Coupled Gating Between Cardiac Calcium Release Channels
(Ryanodine Receptors)

Steven O. Marx,* Jana Gaburjakova,* Marta Gaburjakova,* Charles Henrikson,
Karol Ondrias, Andrew R. Marks

Abstract—Excitation-contraction coupling in heart muscle requires the activation of Ca\(^{2+}\)-release channels/type 2 ryanodine receptors (RyR2s) by Ca\(^{2+}\) influx. RyR2s are arranged on the sarcoplasmic reticulum membrane in closely packed arrays such that their large cytoplasmic domains contact one another. We now show that multiple RyR2s can be isolated under conditions such that they remain physically coupled to one another. When these coupled channels are examined in planar lipid bilayers, multiple channels exhibit simultaneous gating, termed “coupled gating.” Removal of the regulatory subunit, the FK506 binding protein (FKBP12.6), functionally but not physically uncouples multiple RyR2 channels. Coupled gating between RyR2 channels may be an important regulatory mechanism in excitation-contraction coupling as well as in other signaling pathways involving intracellular Ca\(^{2+}\) release. (Circ Res. 2001;88:1151-1158.)

Key Words: ryanodine receptors ■ Ca\(^{2+}\) channels ■ coupled gating ■ FKBP12.6 ■ excitation-contraction coupling

Ryanodine receptors (RyRs) are intracellular ion channels that provide a pathway for the release of Ca\(^{2+}\) from the sarcoplasmic reticulum (SR)/endoplasmic reticulum into the cytosol. The release of intracellular stores of Ca\(^{2+}\) occurs in virtually all types of cells as a means of amplifying external signals that modulate intracellular signaling events. In cardiac myocytes, type 2 RyRs (RyR2s) are activated during excitation-contraction (E-C) coupling by Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) triggered by Ca\(^{2+}\) influx across the sarcolemma.

If RyR2s act independently, then the activation and inactivation of individual channels should be stochastic, based on the probability of an individual channel being open or closed. Although the process is complicated by cellular geometry, the distribution of RyR2s, and local activation of CICR in cardiac myocytes,1,2 the probability that any single RyR2 opens should still be determined in part by CICR.3-6 However, we now present data showing that RyR2 channels are physically and functionally coupled such that the activity of an individual channel is coordinated with that of its neighbors in a manner analogous to that in skeletal muscle.7

Ca\(^{2+}\)-release channels in cardiac muscle comprise four 565-kDa RyR2 subunits and four FK506 binding proteins (FKBP12.6). FKBP12, originally identified as the peptide KC 7 that copurifies with the type 1 RyR (RyR1),8 and FKBP12.6 (found in cardiac muscle9) are members of the immunophilin family of cis-trans peptidyl-prolyl isomerases that serve as cytosolic receptors for the immunosuppressant drugs rapamycin and FK506.10,11 In the absence of FKBP12/12.6, a homotetrameric RyR1 or RyR2 channel can be examined in planar lipid bilayers but reveals subconductance states, consistent with a defect in coordinated activity of the four subunits that form the RyR channel.12,13 The addition of FKBP12 to recombinant RyR1 stabilizes the channel complex, resulting in channels with full conductance.12 These stabilizing effects are reversed by treating the channels with rapamycin or FK506 to remove FKBP12/12.6 from the RyR channels.12,13 From these findings, we hypothesize that the FKBPs serve to stabilize interactions among RyR subunits.12 Skeletal muscle Ca\(^{2+}\)-release channels (RyR1s) exhibit a phenomenon termed “coupled gating.”7 Coupled gating enables RyR1 Ca\(^{2+}\)-release channels in a given SR/T-tubule junction to open and close in a coordinated manner. The spatial organization of RyR1s on the SR junctional membrane into clustered arrays of channels is such that each channel physically contacts four of its neighbors.14 Clustering of channels is critical if coupled gating is to be physiologically important. In the heart, the spatial organizations of RyR2s and the sarcolemmal Ca\(^{2+}\) channels required to activate CICR have no clear relation to one another, as opposed to skeletal muscle, in which every other RyR1 is closely apposed to four Ca\(^{2+}\) channels in the T tubule.15 However, the RyR2s are clustered into arrays on the junctional SR such that each channel physically contacts four of its neighbors.16,17 The...
assembly of multiple RyR2s into functional Ca\textsuperscript{2+}-release units mediated by coupled gating in cardiomyocytes provides a means of regulating the Ca\textsuperscript{2+} signal required for E-C coupling in the heart.

Materials and Methods

Isolation of RyR2s From Cardiac SR

Canine cardiac muscle heavy SR was isolated as described,\textsuperscript{13} incubated with [\textsuperscript{1}H]ryanodine, solubilized with CHAPS, and centrifuged on a 10% to 32% linear sucrose gradient at 26 000 rpm and 2°C in a Sorvall AH-629 rotor.\textsuperscript{7,12} In some experiments, cardiac SR was preincubated with rapamycin (2 \textmu M) to remove FKBP12.6 from RyR2s.

Immunoblots

Immunoblots were performed as described\textsuperscript{18} by use of the following: anti-FKBP12 (1:1000, recognizes both FKBP12 and FKBP12.6) and anti-RyR (5029, 1:3000).\textsuperscript{19} After they were washed, the membranes were incubated with peroxidase-conjugated goat anti-rabbit IgG antiserum (1:3000, Boehringer-Mannheim) for 60 minutes at room temperature, washed 3 times with Tris-buffered saline and 0.1% Tween 20, and developed by use of enhanced chemiluminescence (ECL, Amersham).

Single-Channel Recordings

Cardiac SR microsomes or purified RyR2s (dialyzed into lipid liposomes) were fused to planar lipid bilayers as described.\textsuperscript{7,12} Single-channel experiments were conducted under voltage-clamp conditions. Cardiac SR vesicles or liposomes were added to the cis chamber near the planar lipid bilayer composed of 3:1 phosphatidyl ethanolamine/phosphatidyl serine (Avanti Polar Lipids). The bilayer cup was polystyrene with a 0.15-mm aperture. Fusion of vesicles/liposomes was promoted by KCl added to the cis chamber (∼500 mmol/L). After incorporation of a Ca\textsuperscript{2+} channel, the KCl gradient was eliminated by perfusion of cis chamber with cis solution (10 mL). Solutions were as follows: trans, 250 mmol/L HEPES, 53 mmol/L Ca(OH)\textsubscript{2} or Ba(OH)\textsubscript{2}, and 50 mmol/L KC\textsubscript{l}, pH 7.35; cis, 250 mmol/L HEPES, 125 mmol/L Tris, 1 mmol/L EGTA, 0.5 mmol/L CaCl\textsubscript{2}, and 50 mmol/L KC\textsubscript{l}, pH 7.35. Free Ca\textsuperscript{2+} concentration of cis was calculated by using CHELATOR software.\textsuperscript{20} The trans chamber was connected to the head-stage input of an Axopatch 200 amplifier (Axon Instruments) with Ag/AgCl electrodes and an agar/KCl bridge. The cis chamber was held at ground with a similar electrode. Single-channel currents were filtered at 1 kHz with an 8-pole Bessel filter (Warner Instruments) and digitized at 4 kHz. Data were collected on a Pentium computer with the use of AxoSceopel (Axon Instruments) and a Digidata 1200 (Axon Instruments) interface. pClamp 6.0.1 (Axon Instruments) was used for analyses. Open probability (P\textsubscript{o}) was identified by using >2 minutes of continual recording. Time constants and proportions (area under the curve for each exponential distribution) were calculated by using SigmaPlot 4.0 (time constant exponentials were fitted by using the Levenberg-Marquardt least-squares method). Open-time distributions were generated by pooling data for individual channels (n=29), coupled channels with Ca\textsuperscript{2+} as the charge carrier (n=16), and coupled channels with Ba\textsuperscript{2+} as the charge carrier (n=2). P\textsubscript{o} for individual channels in experiments in which there was more than one channel in the membrane (eg, one set of coupled channels and one or more single channels) was analyzed by manually identifying each opening and by using pClamp to determine its duration. Current-amplitude histograms are all-points histograms. The numbers of channel openings analyzed for each histogram are as follows: Figure 2B, 76; Figure 2D, 84; Figure 2F, 24; Figure 5B, 223; Figure 5G, 216; Figure 6B, 47; Figure 6G, 133; and Figure 6I, 35). Results are mean±SD. Significance of differences were analyzed by the Student t test and are regarded as statistically significant at P<0.05.

Results

RyR2 channels were isolated from myocardium by sucrose density gradient centrifugation. The 30S complex represents single tetrameric RyR2 channels, and the ≈60S complex represents two physically associated RyR2 channels (Figure 1A). Immunoblot analyses showed that RyR2 protein was in both the 30S and 60S complexes as well as in denser complexes, consistent with multiple channels (≥2) being physically associated (Figure 1B, top). Pretreatment of SR microsomes with rapamycin (2 \textmu M) caused dissociation of FKBP12.6 from RyR2s (Figure 1B, bottom) but did not alter sedimentation of RyR2 complexes (Figure 1B, second panel; densitometric analyses of the immunoblots showed no significant differences in the amount of immunoreactive RyR2 protein in each fraction of the sucrose gradient for control versus rapamycin-treated samples). In the rapamycin-treated samples, most of the FKBP12.6 was in upper fractions of the gradient (≥14% sucrose). In the samples not treated with rapamycin, most of the FKBP12.6 was in fractions containing RyR2s (≥18% sucrose, Figure 1B). Removal of FKBP12.6 by rapamycin is such that we cannot detect any FKBP12.6 co-distribution with RyR2s, nor is there any FKBP12.6 detectable in RyR2 immunoprecipitates (data not shown). As noted above, there is no significant reduction in RyR2 protein in the FKBP12.6-stripped RyR2 samples compared with control nonstripped RyR2s on the basis of immunoblotting and [\textsuperscript{1}H]ryanodine binding (Figures 1A and 1B). Thus, removal of FKBP12.6 does not influence the physically associated coupling of RyR2 channels.

Cardiac microsomes containing RyR2 channels, as well as RyR2 channels from the 30S complex isolated by sucrose density gradient centrifugation, were incorporated into planar lipid bilayers and revealed stable openings of ≈4 pA (eg, Figure 2A). A current-amplitude histogram (Figure 2B) revealed two discrete populations of events, closed channels (0 pA) and openings to the full amplitude of a single channel (≈4 pA). Channels similar to these have been well described.

Figure 1. Physical coupling between cardiac RyR2 channels. A, Physical association between RyR2/Ca\textsuperscript{2+}-release channels demonstrated by centrifugation of cardiac muscle heavy SR (incubated with [\textsuperscript{1}H]ryanodine and solubilized with CHAPS) through 10% to 32% linear sucrose density gradients\textsuperscript{12} for 14 hours±preincubation with rapamycin (2 \textmu M) to dissociate FKBP12.6 from RyR2s. The 18% sucrose fraction contains the 30S (arrowhead) RyR2 complex, and the ≈60S (arrow) complexes (two physically coupled RyR2 channels) are in the 22% sucrose fraction. B, Immunoblot analyses of centrifuged cardiac SR with (+) or without (−) preincubation with rapamycin (2 \textmu M) to dissociate FKBP12.6 from RyR2s. Fractions were size-fractionated on SDS-PAGE and immunoblotted by using anti-RyR or anti-FKBP12 (which cross-reacts with FKBP12.6) antisera. Data shown are representative of 4 similar experiments.
in the past and have been shown to be due to the Ca\textsuperscript{2+}-activated RyR2s from the heart. However, some channels from cardiac microsomes as well as RyR2 channels from the 60S complex opened to \( \sim 8 \) pA, twice the normal current amplitude and conductance (eg, Figure 2C), a finding consistent with the simultaneous opening of two RyR2s. Double-amplitude “coupled” channels opening to \( \sim 8 \) pA were observed in 19 of 205 consecutive experiments (9.3%) using canine cardiac microsomes, and triple-amplitude coupled channels opening to \( \sim 12 \) pA were observed in 3 of 205 consecutive experiments (1.5%). A current-amplitude histogram (Figure 2D) revealed closed channels (0 pA) and openings to \( \sim 8 \) pA. The RyR2 has a conductance for Ca\textsuperscript{2+} of \( \sim 100 \) pS.\textsuperscript{4} The conductances were 100\( \pm 6 \) pS for the single-amplitude openings compared with 200\( \pm 17 \) pS for the double-amplitude openings. Double-amplitude coupled channels opening to \( \sim 8 \) pA were also observed by using Ba\textsuperscript{2+} (n=2) as the charge carrier, indicating that Ca\textsuperscript{2+} fluxing through the channel is not required for coupled gating.

Ultrastructural studies suggest that RyR2s in cardiomyocytes are present in dense arrays on the SR, with the four

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**Figure 2.** Coupled gating between individual cardiac RyR2 channels. A, Cardiac microsomes containing RyR2s corresponding to the 30S sucrose gradient fraction exhibiting channels opening to \( \sim 4 \) pA when fused to planar lipid bilayers, with Ca\textsuperscript{2+} used as the charge carrier. Average dwell times are indicated as open time (\( T_o \)) and closed time (\( T_c \)). B, Corresponding current-amplitude histogram. C, Two coupled RyR2 channels opening to \( \sim 8 \) pA, with Ca\textsuperscript{2+} used as charge carrier. D, Corresponding current-amplitude histogram. E, Three coupled channels opening to \( \sim 12 \) pA, with Ca\textsuperscript{2+} used as the current carrier. F, Corresponding current-amplitude histogram. Recordings were at 0 mV, and channel openings are upward. Dashes to the left of the tracings indicate closed state (C) and current levels for single (\( \sim 4\)-pA), two coupled (\( \sim 8\)-pA), and three coupled (\( \sim 12\)-pA) channels.

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**Figure 3.** Ryanodine and ruthenium red modify coupled RyR2 channels. A, Effect of ryanodine on coupled RyR2 channels. Ryanodine induces stable 50% subconductance states sequentially in each of the two coupled RyR2 channels. B, Effect of ruthenium red on coupled RyR2 channels. Coupled RyR2 channels were first activated with caffeine (5 mmol/L), and then ruthenium red (20 \( \mu \)mol/L) was added to inhibit the channels. Recordings were at 0 mV, and channel openings are in the upward direction. Dashes to the left of the tracings indicate closed state (C) and current levels for single (\( \sim 4\)-pA) and two coupled (\( \sim 8\)-pA) channels.

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**Figure 4.** Effects of caffeine and MgCl\textsubscript{2} on coupled RyR2 channels. A, Two coupled cardiac RyR2 channels opening to \( \sim 8 \) pA. Channels are activated with caffeine (5 mmol/L) (middle tracing) and inhibited by MgCl\textsubscript{2} (1 mmol/L) (bottom tracing). B, Comparison of the \( P_o \) of coupled RyR2 channels activated by caffeine (n=5). Recordings were at 0 mV, and channel openings are in the upward direction. Dashes at the left of the tracings indicate closed state (C) and current levels for single (\( \sim 4\)-pA), and two coupled (\( \sim 8\)-pA) channels.
corners of each channel appearing to contact corners of each of four neighboring channels. We estimate that \( \approx 10\% \) of these RyR2 channels remain physically connected in native cardiac SR preparations. This is based, in part, on electron micrographs of isolated cardiac SR showing that \( \approx 10\% \) of the channels remain physically contacting one another after isolation of the vesicles with the use of methods similar to the ones used in the present study. With use of slightly modified conditions for cardiac SR vesicle fusion to the bilayer (see Materials and Methods), coupled RyR2s were observed in \( \approx 11\% \) of our experiments (n = 22 of 205).

RyR2s are organized in large arrays (up to 300); thus, we expect to find interacting RyR2 homotetrameric units with a multiplicity \( \geq 2 \). This is consistent with the presence of RyR2 complexes sedimenting below the 30S complex on the sucrose gradient (Figure 1B, top two panels) and our finding (in planar lipid bilayer experiments) of channels that open and close with current amplitudes equivalent to three RyR2 homotetrameric channels (n = 3, Figures 2E and 2F). The probability of obtaining still larger arrays of interacting and functional RyR2 channels is reduced as the number increases because of the likely instability of these arrays when purified biochemically.

Ryanodine (5 \( \mu \text{mol/L} \)) induced the characteristic 50% subconductance state when it was added to coupled channels (Figure 3A), resulting in a current amplitude of \( \approx 4 \text{ pA} \) (equivalent to two coupled channels, each contributing 50% of the normal single-channel current amplitude of \( \approx 4 \text{ pA} \)). The effect of ryanodine further supports the concept that the \( \approx 8\text{-pA} \) openings represent two coupled RyR2 channels. In this experiment (Figure 3A), ryanodine partially blocks the pore of each channel (resulting in a reduction from \( \approx 8 \text{ pA} \) to \( \approx 6 \text{ pA} \), representing coupled gating of one half-conducting channel and one fully open channel). Subsequently, ryanodine blocks the pore of the second channel, resulting in a final current amplitude of \( \approx 4 \text{ pA} \), representing two half-conducting channels, consistent with the known effect of ryanodine on the RyR2 channel.

Ruthenium red (20 \( \mu \text{mol/L} \)), an RyR2 inhibitor, blocked coupled RyR2 channels (Figure 3B). In this experiment, two coupled RyR2 channels were first activated with caffeine (5 mmol/L) and then blocked with 20 \( \mu \text{mol/L} \) ruthenium red (Figure 3B). In contrast to ryanodine, which sequentially modified each of the two coupled RyR2 channels (Figure 3A), ruthenium red simultaneously blocked both coupled channels (Figure 3B). Coupled RyR2 channels also responded to other known modulators of RyR2 channel function in the same manner as single RyR2 channels. For example, coupled RyR2 channels were activated by 5 mmol/L caffeine (Figures 4A, middle tracing, and 4B), and 1 mmol/L MgCl\(_2\) decreased the \( P_o \) and current amplitude (Figure 4A, bottom tracing). Taken together, these data show that coupled RyR2 channels respond to modulators in the same manner as single RyR2 channels.
One possible explanation for coupled gating between RyR2s is that Ca\(^{2+}\) fluxing through one channel activates a neighboring channel via CICR. To exclude this possibility, we also recorded coupled RyR2 channels by using Ba\(^{2+}\) as the charge carrier (n=2; eg, see Figures 5A and 5B). We also observed coupled gating between RyR2 channels with K\(^+\) used as the charge carrier (n=3; with use of 250 mmol/L KCl trans/50 mmol/L KCl cis, data not shown). Dissociation of FKBP12.6 from RyR2s with rapamycin (2 \(\mu\)mol/L) first caused the \(\sim\)8-pA openings to be halved to \(\sim\)4 pA (two uncoupled channels) and then induced subconductance states in each of the uncoupled channels, as shown in (Figures 5A through 5H). Twenty-five minutes after addition of rapamycin (2 \(\mu\)mol/L), the two coupled channels first uncoupled (Figure 5D); 27 minutes after adding rapamycin, the two RyR2 channels were clearly uncoupled (Figures 5F and 5G); 32 minutes after the addition of rapamycin, the uncoupled RyR2 channels exhibited subconductance states (Figure 5H). In this experiment (Figure 5E), the P_o of the coupled channels decreases to zero after the complete uncoupling that is due to the removal of FKBP12.6 is achieved. We conclude that removal of FKBP12.6 causes RyR2s to gate independently despite the fact that they remain physically associated (see Figure 1B). Interestingly, in some experiments, the open states (but not the closed states or baseline) of the coupled channels were noisier than those of single channels. This was particularly evident during the removal of FKBP12.6 (ie, during the transition from coupled to uncoupled channels, as shown in Figures 5C, 5D, and 6C), consistent with the concept that FKBP12 and FKBP12.6 “stabilize” RyR1s and RyR2s, respectively.\(^{13}\) Longer treatment with rapamycin (2 \(\mu\)mol/L) leads to the appearance of subconductance states of the individual uncoupled RyR2 homotetrameric channels (Figure 5H), similar to those previously reported when single RyR2 channels are treated with rapamycin.\(^{13}\) Thus, we conclude that FKBP12.6 facilitates the coordination of single RyR2 subunits with the other members of the homotetramer and also permits homotetrameric RyR2s to exhibit coupled gating with each other.

An important question is whether dissociation of FKBP12.6 from RyR2s results in functional uncoupling of the channels followed by the induction of subconductance states in each channel versus the induction of subconductance states in each individual channel without functionally uncoupling them. In either case, the result would be the appearance of subconductance states, as shown in Figure 5H. Examination of the transition from two coupled channels to two uncoupled channels after dissociation of FKBP12.6 by rapamycin (2 \(\mu\)mol/L) shows that the two channels first uncouple (\(\sim\)25 minutes after the addition of rapamycin) and gate independently and that they subsequently (\(>\)27 minutes after rapamycin addition) develop subconductance states (Figures 5A through 5H). Because each RyR2 channel binds 4 FKBP12.6 (one per subunit), one possible explanation for this sequence of events is that dissociation of one or two FKBP12.6 molecules is sufficient to uncouple the channels and that subconductance states appear after dissociation of the remaining FKBP12.6.
The role of FKBP12.6 in coupled gating was further supported by experiments showing that two physically coupled channels that are functionally uncoupled because of the removal of FKBP12.6 with rapamycin become functionally coupled with the readdition of FKBP12.6 (Figure 6). In these experiments, two coupled channels were uncoupled by the addition of rapamycin (Figures 6A through 6G). Rapamycin was washed out, and then 30 minutes after addition of FKBP12.6, coupled gating of the two channels was restored (Figures 6H through 6I).

Measured under identical conditions (cis Ca\(^{2+}\) = 150 nmol/L), the \(P_0\) of coupled channels was significantly increased compared with uncoupled channels (two coupled channels \(P_0 = 0.47 ± 0.06\), \(n = 16\) versus single channels \(P_0 = 0.03 ± 0.03\), \(n = 8\), \(P < 0.01\); Figures 7A and 7B). In some experiments (\(n = 9\)), two coupled channels and an uncoupled channel were active in the same bilayer membrane (Figures 7A and 7B). These experiments show the increased \(P_0\) of two coupled channels compared with single channels under the same conditions, ie, when the cis and trans solutions are the same for both the coupled and uncoupled channels (eg, cis Ca\(^{2+}\) = 150 nmol/L). The distributions of open times for individual versus coupled channels are shown in Figure 7C. Fitting with three exponentials yielded mean open times of \(t_1 = 0.48 ± 0.06\) ms, \(t_2 = 1.88 ± 0.24\) ms, and \(t_3 = 13.60 ± 0.91\) ms for the individual channels and \(t_1 = 75.8 ± 2.8\) ms, \(t_2 = 285.7 ± 32.7\) ms, and \(t_3 = 2500 ± 625.0\) ms for the coupled channels. For each of these mean open times, there is a statistically significant difference between individual and coupled channels (\(P < 0.01\)). The open-time distributions for coupled channels recorded with Ba\(^{2+}\) used as the charge carrier were fit with two exponentials, yielding mean open times of \(t_1 = 86.06 ± 34.81\) ms and \(t_2 = 176.90 ± 50.07\) ms. As with the coupled channels recorded with Ca\(^{2+}\) used as the charge carrier, the mean open times for coupled channels recorded with Ba\(^{2+}\) used as the charge carrier were longer than those observed for individual

Figure 7. Coupled RyR2 channels exhibit increased \(P_0\) and \(T_o\) compared with single uncoupled channels under identical conditions. A, Two coupled RyR2 channels and two uncoupled RyR2 channels recorded at cis Ca\(^{2+}\) (150 nmol/L) in the same bilayer. Recordings were at 0 mV, and channel openings are upward. Dashes at left of tracings indicate closed state (C) and current levels for single (~4-pA) and two coupled (~8-pA) channels. B, \(P_0\) of coupled (●) and single (○) RyR2 channels from 9 experiments in which both coupled and single channels were observed in the same bilayer membrane. C, Open-time distributions for individual vs coupled RyR2 channels. Curves were fit with three exponentials to derive \(t_1\), \(t_2\), and \(t_3\). The fraction of time that the channels opened to each of the three classes of open times are represented by \(W_1\), \(W_2\), and \(W_3\).
RyR2 channels with Ba2+ used as the charge carrier. Interestingly, the longest time constant observed for coupled RyR2 channels with Ca2+ used as the charge carrier (τ1 = 2500 ms, Figure 7C) was not observed for coupled channels with Ba2+ used as the charge carrier. These longest openings of coupled channels may reflect the effect of CICR in which Ca2+ fluxing through one open channel could help keep the second channel open. In 8 of 9 such experiments (when two coupled RyR2s and a single RyR2 channel were recorded in the same bilayer), the coupled RyR2 channels exhibited a higher P, than the single RyR2 channels (Figure 7B). This may be due to the fact that two coupled RyR2 channels have twice as many Ca2+-activating sites as single RyR2 channels. Compared with single RyR2 channels, coupled channels also exhibited increased caffeine sensitivity (data not shown). This finding further supports the concept that two coupled RyR2 channels have twice as many Ca2+-activating sites as a single RyR2 channel, inasmuch as caffeine is known to activate RyR2s by increasing the Ca2+ sensitivity of the channel. Moreover, the ability to record coupled and single RyR2 channels in the same bilayer excludes the possibility that recording of double-amplitude RyR2 channels could be due to artifacts or other technical explanations (eg, drift in amplifier gain).

Discussion

Could observations that suggest functionally important coupled gating of RyR2s in the heart be due to some other channel? This is unlikely for several reasons: (1) The pharmacological profile of coupled channels is the same as that of individual RyR2s. The characteristic 50% conductance state was observed in response to appropriate concentrations of ryanodine but scaled by the number of coupled RyR2s (Figure 3A). Additionally, caffeine activates coupled RyR2 channels, MgCl2 inhibits them, and ruthenium red blocks the coupled channels just as they block the single RyR2 channels (Figure 4). (2) Uncoupling of the coupled channels leads to RyR2 channels with half the current of two coupled RyR2 channels (eg, Figure 5). (3) The conversion of coupled RyR2s into uncoupled RyR2s depends on the presence or absence of a specific protein, FKBP12.6 (Figure 5), and the addition of FKBP12.6 can restore coupled gating between channels (Figure 6). (4) When highly purified RyR2 protein containing FKBP12.6 is fused to planar lipid bilayers, we observe coupled channels.

The present study of coupled gating between cardiac RyR2 channels supports the following conclusions: (1) Cardiac RyR2s can exhibit coupled gating. (2) Physical coupling between RyR2s does not require FKBP12.6, whereas functional coupling between RyR2s does require FKBP12.6. (3) Removal of FKBP12.6 from RyR2s with rapamycin first uncouples two coupled channels, and then each individual channel exhibits subconductance states. (4) Two coupled RyR2 channels exhibit an increased P, compared with a single channel.

Taken together, the data of the present study show that two or more physically connected RyR2 channels can gate simultaneously, a phenomenon termed coupled gating.7 Coupled gating between individual RyR2s, which requires FKBP12.6, provides a mechanism for the coordinated activation and inactivation of RyR2/Ca2+-release channels during cardiac muscle E-C coupling. Coupled gating may explain how the coordinated termination of cardiac SR Ca2+ release occurs (Figure 8). Coupled gating provides a mechanism for simultaneously closing all RyR2s in a T-tubule/SR junction, thereby reducing the probability that individual RyR2 channels will be reactivated stochastically by Ca2+ fluxing through their neighbors (Figure 8).

It has been proposed that the Ca2+ efflux via the RyR2s leads to an elevated [Ca2+], that produces RyR2 inactivation.21 However, experiments suggest that inactivation processes dependent on cytosolic [Ca2+] are too slow to account for the termination of Ca2+ release.23–25 A second mechanism proposed for closing RyR2s during cardiac E-C coupling relies on the stochastic closing of RyR2s. However, modeling of the stochastic closing of RyR2s suggests that this mechanism may also be too slow.26 A third possibility is that a reduction in SR Ca2+ (that is due to Ca2+ release) signals the RyR2s to close because the RyR2 P, depends (in part) on SR luminal Ca2+.5 SR Ca2+ depletion combined with coupled gating could account for RyR2 closure and termination of Ca2+ release. Under this proposed model (Figure 8), SR Ca2+ depletion reduces the P, of RyR2s in a T-tubule/SR junction. When the first channel closes because of decreased P, that is signaled by falling SR Ca2+ levels, all RyR2s in the junction close because of coupled gating.

Treatment of cardiomyocytes with FK506, which can dissociate FKBP12.6 from RyR2s, alters Ca2+ sparks.27,28
FK506 prolonged Ca\(^{2+}\) sparks,\(^{29}\) a finding that is consistent with a model in which uncoupling RyR2 channels causes a defect in RyR2 closure. However, another study found that FK506 did not significantly change spark frequency, although spark frequency was increased.\(^{27}\) The fact that Ca\(^{2+}\) sparks are observed despite dissociation of FKBP12.6 from RyR2s indicates that coupled gating is not required for the generation of sparks. There is clear evidence that in vivo FKBP12.6 is physically associated with RyR2s.\(^{9,18}\) Defects in cardiomyocyte Ca\(^{2+}\) signaling were recently reported in an FKBP12.6 knockout mouse model.\(^{29}\) These defects included an increase in the amplitude and duration of Ca\(^{2+}\) sparks in cardiomyocytes from FKBP12.6-deficient mice.\(^{29}\) Thus, although the physiological impact of coupled gating has not been proven, currently available data involving FKBP12.6 knockout mice support the concept that it plays a role in modulating E-C coupling in cardiac muscle. The present findings may have important implications for understanding the mechanisms underlying human disease states, such as heart failure\(^{30}\) and sudden cardiac death. For example, we have recently shown that protein kinase A hyperphosphorylation of RyR2s occurs in failing hearts.\(^{30}\) Protein kinase A hyperphosphorylation dissociates FKBP12.6 from the RyR2 macromolecular complex.\(^{30}\) These data suggest that coupled gating of RyR2s would be reduced in failing hearts because of the dissociation of FKBP12.6. Reduced coupled gating between RyR2s might result in defective closure of RyR2 channels, which could deplete SR Ca\(^{2+}\), reduce E-C coupling gain, and/or contribute to diastolic depolarizations that can initiate fatal cardiac arrhythmias. However, these speculations as to the in vivo consequences of the inhibition of coupled gating will have to be tested with the use of physiological animal models.

Acknowledgments

This study was supported by the National Institutes of Health (Dr Marks), the American Heart Association (Drs Marks and Marx), and Vedecka Granvola Agentura 7019 (Dr Ondrias). Dr Marks is a Doris Duke Distinguished Clinical Scientist. We thank R. Kass and J. Lederer for helpful discussions.

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Circ Res. 2001;88:1151-1158; originally published online May 24, 2001;
doi: 10.1161/01.CIR.88.11.1151

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