane · Ca^{2+} sensitivity · Gating kinetics · Ion conductance · Channel gate

J. Gaburjakova · M. Gaburjakova (✉)
Institute of Molecular Physiology and Genetics,
Slovak Academy of Sciences, Vlarska 5, 833 34 Bratislava,
Slovak Republic
e-mail: marta.gaburjakova@savba.sk

The coupled gating of cardiac ryanodine receptor (RyR2) channels was evidenced for the first time in the work of Marx et al. (2001). This phenomenon is primarily manifested by simultaneous openings and closings of multiple RyR2 channels. Marx et al. (2001) provided provocative new evidence that clusters of RyR2 channels gate in a coordinated fashion to release Ca^{2+} from the sarcoplasmic reticulum (SR), which is required for contraction of the cardiac muscle. The consequence is that the RyR2 channel cluster would speed the local Ca^{2+} release event. Although the physiological relevance of coupled gating of RyR2 channels is largely open to debate at the present time, it has also been considered as one of the termination mechanisms of Ca^{2+} release, ensuring periodic contraction and relaxation of cardiac muscle (Stern and Cheng 2004). The local Ca^{2+} release in cardiac muscle is a self-regenerative process because activated RyR2 channels can reactivate neighboring RyR2 channels via released Ca^{2+}. Thus, the simultaneous closure of all channels in the release unit would break the positive feedback loop and terminate Ca^{2+} release. Furthermore, the coupled gating phenomenon has been included in a number of cardiac excitation-contraction (EC) coupling computational models which faithfully reproduced most of the observed physiological features of cardiac muscle activity (Stern et al. 1999; Sobie et al. 2002; Hinch 2004).

In general, cooperative behavior in biological systems has been the focus of many theoretical and experimental investigations. They have all suggested that neighboring ion channels may interact with each other and that the functional interaction among ion channels can manifest in different ways. The interaction among Na^+ channels from neuroblastoma cells caused a peculiar behavior when the open time of a given channel was influenced by the "neighboring" ones (Kiss and Nagy 1985; Iwasa et al. 1986). Yeramian et al. (1986) demonstrated the functional coupling between two acetylcholine receptors in rat myotubes. They demonstrated that channel gating was modified when there was some increased probability that the opening of a second channel would occur while the first channel was still open. The modified gating behavior of an ion channel by neighboring channels strongly pointed to protein-protein interactions in the membrane matrix. However, the mechanism of such conformational communication among ion channels is still not completely understood. Another type of ion channel interaction has been reported by Neumcke and Stämpfli (1983). In this study, Na^+ channels in a myelinated frog nerve exhibited negative cooperativity; the ion conductance of a single Na^+ channel decreased with increasing numbers of Na^+ channels in a patch. The authors rejected a direct channel-channel interaction as a cause of the investigated phenomenon based on their finding that gating time constants were unaltered. Therefore, a hypothesis has been formulated that Na^+ current flow through individual channels is hindered by neighboring channels. More recently, Taufig-Ur-Rahman et al. (2009) published very interesting results describing a clustering of inositol-1,4,5-trisphosphate receptors (IP_3R) channels induced by IP_3. They demonstrated that a low concentration of IP_3 caused IP_3R channel to aggregate into small clusters and that simultaneous channel gating appeared with increasing concentration of cytosolic Ca^{2+}. The interaction among channels resulted in prolongation of the duration of multiple openings and retuned IP_3 and Ca^{2+} sensitivities. Moreover, authors also brought a new hypothesis that dynamic regulation of functional coupling among IP_3R channels might play a role in hierarchical recruitment of Ca^{2+} release events.

Our goal for this paper was to gain further information about the conformational interaction between two cardiac RyR2 channels. Using a method of reconstitution of an ion channel into a planar lipid membrane (BLM), we systematically examined the cytosolic Ca^{2+} sensitivity and permeation properties of coupled RyR2 channels isolated from the rat heart. By comparing these data to those for the single RyR2 channel, we discovered that the individual gates of coupled RyR2 channels may be one of the main subjects affected by a channel interaction.

Materials and Methods

Isolation of SR Microsomes

Cardiac SR microsomes were isolated from Wistar rat heart according to Buck et al. (1999) with a few modifications. The isolated left ventricles from four hearts (4 g) were homogenized with a Tissue Tearor (Biospec Products, Inc.) in 5 vol of homogenization buffer (1 M KCl, 10 mM Tris-maleate) and a cocktail of protease inhibitors (Roche Applied Science, Mannheim, Germany; 1 mM benzamidin, 5 μg/ml pepstatin, 5 μg/ml leupeptine, 1 μM calpain inhibitor I, 1 μg/ml aprotinin, 1 mM AEBSF). The homogenate was centrifuged for 20 min at $10{,}000g_{max}$ 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 $6000g_{max}$ and 4°C. The supernatant was centrifuged for 25 min at $24{,}000g_{max}$ at 4°C and the resulting supernatant was further centrifuged for 120 min at $41{,}000g_{max}$ 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 the BLM and single-channel currents were recorded under voltage-clamp conditions. Cardiac SR microsomes were added to the *cis* chamber near the BLM formed from a 3:1 mixture of DOPE/DOPS (Avanti Polar Lipids, Inc., Alabaster, AL, USA) across a 50-μm aperture in the wall of a polystyrene cup. Fusion of microsomes was promoted with 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 HEPES, 125 mM Tris, 50 mM KCl, 1 mM EGTA, 0.5 mM $CaCl_2$ (pH 7.40), and the *trans* chamber (corresponding to the lumen of SR) was filled with 1 ml of 53 mM $Ca(OH)_2$, 50 mM KCl, 250 mM HEPES (pH 7.40). In experiments where the ion selectivity of the RyR2 channel was determined, the *trans* chamber was filled with 1 ml of 20 mM HEPES, 50 mM KCl, and either 8 mM $Ca(OH)_2$ or 8 mM $Ba(OH)_2$, pH 7.40. The concentration of ions in the *trans* chamber was manipulated during the experiment by addition of aliquots of stock solution composed of 20 mM HEPES, 50 mM KCl, and either 8 mM $Ba(OH)_2$ or Ca $(OH)_2$, pH 7.40. The free cytosolic Ca^{2+} concentration was calculated by WinMaxc32 version 2.50 (http://www.stanford.edu/~cpatton/maxc.html). At the end of experiments, ryanodine was applied to confirm channel identity. Experiments were carried out at room temperature

(21–23°C). The *trans* chamber was connected to the headstage input of a Bilayer Clamp Amplifier BC-525D (Warner Instrument, Hamden, CT, USA). The *cis* chamber was grounded. Single-channel currents were filtered at 1 kHz and digitized at 4 kHz. Data were collected on a Pentium computer using an AxoScope7.0 (Axon Instruments, Burlingame, CA, USA) and DigiData 1322A (Axon Instruments) interface. pClamp5.5 (Axon Instruments) was used for analysis. The open probability (P_o) of Ca^{2+}-activated coupled RyR2 channels was calculated from the current recordings with a duration >2 min. Ca^{2+} sensitivity curve was fitted with the Hill equation (SigmaPlot 8.02; Systat Software Inc., Richmond, CA, USA) and resulting EC_{50} and $P_{o\ max}$ values from individual experiments were averaged (Fig. 1). To analyze the gating kinetics of coupled RyR2 channels as a single functional unit, the records were divided into 60-s intervals and flicker gating manifested by brief transitions from the main open current level were ignored. The average open and closed times and the frequency of opening were calculated on these intervals as a standard arithmetic average. The resulting values were further averaged on the defined intervals of P_o and statistically compared (Fig. 2). The flicker gating of coupled channels was analyzed as a function of cytosolic Ca^{2+} concentration. The average closed time and the frequency of closing were determined for each opening of a channel complex and then averaged for a given cytosolic Ca^{2+} concentration using data collected from more than three experiments (Fig. 3). The current amplitude of channel openings was determined from the differences in medians of the best-fit Gaussians for the baseline and the main open current level. The ion conductance was calculated from a linear regression of the points in the current-voltage relationship between –30 and +30 mV. Current amplitude-$[Ca^{2+}]_{lum}$ dependence was fitted with a Michaelis-Menten-type saturation curve (Figs. 4, 5). Data are presented as mean ± SD unless otherwise stated. Differences were statistically evaluated by a Student's *t*-test or Welch approximation of the *t*-test or by one-way ANOVA test and are regarded as statistically significant at $P < 0.05$.

Results

Cardiac SR microsomes were fused into the BLM, and single-channel currents were recorded under asymmetric conditions with either luminal Ca^{2+}, luminal Ba^{2+}, or a mixture of Ca^{2+} with Ba^{2+} as charge carriers. Although the majority of reconstituted RyR2 channels were single, in 13% of experiments we observed coupled RyR2 channels. From that, the efficiency to reconstitute the complex of two RyR2 channels from our SR microsome sample was 11 times higher than those obtained for three or more coupled channels (11% for two coupled, 1% for three coupled, and 1% for four coupled channels). Therefore, for this first attempt at assessing how coupled gating modifies the functional profile of RyR2 channels, we limit our attention only to the simplest functional complex composed of two RyR2 channels, which demonstrated the highest level of successful reconstitution. Hereafter, we use "coupled RyR2 channels" to refer specifically to this complex of two RyR2 channels.

Previously, we reported that coupled RyR2 channels showed a wide variation of activity at diastolic Ca^{2+} concentrations after incorporation into the BLM, indicating a heterogeneous population (Marx et al. 2001; Gaburjakova and Gaburjakova 2008). This range of activities has not been observed for single RyR2 channels, which displayed very negligible activity during the diastole. For simplification, we classified coupled RyR2 channels as low-activity (LA) and high-activity (HA) channels. At 53 mM luminal Ca^{2+}, LA coupled channels displayed an open probability of 0.014 ± 0.013 ($n = 12$). This value is similar to that determined for the single RyR2 channel recorded at 90 nM cytosolic Ca^{2+} ($P_o = 0.013 \pm 0.021$; $n = 170$). The remaining coupled channels were classified as HA coupled channels, characterized by higher diastolic activity, with $P_o = 0.59 \pm 0.20$ ($n = 11$), and broader heterogeneity. In the present study, we characterized both groups of coupled RyR2 channels separately and compared their functional and permeation properties with those of the single RyR2 channel.

Sensitivity of Coupled RyR2 Channels to Cytosolic Ca^{2+}

The major physiological activator of the RyR2 channel in cardiac muscle is cytosolic Ca^{2+}. Therefore, we focused our effort on testing whether the coupling between RyR2 channels influenced this property. The representative current traces of the LA and HA coupled RyR2 channels recorded in the presence of various concentrations of cytosolic Ca^{2+} are shown in Fig. 1a, b, respectively. The concentration of luminal Ca^{2+} was kept constant at 53 mM. In both groups, an elevation of cytosolic Ca^{2+} from 70 nM up to 5 μM resulted in the sequential activation of coupled RyR2 channels to a near-maximum extent. Figure 1c summarizes this set of experiments by plotting P_o against cytosolic Ca^{2+} concentration for each experiment. Individual curves were fitted with the Hill equation and the yielded values of EC_{50} and $P_{o\ max}$ were further averaged in each group of coupled channels. It is apparent from Fig. 1c that there is not a significant difference in the sensitivity of LA versus HA coupled RyR2 channels to cytosolic Ca^{2+}. The EC_{50} of 0.190 ± 0.078 μM ($n = 8$) and $P_{o\ max}$ of 0.952 ± 0.041 ($n = 8$) were determined for

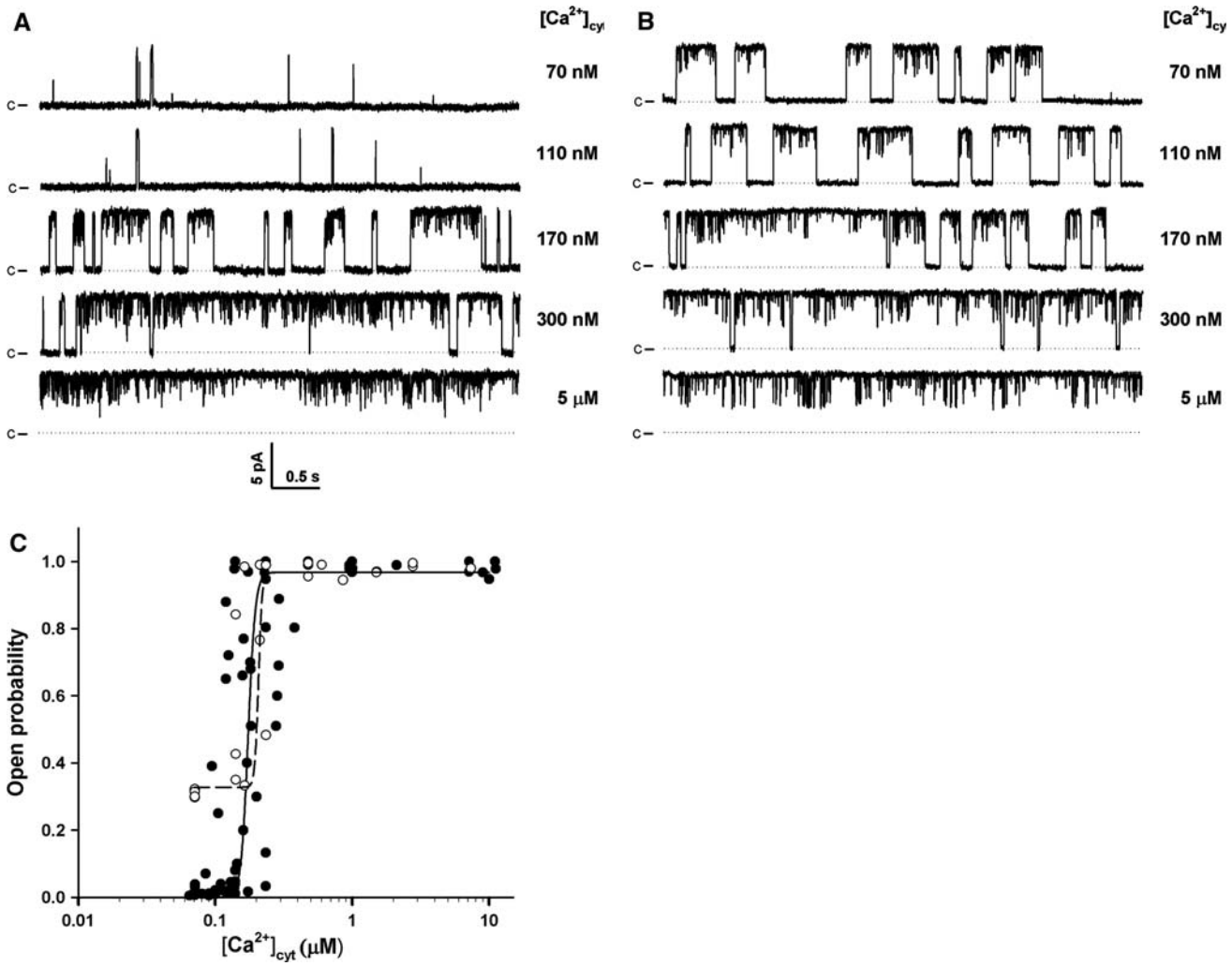

Fig. 1 Response of coupled RyR2 channels to cytosolic Ca^{2+}. Representative current traces of LA (**a**) and HA (**b**) coupled RyR2 channels activated by cytosolic Ca^{2+} ranging from 70 nM to 5 μM. Recordings were conducted under steady-state conditions at 0 mV. Channel openings are in the upward direction. Dashes at the left of the tracings indicate the closed (C) state of coupled channels. **c** Relationships between the P_o of LA (●) and HA (○) coupled RyR2 channels and $[Ca^{2+}]_{cyt}$ at constant $[Ca^{2+}]_{lum} = 53$ mM. Data points displayed are individual P_o measurements from more than five experiments. Each relationship was fitted using the Hill equation and EC_{50} and $P_{o\ max}$ values were further averaged. An EC_{50} of 0.190 ± 0.078 μM ($n = 8$) and a $P_{o\ max}$ of 0.952 ± 0.041 ($n = 8$) were determined for LA coupled RyR2 channels and an EC_{50} of 0.207 ± 0.075 μM ($n = 3$) and $P_{o\ max}$ of 0.991 ± 0.010 ($n = 3$) were determined for HA coupled channels

LA coupled channels. Similar values for these parameters were obtained for HA coupled channels [$EC_{50} = 0.207 \pm 0.075$ μM ($n = 3$); $P_{o\ max} = 0.991 \pm 0.010$ ($n = 3$)]. These results indicate that the high diastolic activity of HA coupled RyR2 channels is Ca^{2+} independent and is not caused by the enhanced sensitivity to cytosolic Ca^{2+}.

Gating Kinetics of Coupled RyR2 Channels

In our previous work (Marx et al. 2001) we showed that caffeine-activated coupled RyR2 channels exhibited longer open time constants in comparison with those determined for the single RyR2 channel under the same experimental conditions. At that time, the open event was considered as an interval between two closed events where both RyR2 channels coupled to a functional complex were closed. In other words, we ignored the flicker gating, which likely reflects the thermodynamic stability of channel coupling and is the result of alternate brief transitions of individual RyR2 channels between open and closed states. In the present work, we focused on a more detailed analysis of gating kinetics and we provided a separate characterization of LA and HA coupled RyR2 channels activated by a physiological activator, cytosolic Ca^{2+}. First, we described the gating behavior of coupled channels as a single functional unit. We constructed an ideal current trace by

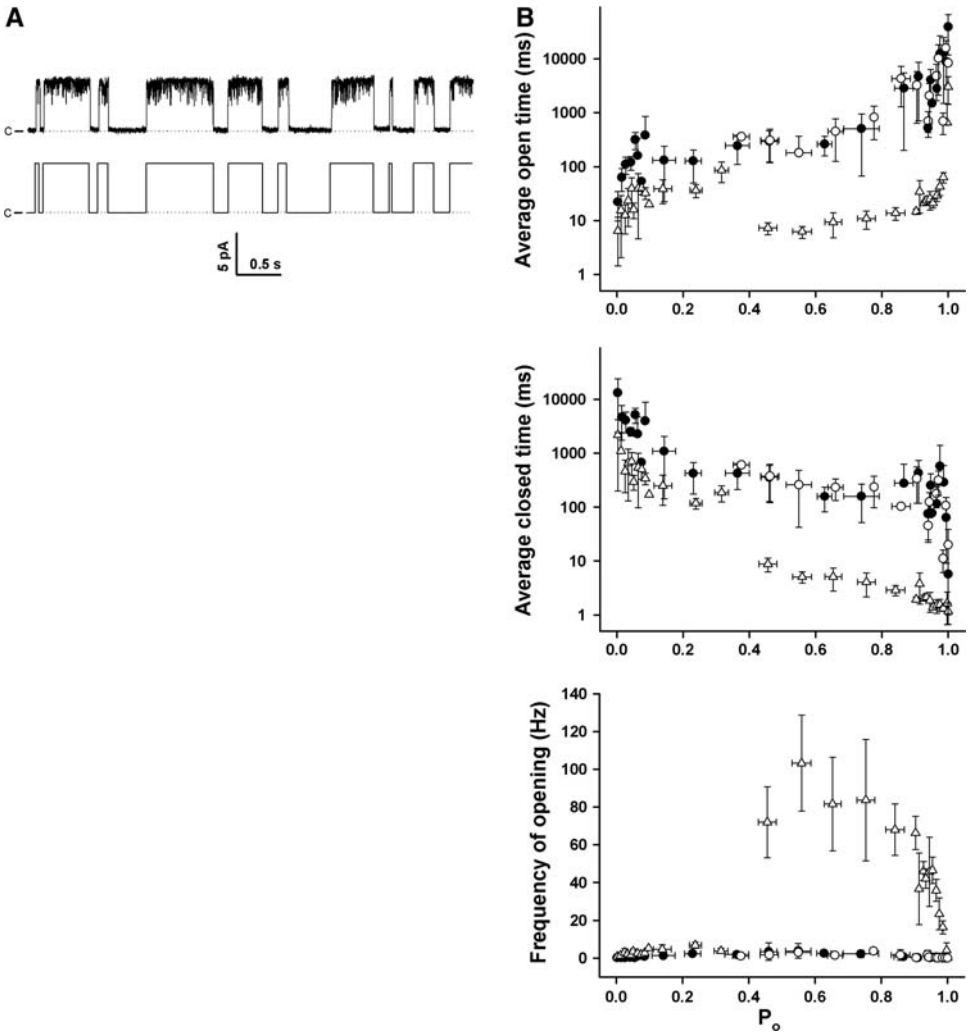

Fig. 2 Comparison of the gating behavior of coupled RYR2 channels as a single functional unit and the single RyR2 channel. **a** Representative current trace of LA coupled RYR2 channels activated by cytosolic Ca^{2+}. Recordings were conducted under steady-state conditions at 0 mV. Channel openings are in the upward direction. The dash at the left of the tracings indicates the closed state of the channel (C). The lower trace represents the corresponding ideal current trace when flicker gating inside the main open events were eliminated. **b** Average open time, closed time, and frequency of opening accumulated from 60-s recordings for LA (●) and HA (○) coupled RyR2 channels were further averaged on defined intervals of P_o and compared with corresponding parameters previously obtained for the single RyR2 channel (Δ) (data replotted from Gaburjakova and Gaburjakova 2006). Statistically significant differences between gating kinetics parameters of LA and HA coupled, as well as single, RyR2 channels were reliably shown for all tested intervals of $P_o > 0.4$ ($P<0.05$). At intervals of $P_o < 0.4$, the differences were too small to achieve statistical significance if fewer than 10 points were averaged and this criterion was met only half of the tested P_o intervals. All gating kinetics parameters of LA and HA coupled channels were similar for all tested intervals of P_o. Error bars represent SD

filtering out all short closures during the main open events (Fig. 2a). Upon initial inspection, Fig. 1a, b seem to suggest that the activation of both LA and HA coupled RyR2 channels by cytosolic Ca^{2+} is manifested by a prolongation of the open time and a shortening of the closed time. To prove this subjective impression we calculated several gating kinetics parameters, including 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 a previous work (Gaburjakova and Gaburjakova 2006), and Fig. 2b summarizes the results obtained. For both LA and HA coupled channels, the increase in P_o arose from a prolongation of the average open time and a decrease in the average closed time. These changes resulted in a biphasic dependence of the frequency of opening. The maximum of 3.87 Hz for LA and 3.05 Hz for HA coupled channels was reached at $P_o \sim 0.5$; at full channel activation, the frequency of opening went down to the initial value. Similar behavior was observed for the single RyR2 channel examined under the same experimental conditions with one exception (Gaburjakova and Gaburjakova 2006). We did not detect the sudden switch to a different mode of gating

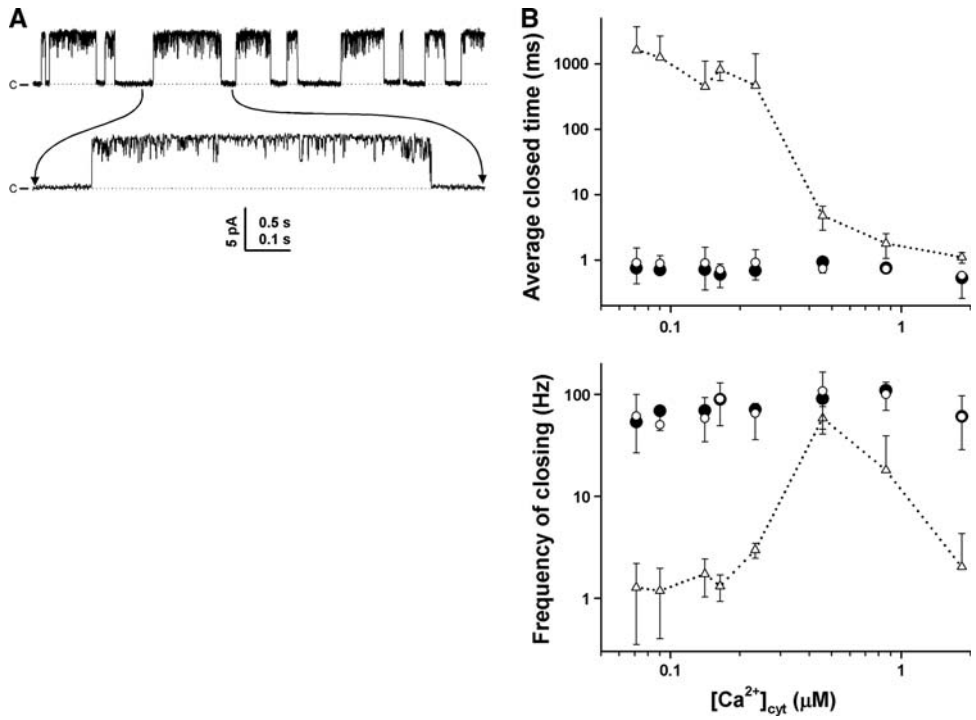

Fig. 3 Comparison of flicker gating kinetics of coupled RyR2 channels and gating behavior of the single RyR2 channel. **a** Representative current trace of LA coupled RYR2 channels activated by cytosolic Ca^{2+}. The lower trace shows the detailed flicker gating behavior inside the selected main open event. Recordings were conducted under steady-state conditions at 0 mV. Channel openings are in the upward direction. The dash at the left of the tracings indicates the closed state of the channel (C). **b** The average closed time and the frequency of closing were determined for each main opening of LA (●) and HA (○) coupled channels and each opening of the single channel (Δ), then averaged for a given cytosolic Ca^{2+} concentration using data collected from more than three experiments. Statistically significant differences between gating kinetics parameters of LA and HA coupled, as well as single, RyR2 channels were reliably shown for all tested concentrations of cytosolic Ca^{2+} (except the point where the frequency of closing was peaked). All gating kinetics parameters of LA and HA coupled channels were similar for the whole tested interval of cytosolic Ca^{2+} concentration. Error bars represent SD and they are displayed in the negative direction for LA (●) coupled channels and in the positive direction for HA (○) coupled channels

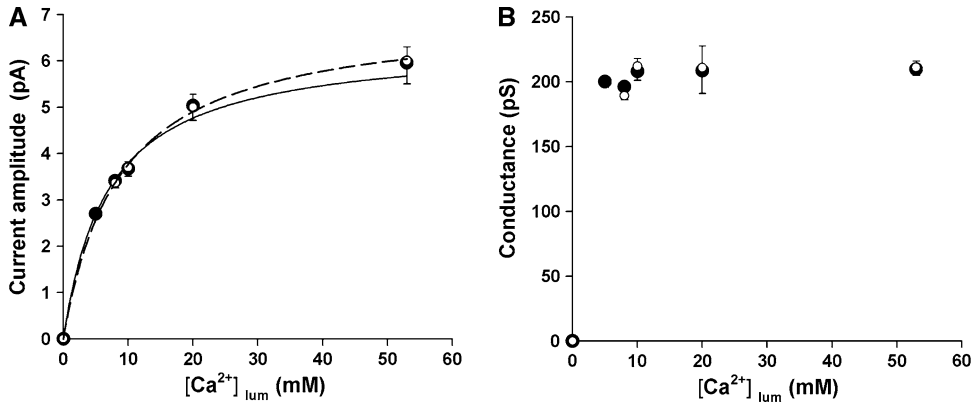

Fig. 4 Permeation properties of coupled RyR2 channels with respect to $[Ca^{2+}]_{lum}$. Channel activity was recorded at different $[Ca^{2+}]_{lum}$ ranging from 5 to 53 mM. LA coupled channels were activated by 5 mM caffeine. **a** Current amplitude and **b** ion conductance for LA (●) and HA (○) (replotted from Gaburjakova and Gaburjakova 2008) coupled RyR2 channels are plotted as a function of $[Ca^{2+}]_{lum}$. Ion conductance was independent of $[Ca^{2+}]_{lum}$ in the studied range (ANOVA). Current data were fitted by a Michaelis-Menten curve with a K_D of 8.46 ± 0.59 mM and an I_{max} of 6.96 ± 0.16 pA for LA coupled channels (solid line) and a K_D of 8.68 ± 0.45 mM and an I_{max} of 7.03 ± 0.12 pA for HA coupled channels (dashed line). Corresponding parameters are not significantly different. The current amplitude was determined at 0 mV potential. Data are presented as mean ± SE. Error bars are displayed in the negative direction for LA (●) coupled channels and in the positive direction for HA (○) coupled channels. More than three experiments were used to calculate average values

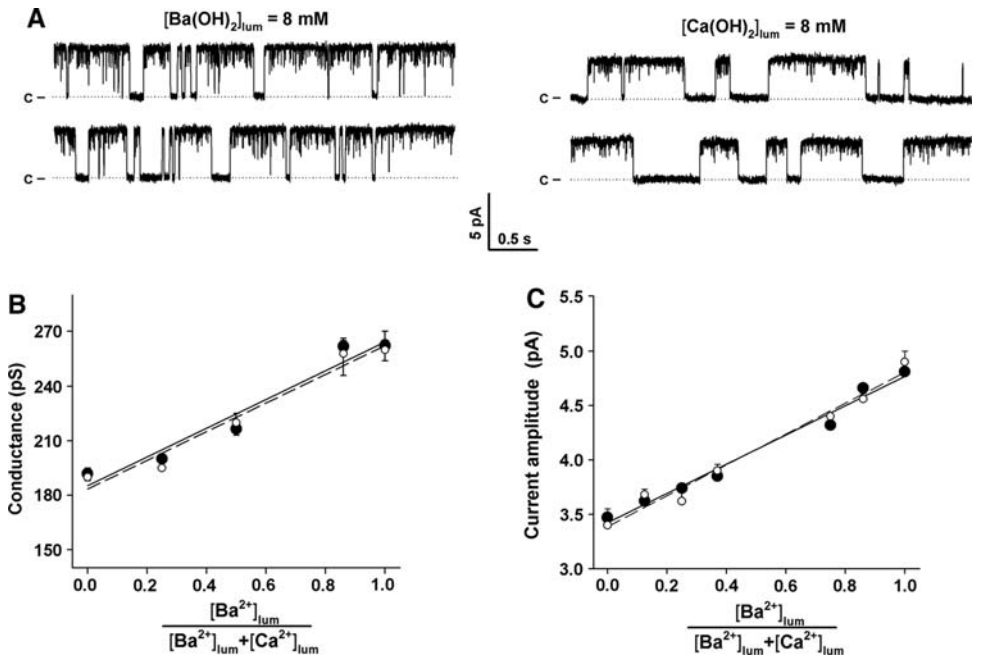

Fig. 5 Ba^{2+}-Ca^{2+} selectivity of coupled RyR2 channels. **a** Representative current traces for LA (upper traces) and HA (lower traces) coupled RyR2 channels when either 8 mM Ba^{2+} or 8 mM Ca^{2+} was used as charge carrier and was present on the luminal side of channels. Recordings were conducted under steady-state conditions at 0 mV. Channel openings are in the upward direction. The dash at the left of the tracings indicates the closed state of the channel (C). **b** Ion conductance and **c** current amplitude of LA (●) and HA (○) coupled RyR2 channels are plotted as a function of the mole fraction of Ba^{2+} in the mixture with Ca^{2+}. The dependences were satisfactorily fit using a linear function (solid line for LA coupled channels and dashed line for HA coupled channels). The total concentration of permeant ions was adjusted to 8 mM. The LA coupled RyR2 channels were activated with 5 mM caffeine. The current amplitude was determined at 0 mV potential. Individual points are the average of more than three experiments and the SE is indicated by error bars. Error bars are displayed in the negative direction for LA (●) coupled channels and in the positive direction for HA (○) coupled channels

at $P_o \sim 0.5$ in the presence of luminal Ca^{2+}, which is characteristic of the single RyR2 channel. This resulted in more pronounced differences in the corresponding gating kinetics parameters obtained for coupled versus single RyR2 channels for P_o intervals where $P_o > 0.4$. For $P_o < 0.4$, the differences were too small to achieve statistical significance if fewer than 10 points were averaged and this criterion was met for only half of the tested P_o intervals. Nevertheless, we can state that over the whole range of P_o, both LA and HA coupled RyR2 channels either exhibited or had a tendency toward longer average open and closed times and a lower frequency of opening.

As a further step in the characterization of the gating behavior of coupled RyR2 channels, we concentrated on the flicker gating. Without any evidence that one of the channels recruited to a functional complex is superior and the other inferior, we considered that both channels are equal and both channels equally contributed to the flickering. These assumptions restricted our analysis only with regard to the average closed time and the frequency of closing. The average open time could not be determined because it was not possible to unambiguously identify whether the subsequent closure was the closing of the same channel. Gating kinetics parameters were calculated for each main opening of a channel complex (Fig. 3a), averaged, and plotted as a function of cytosolic Ca^{2+} concentration instead of P_o. Our aim was to assess the potential effect of functional coupling on the intrinsic gating of individual RyR2 channels. During all main openings of a channel complex, the P_o corresponding to individual RyR2 channels was very high and in the narrow range from 0.8 to 0.9 (data not shown). Thus, the data for the whole P_o interval from 0 to 0.8 would not have been available. Therefore, we chose the alternative approach and we plotted parameters of flicker gating kinetics against cytosolic Ca^{2+} concentration. Figure 3b summarizes our results. For both LA and HA coupled RyR2 channels, the average closed time and the frequency of closing were independent of cytosolic Ca^{2+}. Importantly, there was no significant difference between these two groups of coupled channels. In contrast, a strong relationship was observed between the analyzed parameters and cytosolic Ca^{2+} with regard to the single RyR2 channel. At low, subactivating concentrations of cytosolic Ca^{2+}, the average closed time was gradually reduced, and a sharp decline appeared between 0.229 and 0.456 nM cytosolic Ca^{2+}. This likely corresponds to the reported sudden transition between two distinct gating modes (Gaburjakova and Gaburjakova

2006). This change is also documented by a considerable increase in the frequency of closing from plateau to the maximal value at the same cytosolic Ca^{2+} concentrations. At higher concentrations, the frequency of closing dropped to the initial value. Over the whole examined interval of cytosolic Ca^{2+}, the average closed time was significantly smaller and the frequency of closing was significantly higher (except the point where this parameter reached the maximum) for both groups of coupled RyR2 channels compared with the single RyR2 channel.

Taken together, these results support our previously published data, which demonstrated slower gating kinetics of caffeine-activated coupled RyR2 channels compared with that of the single RyR2 channel. Importantly, the gating kinetics of individual RyR2 channels that were part of a single functional unit was different from that obtained for the single RyR2 channel. The combination of these results strongly suggests that there is a direct conformational interaction between the individual RyR2 channels in a channel complex.

Permeation Properties of Coupled RyR2 Channels

When ion flux through channel pores is strongly regulated, as in the case of coupled RyR2, it is feasible to assume that interactions between channels might also influence some permeation properties of individual channels recruited into a functional complex. First, we investigated the relation between current amplitude/ion conductance and concentration of luminal Ca^{2+}, ranging from 5 to 53 mM. Experiments were performed only for LA coupled channels, because the data for HA coupled channels were replotted from Gaburjakova and Gaburjakova (2008). Cytosolic Ca^{2+} was kept constant at 70 nM and the LA coupled channels were activated by 5 mM caffeine to obtain a sufficient number of main open events long enough for determining the current amplitude. For both groups of coupled channels, the current amplitude gradually declined with reducing luminal Ca^{2+} as a response to attenuation of the driving force for Ca^{2+} ions across the BLM (Fig. 4a). At luminal Ca^{2+} concentrations below 5 mM, we were unable to reliably determine the current amplitude under our experimental conditions. In contrast, the ion conductance was insensitive to changes in luminal Ca^{2+} concentrations (Fig. 4b). For each group of coupled channels, the average values of current amplitude and ion conductance were plotted as a function of luminal Ca^{2+}, and current amplitude points were fitted using a Michaelis-Menten curve (Fig. 4a). This analysis yielded a K_D of 8.46 ± 0.59 mM and an I_{max} of 6.96 ± 0.16 pA for LA coupled channels and a K_D of 8.68 ± 0.45 mM and an I_{max} of 7.03 ± 0.12 pA for HA coupled channels. Corresponding parameters were not significantly different.

Another permeation profile characteristic of coupled RyR2 channels that we investigated was ion selectivity. From the three experimental protocols used for the estimation of ion selectivity, we chose the mole fraction experiment (Gillespie and Eisenberg 2002). This experimental approach is based on the determination of the current amplitude and the ion conductance as a function of the mole fraction of one ion in mixture with another. Theoretically, if the total current amplitude/ion conductance were a linear function of a mole fraction, than the contribution of given ions to the overall current amplitude/conductance would be additive. In that case, an ion channel would not discriminate between tested ions. In our study, we examined coupled RyR2 channels in the presence of varied molar ratios of Ba^{2+} and Ca^{2+} ions at a total concentration of 8 mM. This value was chosen to be close to the determined value of K_D in order to minimize a potential masking effect of the saturating ion concentration on the shape of the concentration dependence of studied permeation parameters (Rodriguez-Contreras et al. 2002). The fact that the coupled gating phenomenon is not fixed to Ca^{2+} as a permeant ion (Marx et al. 2001; Gaburjakova and Gaburjakova 2008) allowed us to examine the complete molar ratio dependence of the studied permeation properties. For illustration, representative current traces of LA (upper traces) and HA (lower traces) coupled RyR2 channels using either 8 mM Ba^{2+} (Fig. 5a, left) or 8 mM Ca^{2+} (Fig. 5a, right) as charge carrier are shown. Figure 5b, c summarize the behavior of the ion conductance and the current amplitude at 0 mV with varying mole fraction of Ba^{2+} in the mixture with Ca^{2+}. Both parameters determined for LA and HA coupled RyR2 channels changed monotonically. Linear regression provided satisfactory data fitting, indicating that Ba^{2+} and Ca^{2+} ions are similarly permeant in the pores of both groups of coupled RyR2 channels.

Discussion

In this study, we decided to focus our efforts on a systematic characterization of specific functional properties of coupled RyR2 channels in order to identify some attributes that could help us to answer the question: What is the nature of functional interaction between RyR2 channels? The main role of the RyR2 channel in cardiac muscle is to allow massive release of Ca^{2+} from the SR upon channel activation by Ca^{2+} entering the cell from the extracellular space. In this respect, the main channel characteristics of the RyR2 channel that are feasible in vivo appear to be its cytosolic Ca^{2+} activation and permeation properties. Therefore, in our study we addressed the following issues: first, how coupled RyR2 channels respond to cytosolic

Ca^{2+}; second, how the ion conductance and the Ca^{2+} current amplitude of coupled RyR2 channels change with luminal Ca^{2+} concentration; and third, how coupled RyR2 channels discriminate between divalent cations such as Ba^{2+} and Ca^{2+}.

A further issue that we attempted to address in the present work, although of secondary importance, was the wide functional heterogeneity of coupled RyR2 channels with regard to diastolic activity. In our previous work we mentioned that a significant percentage of coupled RyR2 channels exhibited higher activity at diastolic cytosolic Ca^{2+} concentrations in comparison with the single RyR2 channels (Marx et al. 2001; Gaburjakova and Gaburjakova 2008). However, we have yet to address the question: What is the molecular basis of channel heterogeneity? To begin to resolve this issue, we divided coupled channels into distinct groups. For simplification, only two groups were created. At 90 nM cytosolic Ca^{2+}, LA coupled channels exhibited comparable activity as the single RyR2 channel. In contrast, HA coupled channels displayed significantly higher activity compared with the single RyR2 channel. Although the major subject of our attention was the comparison of coupled and single RyR2 channels, we also compared the functional profiles of LA and HA coupled channels with each other. With respect to the tested functional and conductive properties, we did not find any difference between these two groups. Unexpectedly, even the response to cytosolic Ca^{2+} was similar. The most obvious interpretation of this result is that the potentiation of HA coupled channels at low cytosolic Ca^{2+} is Ca^{2+} independent. In fact, this interpretation is consistent with our previous finding that Ca^{2+} does not occupy activation binding sites on the cytosolic side of HA coupled RyR2 channels because the channels were blocked by 1 mM Mg^{2+} in the absence of any activator (Gaburjakova and Gaburjakova 2008). It is obvious that further experiments will be required to reveal the molecular basis of observed variability in diastolic activity of coupled RyR2 channels.

Differences in the Functional Profile of Coupled vs. Single RyR2 Channels

Our initial intention was to compare the properties of coupled RyR2 channels with those we reported for the single RyR2 channel. Therefore, we performed all experiments under the same conditions that we used in our previous studies (Gaburjakova and Gaburjakova 2006; Tomaskova and Gaburjakova 2008). Both LA and HA coupled and single RyR2 channels were strongly activated by cytosolic Ca^{2+}, with the onset of activation at 150 nM. We determined the EC_{50} to be 0.190 ± 0.078 μM ($n = 8$) for LA coupled channels and 0.207 ± 0.075 μM ($n = 3$) for HA coupled channels. These values are similar to the EC_{50} value of 0.191 ± 0.042 μM ($n = 11$) reported for the single RyR2 channel (Gaburjakova and Gaburjakova 2006). Furthermore, interaction between RyR2 channels did not influence the ability of cytosolic Ca^{2+} to induce maximal channel activation. Both LA and HA coupled and single channels were activated to a comparable extent, close to the maximum [$P_{o\ max} = 0.952 \pm 0.041$ ($n = 8$) for LA coupled channels, $P_{o\ max} = 0.991 \pm 0.010$ ($n = 3$) for HA coupled channels, and $P_{o\ max} = 0.974 \pm 0.071$ ($n = 11$) for the single channel). It is apparent from our results that coupled RyR2 channels did not exhibit a significantly stronger sensitivity to cytosolic Ca^{2+}, thus, it is unlikely that the individual RyR2 channels communicate through an allosteric interaction between Ca^{2+} binding sites on the cytosolic domain of each RyR2 channel. The next information we obtained from the analysis was regarding gating kinetics. We first described the gating behavior of coupled RyR2 channels as a single functional unit, ignoring flicker gating. In comparison with the single RyR2 channel, both LA and HA coupled channels either exhibited or had a tendency toward the significantly lower frequency of opening caused by prolongation of the average open and closed times over the whole range of P_o. At $P_o \sim 0.5$, the single RyR2 channel activated by cytosolic Ca^{2+} switched suddenly to a different mode of gating characterized by the shorter average open and closed times, which resulted in a striking increase in the frequency of opening (Gaburjakova and Gaburjakova 2006). The reason for this special behavior is still not understood. However, it seems that it is specific only for the single RyR2 channel; coupled channels, as a single functional unit, did not display this abnormal behavior. On the contrary, the average open and closed times of coupled channels displayed monotonic dependence and the frequency of opening was biphasic without any visible break. Importantly, our results are in agreement with the outcomes of an allosteric energy model that predicted significantly slowed gating transitions for a cluster composed of several ion channels. Thus, the gating kinetics of the coupled RyR2 channels is not thermodynamically paradoxical as concluded by Stern and Cheng (2004). They extrapolated results determined for a skeletal isoform of the RyR (RyR1) channel (Marx et al. 1998) to the cardiac isoform. We clearly showed in our previous work (Marx et al. 2001), however, that coupled RyR2 channels activated by caffeine exhibited significantly longer mean open and closed times (dwell times were collected from records where P_o was ~0.5 to ensure the sufficient amount of data for the fitting of exponential functions). In the present work, we determined the entire relationship between the main gating kinetics parameters and P_o. These data indicated that differences between single and coupled RyR2 channels depended on the channel activity. This finding may explain why Marx et al.

(1998) reported that coupled and uncoupled RyR1 channels gated synchronously with the similar opening and closing rates. They likely collected data from the channels with activity less than 0.5, where the gating kinetics parameters of coupled and single RyR1 channels were only marginally different.

The next important result of our work was the comparison between flicker gating during the main open events of coupled channels and gating behavior of the single RyR2 channel. Previously, we described this flickering as a reflection of the thermodynamic stability of the functional interaction between RyR2 channels when individual channels exhibited a higher tendency to escape from a synchronized regime of their operation (Gaburjakova and Gaburjakova 2008). However, in the present work, we provide a new view of this characteristic in order to gain further evidence in support of the coupled gating phenomenon. It is clear from our results that the flicker gating of both LA and HA coupled RyR2 channels was resistant to cytosolic Ca^{2+}, in contrast to the strong Ca^{2+} dependence of the gating behavior of the single RyR2 channel. In addition, across the whole range of tested concentrations, the average closed time was considerable shorter, which inevitably caused a significantly higher frequency of closing for coupled channels. This finding provides strong evidence that individual RyR2 channels coupled into a single functional unit influence the gating behavior of one another. Our conclusion is supported by mathematical models of cardiac EC coupling, where the allosteric interaction between neighboring RyR2 channels was presented through interaction energy (Stern et al. 1999; Sobie et al. 2002; Hinch 2004). Thus, when channels are tightly coupled, the transition of one closed channel to an open state will be effectively prevented by an energy barrier when that channel is surrounded by several other closed channels. In other words, the neighbors in the same state as the central channel stabilize the central one. However, when the interaction between channels is attenuated, then the probability that interacting channels could be found in a different functional state at a given time is enhanced.

In general, an ion channel possesses two basic characteristics. It is ion selective and is able to control movement of ions by opening or closing the ion conduction pathway at the desired time. Therefore, we investigated the concentration dependence of the Ca^{2+} current and the Ba^{2+}-Ca^{2+} selectivity of coupled RyR2 channels in order to compare these properties with those of the single RyR2 channel. Ultimately, these studies are aimed at gaining insight into the ion translocation and selection processes in conductive pathways of individual RyR2 channels.

The concentration dependence of Ca^{2+} current flowing through the channel pores was saturated and fitted using a Michaelis-Menten curve with similar values of K_D for LA and HA coupled as well as single RyR2 channels [$K_D = 8.46 \pm 0.59$ mM for LA coupled channels, 8.68 ± 0.45 mM for HA coupled channels, and 8.8 mM for the single channel (Tomaskova and Gaburjakova 2008)]. In order to test the ion selectivity, we decided to use the mole fraction experiment with various mixtures of Ba^{2+} and Ca^{2+} ions present on the luminal side of the channels. This was possible only because the coupled gating phenomenon is not fixed to Ca^{2+} flux through RyR2 channels and is stable also when pure Ba^{2+} ion is used as a charge carrier. Under these conditions neither LA nor HA coupled RyR2 channels discriminated between Ba^{2+} and Ca^{2+} ions. The same characteristic was reported for the single RyR2 channel (Tomaskova and Gaburjakova 2008). In general, discrimination between ions takes place in a selectivity filter of a channel where the strong electrostatic interaction of ions with residues lining the filter takes place. Thus, the composition and the architecture of this narrowest region of the conduction pathway determine the specific mechanism of ion translocation and selection in a channel pore. From this, it is clear that disruption of the electrostatic environment in the selectivity filter results in an alteration in ion selectivity. It is reasonable to consider the existence of a potential electrostatic interaction between individual RYR2 channels coupled into a functional complex. Then we could imagine that the conductive pathway is not insulated, and when coupled channels get closer to each other the electrostatic fields governing ion discrimination may overlap, resulting in an increase or decrease in ion selectivity. However, it seems from our results that this is not the case for coupled RyR2 channels. Overall, the coupling between RyR2 channels does not interfere with the studied permeation properties, and each channel recruited into a functional complex retains the conductive properties of the single RyR2 channel.

Conclusions

In light of the above arguments, we suggest that, of the whole conductive pathway, only gates may be affected by the coupling between RyR2 channels. Currently, the precise location of the RyR2 channel gate there is not known due to the absence of the channel crystal structure. We have some suggestions based on a model of the putative pore region of the RyR2 channel constructed using the known tertiary structure of the bacterial K^+ channel (Welch et al. 2004). This model predicted that the last two transmembrane helices (outer and inner helix) and their linking luminal loop comprise the putative channel pore. In the case of the K^+ channel, bringing the inner helices close together blocks the ion conduction pathway, and moving them apart opens the channel. It is apparent that when the

channel moves between the closed and the open states, substantial transmembrane helical movements are required (Doyle 2004). Considering all of these facts, the region of inner helix crossover was suggested to operate as the RyR2 channel gate (Welch et al. 2004). It is questionable how information about the position of one channel gate could be transferred to the gate of an adjacent channel. Evidently, some form of crosstalk must take place between gates. In support of this hypothesis, the RyR2 channels were found to be clustered into arrays on the membrane of the SR such that each channel physically contacts four of its neighbors (Flucher and Franzini-Armstrong 1996; Franzini-Armstrong et al. 1999). Therefore, it is possible that the conformation of each channel influences the conformational state of its neighbors. It is obvious that the rate of conformational spreading should be fast enough to synchronize the gating of channels in order to correlate the Ca^{2+} fluxes.

Taken together, our data suggest that the main purpose of coupling between RyR2 channels is synchronization of the channel gates, while the functional and the permeation properties of individual RyR2 channels remain unchanged.

Acknowledgments We thank Lubica Malekova for technical assistance. This work was supported by the Slovak Scientific Grant Agency (Grant VEGA 2/0118/09) and the Government of Slovak Republic (Grants SP 51/0280800/0280802 and SP 51/0280900/0280901).

References

Buck ED, Lachnit WG, Pessah IN (1999) Mechanisms of δ-hexachlorocyclohexane toxicity: I. Relationship between altered ventricular myocyte contractility and ryanodine receptor function. J Pharmacol Exp Ther 289:477–485

Doyle DA (2004) Structural changes during ion channel gating. Trends Neurosci 27:298–302

Flucher BE, Franzizni-Armstrong C (1996) Formation of junctions involved in excitation-contraction coupling in skeletal and cardiac muscle. Proc Natl Acad Sci USA 93:8101–8106

Franzini-Armstrong C, Protasi F, Ramesh V (1999) Shape, size, and distribution of Ca^{2+} release units and couplons in skeletal and cardiac muscles. Biophys J 77:1528–1539

Gaburjakova J, Gaburjakova M (2006) Comparison of the effects exerted by luminal Ca^{2+} on the sensitivity of the cardiac ryanodine receptor to caffeine and cytosolic Ca^{2+}. J Membr Biol 212:17–28

Gaburjakova J, Gaburjakova M (2008) Effect of luminal Ca^{2+} on the stability of coupled gating between ryanodine receptors from the rat heart. Acta Physiol 193:219–227

Gillespie D, Eisenberg RS (2002) Physical descriptions of experimental selectivity measurements in ion channels. Eur Biophys J 31:454–466

Hinch R (2004) A mathematical analysis of the generation and termination of calcium sparks. Biophys J 86:1293–1307

Iwasa K, Ehrenstein G, Moran N, Jia M (1986) Evidence for interactions between batrachotoxin-modified channels in hybrid neuroblastoma cells. Biophys J 50:531–537

Kiss T, Nagy K (1985) Interaction between sodium channels in mouse neuroblastoma cells. Eur Biophys J 12:13–18

Marx SO, Gaburjakova J, Gaburjakova M, Ch Henrikson, Ondrias K, Marks AR (2001) Coupled gating between cardiac calcium release channels (ryanodine receptors). Circ Res 88:1151–1158

Neumcke B, Stampfli R (1983) Alteration of the conductance of Na^{+} cannels in the nodal membrane of frog nerve by holding potential and tetrodotoxin. Biochim Biophys Acta 727:177–184

Rodriguez-Contreras A, Nonner W, Yamoah EN (2002) Ca^{2+} transport properties and determinants of anomalous mole fraction effects of single voltage-gated Ca^{2+} channels in hair cells from bullfrog saccule. J Physiol 538:729–745

Sobie EA, Dill KW, dos Santos Cruiz J, Lederer WJ, Jafri MS (2002) Termination of cardiac Ca^{2+} sparks: an investigative mathematical model of calcium-induced calcium release. Biophys J 83:59–78

Stern MD, Song LS, Cheng H, Sham JS, Yang HT, Boheler KR, Ríos E (1999) Local control models of cardiac excitation-contraction coupling. A possible role for allosteric interactions between ryanodine receptors. J Gen Physiol 113:469–489

Stern MD, Cheng H (2004) Putting out the fire: what terminates calcium-induced calcium release in cardiac muscle? Cell Calcium 35:591–601

Tomaskova Z, Gaburjakova M (2008) The cardiac ryanodine receptor: looking for anomalies in permeation properties. Biochim Biophys Acta 1778:2564–2572

Welch W, Rheault S, West DJ, Williams AJ (2004) A model of the putative pore region of the cardiac ryanodine receptor channel. Biophys J 87:2335–2351

Yeramian E, Trautmann A, Claverie P (1986) Acetylcholine receptors are not functionally independent. Biophys J 50:253–263