

Coupled Gating Between Individual Cardiac Ryanodine Calcium Release Channels

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Abstract. In order to study interactions between ryanodine receptor calcium release (RyR2) channels during excitation-contraction coupling in cardiac muscle, we used bilayer lipid membrane (BLM) and improved the method of cardiac sarcoplasmic vesicle fusion into BLM. We increased fusion gradient for the vesicles, used chloride ions for fusion up to concentration of 1.2 mol/l and fused the vesicles by adding them directly to the forming BLM. Under these conditions, increased probability of fusion of vesicles containing 2–7 ryanodine channels into BLM was observed. Interestingly about 10% of the channels did not gate into BLM independently, but their gating was coupled. At 53 mmol/l calcium solution, two coupled gating channels had double conductance (191 ± 15 pS) in comparison with the non-coupled channels (93 ± 10 pS). Activities of the coupled channels were decreased by 5 μ mol/l ryanodine and inhibited by 10 μ mol/l ruthenium red similarly as single RyR2 channels. We suppose that cardiac sarcoplasmic vesicles contain single as well as coupled RyR2 channels.

Key words: Coupled gating — Ryanodine channel — Cardiac sarcoplasmic reticulum

Introduction

During excitation-contraction coupling in cardiac muscle, calcium (Ca^{2+}) is released by a process of Ca^{2+} -induced Ca^{2+} release from the sarcoplasmic reticulum (SR) *via* ryanodine receptor/calcium release (RyR2) channels. On the basis of several studies of single RyR2 channels in bilayer lipid membrane (BLM) (Ondrias et al. 1990), it has been assumed that the channels gate independently. However, studies of the amplitude of Ca^{2+} sparks in cardiac muscle and BLM experiments of RyR2 at a physiologic luminal Ca^{2+} (2 mmol/l) indicated the current value less

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then 0.6 pA (Mejia-Alveraz et al. 1999), suggesting that the Ca^{2+} release in cardiac muscle arises from the coordinated opening of a clusters ($\sim 5\text{--}20$) of RyR2 channels. Morphological studies indicate that cardiac RyR2 are arranged in junctional arrays of several tens of channels (Sun et al. 1995; Franzini-Armstrong et al. 1999), and that cardiac muscle RyR2 are organized at regular intervals (approximately at 29 nm). Based upon these data, heart SR membrane vesicle of 50 nm in diameter derived from the junctional arrays should contain approximately 9 RyR2 channels. However, after fusion of cardiac vesicle into BLM, typically one, but occasionally two or more independent channels are recorded; it is extremely rare to observe more than four channels. Recently, it has been reported that skeletal and cardiac muscle RyR demonstrated the coordinated gating of two channels, termed “coupled gating” (Marx et al. 1998; Marx et al. 2001).

In the present work modified method of cardiac SR vesicle fusion into BLM was described. Using the method we were able to incorporate 2–7 RyR2 channels into BLM; some of them demonstrated coupled gating properties.

Materials and Methods

All chemicals were purchased from Sigma (St. Louis, USA) and the lipids were supplied by Avanti Polar Lipids or Sigma. The SR vesicles were prepared from rat or dog hearts according to Webster et al. (1994). In some cases for the modified fusion method, the vesicles were resuspended in 0.4 mol/l sucrose.

BLM were formed across an aperture (diameter 0.1–0.2 mm) separating the *cis* and *trans* chambers, using a mixture of dioleoyl-glycero-phosphatidylcholine, dioleoyl-glycero-phosphatidylserine, and dioleoyl-glycero-phosphoethanolamine at a molar ratio of 3:2:1 in *n*-decane (20 mg/ml), similar to the method used in previous studies (Brillantes et al. 1994; Jayaraman et al. 1996; Marx et al. 1998). The composition of the classical solutions (in mmol/l) was as follows: *trans*-lumenal – 53 $\text{Ca}(\text{OH})_2$, HEPES (~ 250), pH 7.4; *cis*-cytosolic solution – 250 HEPES, Tris (~ 113), 1 EGTA, 0.7 CaCl_2 , (350 nmol/l “ Ca^{2+} -free”; Schoenmakers et al. 1992), pH 7.4. For the modified fusion procedure, the *trans* and the *cis* solution contained 50 mmol/l KCl after perfusion. Voltage was applied to the *trans* side of the chamber and the *cis* side was grounded.

We utilized two approaches to fuse the SR vesicles into BLM. In the classical fusion technique, the vesicles (1–5 μl of 1 mg/ml protein) and 800 mmol/l KCl were added to *cis* solution, which was stirred for 5–20 minutes until the vesicle(s) fused into BLM. Fusion events were monitored by current shifts from baseline. In the modified fusion technique, vesicles (0.1–0.5 μl of 0.1–1 mg/ml protein) were added directly into forming BLM by pipette and the *cis* concentration of KCl was gradually increased by additions of 50 μl of saturated KCl solution from 0.6 to 1 mol/l with combination of 0.4–1 mol/l N-methyl-d-glucamine chloride until a fusion event(s) occurred. After fusion of the vesicles, the osmotic gradient was removed by perfusion of the *cis* chamber with the *cis*-cytosolic solution (in mmol/l; 250 HEPES, Tris (~ 113), 50 KCl, 1 EGTA, 0.7 CaCl_2 , (350 nmol/l “ Ca^{2+} -free”). Single channel

currents were acquired using an amplifier (Axoclamp 1C, Axon Instruments, Foster City, USA), an analog to digital converter (Labmaster) and acquisition software (PCLamp5, Axon Instruments). The single channel data were filtered at 1 kHz, digitized at 4 kHz and stored in an IBM-compatible computer. Data for the figures were filtered at 200 or 500 Hz.

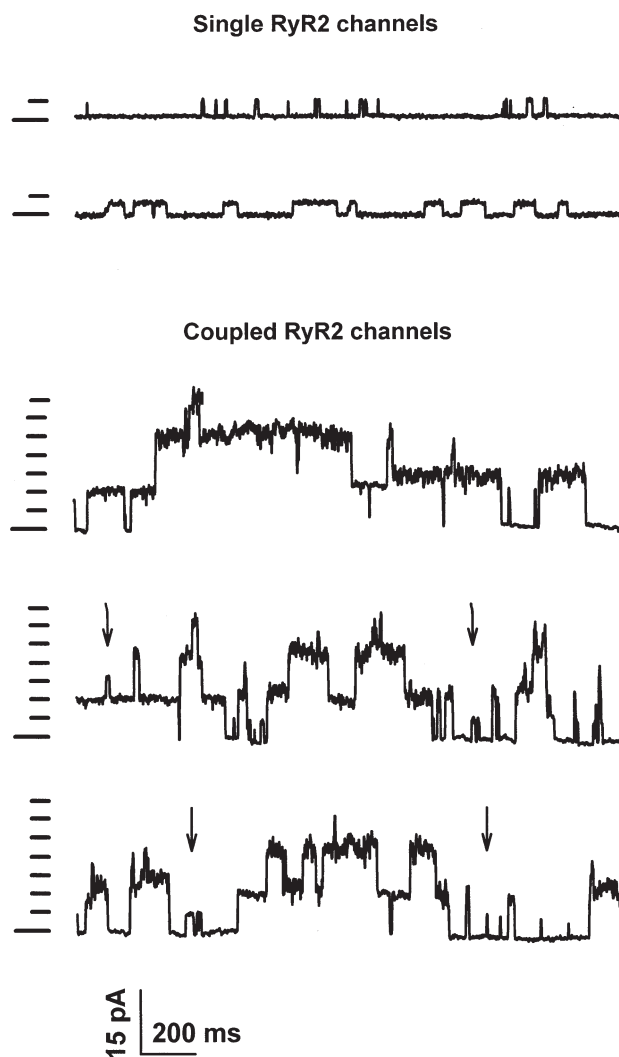


Figure 1. Calcium current of single RyR2 (top two traces), and coupled RyR2 channels (bottom three traces) in BLM. The lines on the left indicate conductance of single channels. The longer left lines mark the closed state of the channels. Arrows indicate single channels.

Results

When vesicle fused into BLM using the classical technique, leak current was close to zero or less than 30 pA and single K^+ and/or Cl^- channels were usually seen. After perfusion of the *cis* chamber the leak current decreased to 0–2 pA and in most cases, only one RyR2 channel in BLM was observed. These single channels typically exhibited fast or slow gating kinetics (Fig. 1, top two traces). The single channel properties were typical for RyR2 in the given solutions; the current was within the range of 3–5 pA at 0 mV ($n = 50$), conductance in the range 90–110 pS and the channels were locked to subconductance state by 1–20 $\mu\text{mol/l}$ ryanodine and inhibited by 10–20 $\mu\text{mol/l}$ ruthenium red (data not shown).

Using the modified technique to achieve fusion of SR vesicles increased the probability of fusion of vesicles into the BLM, which frequently contained more than one RyR2. Using this technique, leak current after fusion was in the range of 10–100 pA with large current fluctuations, suggesting either a fusion of a large vesicle and/or multiple vesicle fusions. After perfusion of the *cis* chamber, the leak current was usually higher than 1 pA, in some cases 10–30 pA. Under these conditions, we recorded several RyR2 channels (2–7 channels) in the BLM in about 10% of experiments. The simultaneous opening or closing of the two channels was seen from the increase or decrease of the current steps which were two times higher than that for single channel (4–5 pA). For comparison, single channels in BLM are marked by arrows (Figs. 1, 3 and 5). In one case, we observed the incorporation of both 2 coupled channels and 3 coupled channels in the bilayer, each group of channels gating independently (Fig. 1, bottom three traces), or the incorporation of 2 coupled channels and an additional partially coupled channel in the bilayer (Figs. 1, 5 and 6). In case of the partially coupled channels, they were coupled only in some openings and decoupled in others. In the coupled channels noise current significantly increased when 2–3 coupled channels opened (Figs. 1, 3, 4) as compared to the noise of the open single channel.

We compared the current amplitude of single (Fig. 2A), two coupled channels (Fig. 2B), and two sets of double coupled channels incorporated in the same BLM (Fig. 2C), recorded at 0 mV. As seen from the all points histogram, the current recorded from a single RyR2 channel was 5 pA, and from the coupled channels 10 pA, which is double the current of a single channel, suggesting that the current may be derived from two RyR2 channels gating simultaneously (coupled channels). This was also confirmed by comparison of the conductance of single and coupled channels observed in the same experiment (Fig. 3). The conductance of the coupled channel was double (191 ± 15 pS) of the single channel conductance (93 ± 10 pS). The activity of the coupled channels were modulated by known modulators of RyR2 channels, including 5–20 $\mu\text{mol/l}$ ryanodine, and 10 $\mu\text{mol/l}$ ruthenium red (Fig. 4). Ryanodine (5–20 $\mu\text{mol/l}$) decreased the activity of the channels and ruthenium red (10 $\mu\text{mol/l}$) inhibited the channels similarly as single RyR2 channels. These findings suggest that the coupled channels are indeed those RyR2 channels that

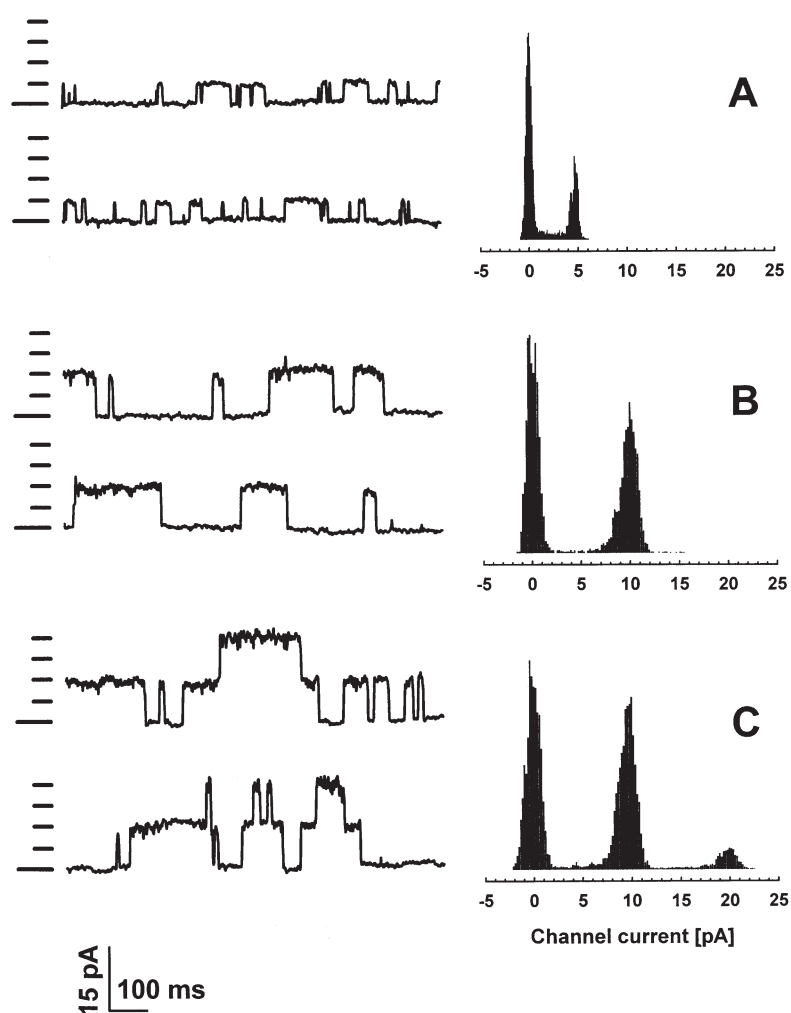


Figure 2. Calcium current traces (left) and all points histograms (right) of single (A) and coupled (B, C) RyR2 channels. Details are the same as described in Fig. 1.

gate in a simultaneous fashion, as has been previously described for RyR1 (Marx et al. 1998).

Using the modification of the fusion technique, we observed fusion of multiple channels in the BLM, consisting of both single and coupled RyR2 channels from vesicles isolated from dog and rat hearts (Fig. 5). In Fig. 5, top two traces demonstrate three channels incorporated into the bilayer; one single channel (marked by arrows) and one coupled channel. In the single channel recordings of RyR2 obtained

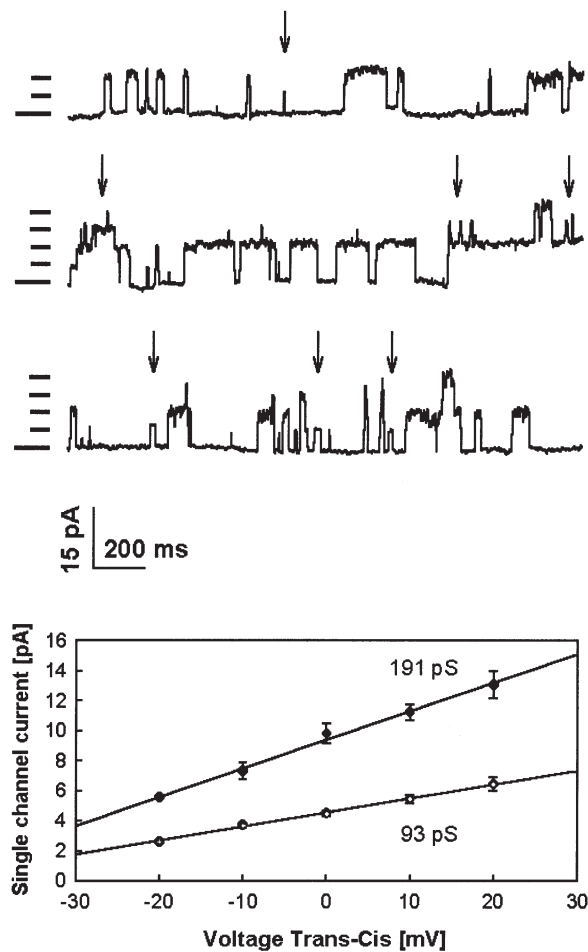


Figure 3. Calcium current traces (top) and current-voltage relationship (bottom) of single and coupled RyR2 channels. Details are the same as described in Fig. 1.

from rat heart, four channels were incorporated into the BLM, which demonstrates partially coupling of the channels (Fig. 5, bottom three traces). Current steps originated from single, two, three and four coupled channels are seen. Some channels were only partially coupled as shown in Fig. 6. In this experiment, two single channels were coupled as follows: when first channel of slow kinetics was opened, the second channel showed flickering of fast kinetics. The flickering of the second channel could occur only when the first channel opened, as it is clearly seen from the traces shown in Fig. 6. However, we can not exclude Ca^{2+} induced Ca^{2+} release mechanism in this kind of coupling.

In some of the experiments, we observed coupling of the channels for more than

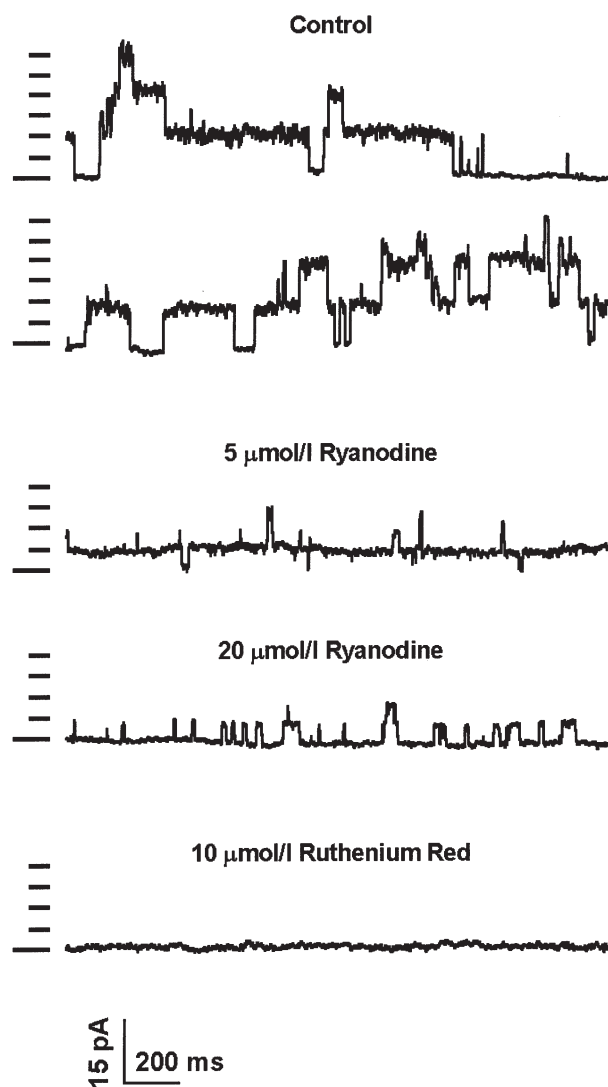


Figure 4. Control coupled RyR2 channels. Effect of 5 and 20 $\mu\text{mol/l}$ ryanodine and 10 $\mu\text{mol/l}$ ruthenium red on coupled RyR2 channels. Details are the same as described in Fig. 1.

one hour; however, in others the coupled channels “ran down” to single uncoupled channels, as seen in Fig. 6A and Fig. 7. Two coupled channels were seen only 50 seconds after *cis* perfusion, later they decoupled and only single channel(s) was seen (Fig. 7, top trace). Interestingly, the coupled channel was sometimes followed by single channel (Fig. 7, bottom 5 traces).

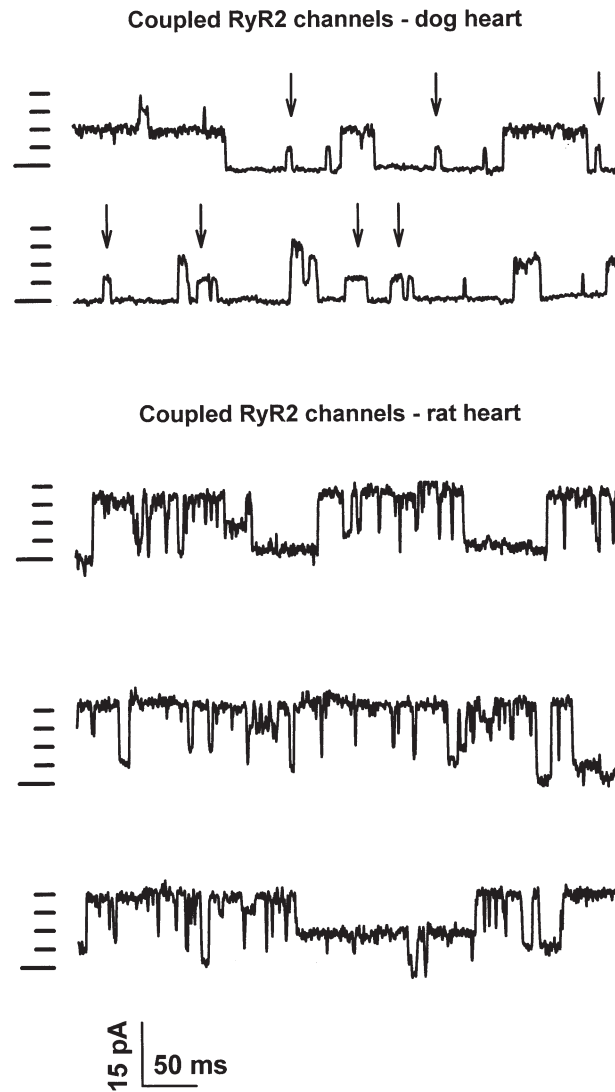


Figure 5. Calcium current traces of mixture of single and coupled RyR2 channels containing 2 channels (top two traces) or 2, 3, 4 channels (bottom three traces). Details are the same as described in Fig. 1.

Discussion

In the heart and skeletal muscle, coordinated activation and termination of RyR2 mediated Ca^{2+} release is required for contraction and relaxation. We hypothesized that in order to obtain several RyR2 channels in BLM, the fusion of large and/or

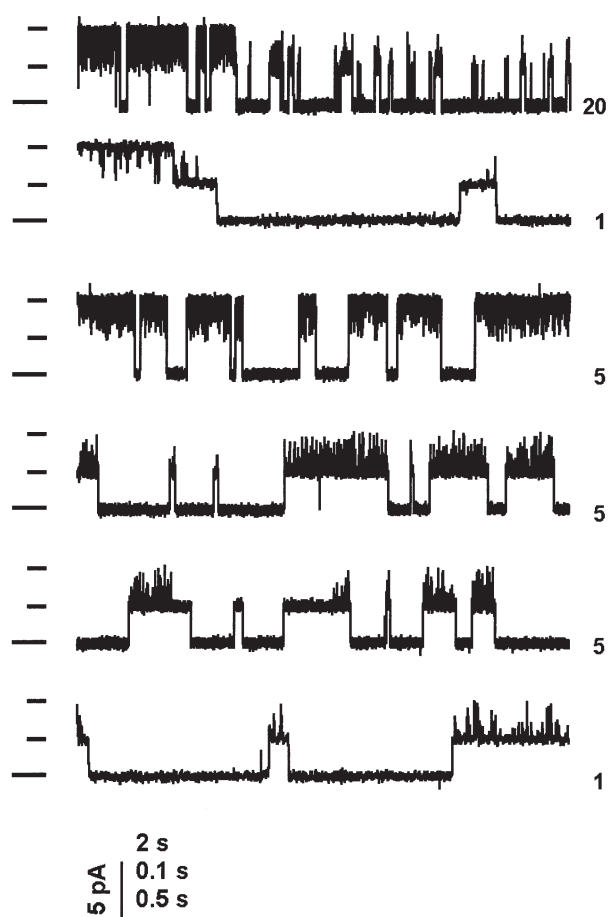


Figure 6. Calcium current traces of partially coupled RyR2 channels. Details are the same as described in Fig. 1, but in the *trans* solution, 53 mmol/l Ba(OH)₂ was used instead of 53 mmol/l Ca(OH)₂. Numbers on the right indicate trace length in seconds.

multiple vesicles is necessary. Therefore, we utilized a technique that was based upon an increased *cis-trans* gradient and vesicles were applied directly into forming BLM. Fusion of large vesicles into BLM was indicated by several times higher fluctuating current, than in the cases of fusion of “single channel” vesicles. To preserve the channel coupling we used simple and fast method for vesicle isolation (Webster et al. 1994). Using these conditions for fusion of large vesicles, coupled channels were only observed in about 10% of experiments in which large vesicle fused. This may indicate that physical coupling of RyR2 is disrupted during vesicle isolation, a significant percentage of channels do not “survive” the isolation process

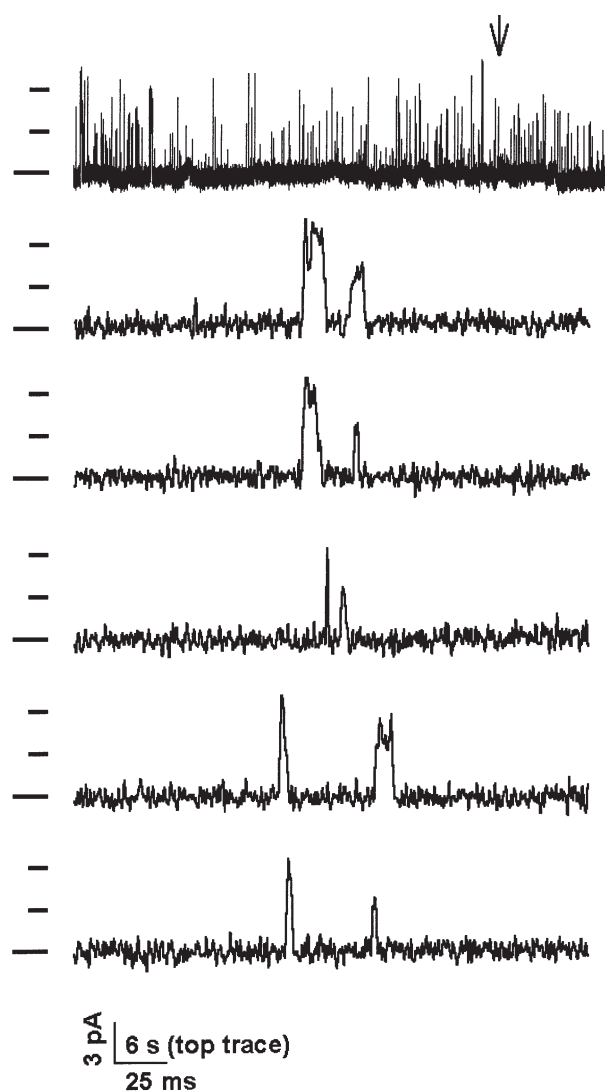


Figure 7. Calcium current traces of single and coupled RyR2 channels. The top trace is at compressed scale and bottom five traces are at high resolution. Arrow indicates “run down” the coupled channels. Details are the same as described in Fig. 1.

and/or that only fraction of RYR2 channels are coupled *in situ*. Similar results have been obtained for coupling of skeletal ryanodine calcium release channels RyR1 in BLM (Marx et al. 1998).

We found that the techniques that increase fusion of large vesicles correspondingly increase the number of coupled channels identified in BLM. The coupled

gated channels result from simultaneous opening and closing of structurally and functionally linked channels (Marx et al. 2001). This assumption is based on the findings that the Ca^{2+} current and their conductance of the coupled channels were multiples of single RyR2 current and single RyR2 conductance (Figs. 2, 3). We observed uncoupling of the coupled channel in BLM (Figs. 6A,B and Fig. 7-top trace), and a mix of single RyR2 and coupled channels in BLM (Figs. 1, 3, 5, 7). Partial coupling of RyR2 channel in the bilayer may be the consequence of the partial, but not complete disruption of the physical and/or functional linkage of the channels or may be physiologic in order to regulate channel gating. Based upon sucrose gradients, it was suggested that the functional, but not the structural coupling is due to the binding of the FK506 binding protein (FKBP12 in RyR1 and FKBP12.6 in RyR2) (Marx et al. 2001). Release of FKBP12/FKBP12.6 from the complex may disrupt coupled gating of RyR1 and RyR2, respectively (Marx et al. 1998; Marx et al. 2001).

Our results support the concept that coupled gating plays an important role in regulating the gating properties of RyR2 and consequently Ca^{2+} release *in vivo*.

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