Unitary Ca^{2+} Current through Cardiac Ryanodine Receptor Channels under Quasi-Physiological Ionic Conditions

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ABSTRACT Single canine cardiac ryanodine receptor channels were incorporated into planar lipid bilayers. Single-channel currents were sampled at 1–5 kHz and filtered at 0.2–1.0 kHz. Channel incorporations were obtained in symmetrical solutions (20 mM HEPES-Tris, pH 7.4, and pCa 5). Unitary Ca^{2+} currents were monitored when 2–30 mM Ca^{2+} was added to the lumenal side of the channel. The relationship between the amplitude of unitary Ca^{2+} current (at 0 mV holding potential) and lumenal [Ca^{2+}] was hyperbolic and saturated at ~4 pA. This relationship was then defined in the presence of different symmetrical $CsCH_3SO_3$ concentrations (5, 50, and 150 mM). Under these conditions, unitary current amplitude was 1.2 ± 0.1, 0.65 ± 0.1, and 0.35 ± 0.1 pA in 2 mM lumenal Ca^{2+}; and 3.3 ± 0.4, 2.4 ± 0.2, and 1.63 ± 0.2 pA in 10 mM lumenal Ca^{2+} ($n > 6$). Unitary Ca^{2+} current was also defined in the presence of symmetrical [Mg^{2+}] (1 mM) and low [Cs^+] (5 mM). Under these conditions, unitary Ca^{2+} current in 2 and 10 mM lumenal Ca^{2+} was 0.66 ± 0.1 and 1.52 ± 0.06 pA, respectively. In the presence of higher symmetrical [Cs^+] (50 mM), Mg^{2+} (1 mM), and lumenal [Ca^{2+}] (10 mM), unitary Ca^{2+} current exhibited an amplitude of 0.9 ± 0.2 pA ($n = 3$). This result indicates that the actions of Cs^+ and Mg^{2+} on unitary Ca^{2+} current were additive. These data demonstrate that physiological levels of monovalent cation and Mg^{2+} effectively compete with Ca^{2+} as charge carrier in cardiac ryanodine receptor channels. If lumenal free Ca^{2+} is 2 mM, then our results indicate that unitary Ca^{2+} current under physiological conditions should be <0.6 pA.

KEY WORDS: Ca^{2+} release • sarcoplasmic reticulum • Ca^{2+} spark • excitation–contraction coupling • planar bilayers

INTRODUCTION

Muscle contraction is activated by a sudden increase in cytosolic [Ca^{2+}] caused by Ca^{2+} release from the sarcoplasmic reticulum (SR).[1] Ca^{2+} release from the SR is mediated by a large tetrameric Ca^{2+} channel known as the ryanodine receptor (RyR). Ca^{2+} release during normal excitation–contraction coupling is triggered in the heart by a small extracellular Ca^{2+} influx through voltage-dependent L-type Ca^{2+} channels and in skeletal muscle by a presumably mechanical action of dihydropyridine receptors in the transverse tubule. A complete understanding of these control mechanisms has been prevented, among other reasons, by the paucity of knowledge concerning unitary Ca^{2+} current through single RyR channels under physiological conditions. A better definition of this current has become critically necessary to interpret the local Ca^{2+} release events termed Ca^{2+} sparks (Cheng et al., 1993), and in particular whether they arise from the opening of a single RyR channel or the concerted opening of several RyR channels.

Unitary Ca^{2+} currents through the RyR channel have been measured in lipid bilayer studies under relatively simple ionic conditions (Smith et al., 1988; Fill and Coronado, 1988). To optimize the signal-to-noise ratio, unitary Ca^{2+} current is typically recorded in the presence of large Ca^{2+} concentrations (e.g., ~50 mM). In cells, however, the intralumenal SR Ca^{2+} concentration is thought to be near 1 mM (Bers, 1991; Chen et al., 1996; Shannon and Bers, 1997). Single RyR channel studies have also revealed that the RyR channel is a rather poorly selective Ca^{2+} channel. Consequently, unitary Ca^{2+} currents are usually recorded in the absence of competing ions. In cells, however, Mg^{2+} and K^+ are found at concentrations that allow them to compete effectively with Ca^{2+} for occupancy of the RyR pore. These considerations indicate that the unitary Ca^{2+} current in the cell must be considerably less than that predicted from measurements of unitary Ca^{2+} cur-

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[1]*Abbreviations used in this paper:* RyR, ryanodine receptor; SR, sarcoplasmic reticulum.

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rent under the typical RyR channel recording conditions.

In this study, unitary Ca^{2+} currents through single cardiac RyR channels were measured in the presence of competing ions at lumenal Ca^{2+} concentrations ranging from 2 to 30 mM. Unitary Ca^{2+} currents were recorded in the presence of different concentrations of competing cations (Cs^+ and/or Mg^{2+}). Competing cation concentration was symmetrical across the membrane and the Ca^{2+} currents were recorded at 0 mV. Our data indicate that, under conditions that roughly mimic the physiological condition (i.e., 1–2 mM lumenal Ca^{2+}, 1 mM Mg^{2+}, 150 mM monovalent salt, 0 mV membrane potential), the amplitude of the Ca^{2+} current through a single RyR channel will be considerably <0.6 pA.

METHODS

Heavy SR microsomes were isolated from canine ventricular muscle using previously described methods (Tate et al., 1985). Single SR Ca^{2+} release channels were reconstituted by fusing heavy SR microsomes into artificial planar lipid bilayers. Channel incorporation was evidenced by the sudden appearance of single-channel activity.

Planar bilayers were formed across a 150-μm diameter aperture in a Delrin partition. Bilayer-forming solution contained a mixture of phosphatidylethanolamine and phosphatidylcholine (7:3, 50 mg/ml of decane; Avanti Polar Lipids). Heavy SR microsomes were added to one side of the bilayer (cis). The cis chamber contained the cytoplasmic side of the channel (Tu et al., 1994). The other side of the bilayer, trans, was held to ground. The transmembrane potential was always held at 0 mV. The standard solution contained 20 mM HEPES-Tris, pH 7.4, and 10 μM added free Ca^{2+}. Unitary currents were measured after addition of different Ca^{2+} (or Ba^{2+}) concentrations (2–30 mM) to the lumenal side of the channel. The attenuating effect on unitary Ca^{2+} currents induced by symmetric concentrations of Mg^{2+} (1 mM) and/or Cs^+ (5, 50, and 150 mM) was defined. The buffering effect of $CH_3SO_3^-$ on free [Ca^{2+}] was quantified with a Ca^{2+}-selective electrode. Those measurements were conducted under conditions of constant ionic strength (with equivalent [Cl^-]'s).

Unitary currents were recorded with a conventional patch clamp amplifier (Axopatch 200B; Axon Instruments). The current signal was digitized at a rate of 4 kHz with a 32 bit AD/DA converter (Digidata 1200; Axon Instruments), filtered with a Bessel filter at 1 kHz, and stored for later analysis. Current amplitudes were determined either by measuring individually long opening events or by fitting gaussian functions to the total amplitude histograms. Data acquisition, unitary current measurements, statistical analysis, and data processing were performed using commercially available software packages (pClamp V6.0; Axon Instruments, Excel 97; Microsoft Corp., and Origin V5.0; Microcal Software, Inc.). Experimental data shown here as mean ± SEM were obtained from a total of 53 bilayers. Opening events are shown as downward deflections.

RESULTS

Unitary Ca^{2+} Current in the Absence of Competing Ions

Single RyR channel activity was measured in the presence of different lumenal Ca^{2+} concentrations ranging from 2 to 30 mM. A sample single-channel recording in the presence of 2 mM Ca^{2+} is illustrated in Fig. 1 A. The free Ca^{2+} concentration on the cytoplasmic side of the channel was ~10 μM and no competing ions (Cs^+ or Mg^{2+}) were present. The recording shown here demonstrates that under these relatively simple ionic conditions, the signal-to-noise ratio of the recording system is sufficient to clearly resolve single-channel openings in the presence of such low lumenal Ca^{2+} concentrations. The unitary current amplitude was determined from the corresponding total amplitude histogram (Fig. 1 A, right). The histogram was fit by the sum of two gaussian functions (describing the closed and open current levels). The difference between the means of the gaussian components (i.e., unitary current amplitude) was 1.37 pA. To confirm the identity of the ion channel responsible for the single-channel activity recorded, we tested its sensitivity to the plant alkaloid ryanodine and to ruthenium red. Fig. 1 B illustrates an experiment where addition of 20 μM ryanodine to the cytoplasmic side of a channel induced the typical effects described for the cardiac RyR channel on both permeation and gating kinetics. The addition of 5 μM ruthenium red to the cytoplasmic side of a channel induced a significant decrease of the RyR channel activity (Fig. 1 C). These results confirmed that the channel activity we recorded in the presence of 2 mM lumenal Ca^{2+} arose from a typical cardiac RyR.

Unitary Ca^{2+} Current Attenuation Induced by Symmetrical Monovalent Cations

To estimate the impact of monovalent cations on Ca^{2+} permeation through the RyR channel, unitary Ca^{2+} current carried by 2 mM Ca^{2+} was recorded in the presence of 150 mM symmetrical Cs^+ and without Mg^{2+} (Fig. 2). A sample single-channel recording obtained under these conditions is illustrated in Fig. 2 A. In this case, since the current signal was filtered at 300 Hz, fast single-channel openings were significantly attenuated; however, the amplitude of long single-channel events was essentially unaffected. The selected sample record illustrates such long single-channel openings. The corresponding total amplitude histogram (Fig. 2 B) indicates that the unitary Ca^{2+} current amplitude was 0.35 pA. This result and that shown in Fig. 1 A indicate that the presence of permeable monovalent cations (i.e., Cs^+) significantly attenuates the unitary Ca^{2+} current (from 1.37 to 0.35 pA). Current attenuation is most likely due to competition between Cs^+ and Ca^{2+} ions for occupation of the RyR pore. Unitary Ca^{2+} current attenuation induced by Cs^+ depended on both the lumenal Ca^{2+} and Cs^+ concentrations. The relationship between the lumenal Ca^{2+} and Cs^+ concentration is illustrated in Fig. 3. At every Cs^+ concentration (5, 50, and 150 mM), unitary Ca^{2+} current amplitude could be

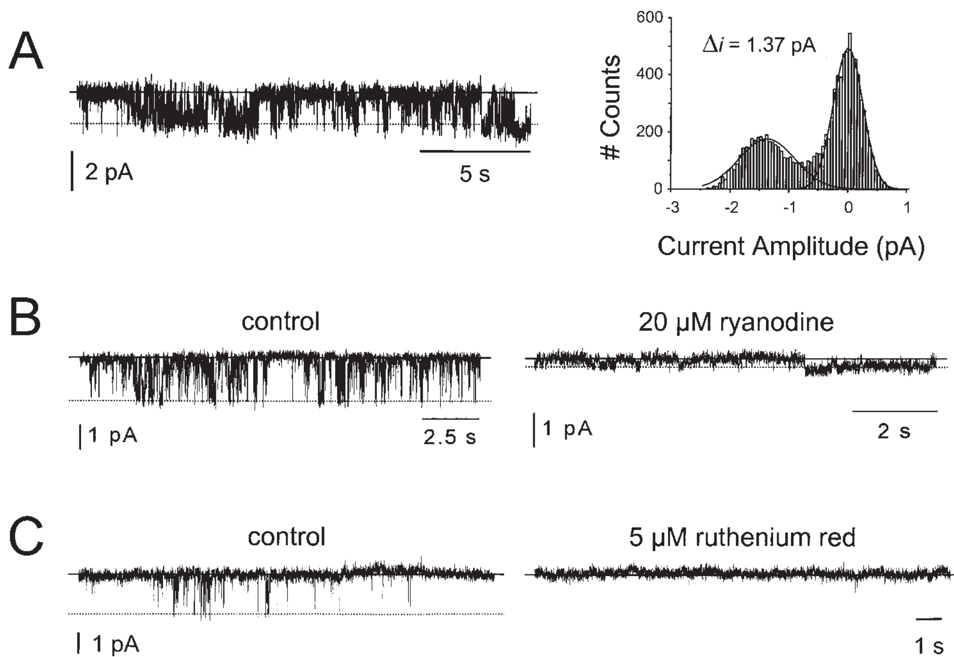

FIGURE 1. Unitary Ca^{2+} current in absence of competing ions. Single-channel current was recorded at 0 mV holding potential, in absence of monovalent ions and using 2 mM Ca^{2+} as current carrier. (A) Representative trace of the single-channel activity recorded under these conditions. Opening events are displayed as downward deflections. The continuous line represents the zero current level, while the dotted line represents the mean opening level. Current signal was filtered at 1 kHz. (Right) Total amplitude histogram obtained from the same experiment. Continuous lines were generated by fitting a double Gaussian function. The opening distribution was centered at −1.37 pA. (B) Single-channel activity recorded under conditions similar to A, before and after addition of 20 μM of ryanodine to the cytoplasmic side of the channel. Traces after ryanodine were filtered at 150 Hz to resolve the squared openings. (C) Single-channel activity recorded under control conditions and after addition of 5 μM ruthenium red to both sides of the channel.

fitted by a hyperbolic function of lumenal Ca^{2+} concentration (between 0 and 10 mM). Higher Cs^+ concentrations resulted in smaller unitary Ca^{2+} currents. To investigate how much of this effect resulted from $[Ca^{2+}]$ buffering by $CH_3SO_3^-$, we measured the free $[Ca^{2+}]$ in our solutions at different $[CH_3SO_3^-]$'s (data not shown). Our results indicated that, acting as a low affinity Ca^{2+} buffer, $CH_3SO_3^-$ did not induce a significant decrease of the Ca^{2+} current amplitude when measured at low lumenal $[Ca^{2+}]$ (between 0 and 2 mM).

At high Cs^+ concentrations (150 mM; Fig. 3, △), it was impossible to reliably record single-channel events at lumenal Ca^{2+} concentration below 2 mM. The data were plotted as a function of lumenal Ca^{2+} to facilitate extrapolation to lower Ca^{2+} concentrations. For example, it is clear that unitary Ca^{2+} current at 1 mM lumenal Ca^{2+} in the presence of 150 mM Cs^+ will be <0.5 pA.

Cs^+ Attenuates Unitary Current Regardless of Current Carrier Identity

If the mechanism of Cs^+ attenuation is competition for occupation of the RyR pore, then Cs^+ should attenuate unitary current in a similar way for other current carriers. Therefore, we measured unitary Ba^{2+} currents in the presence of different Cs^+ symmetrical concentrations. The relationship of the current amplitude with Ba^{2+} and Cs^+ concentrations is illustrated in Fig. 4. Unitary Ba^{2+} current was attenuated by Cs^+ in a dose-dependent fashion. At all Cs^+ concentrations (5, 50, and 150 mM), unitary Ba^{2+} current was a hyperbolic function of lumenal Ba^{2+} concentration (0–10 mM). Like the Ca^{2+} data presented in Fig. 3, unitary Ba^{2+} current was scaled by the competing Cs^+ concentration. High Cs^+ concentrations resulted in smaller unitary Ba^{2+} currents. These data indicate that physiological levels of a permeable monovalent cation are sufficient to attenuate unitary Ca^{2+} or Ba^{2+} to <0.5 pA. The Ba^{2+} data (Fig. 4) also indicate that the competition between Cs^+ and the current carrier was independent of the ionic species used.

Unitary Ca^{2+} Current Attenuation by Symmetrical Mg^{2+}

It has been reported that the concentration of free Mg^{2+} in muscle cells falls in the millimolar range, and that the RyR channel is permeable to Mg^{2+} (Smith et al., 1988). Thus, the unitary Ca^{2+} current through the RyR channel is also likely to be attenuated by Mg^{2+}. The extent of current attenuation induced by 1 mM symmetri-

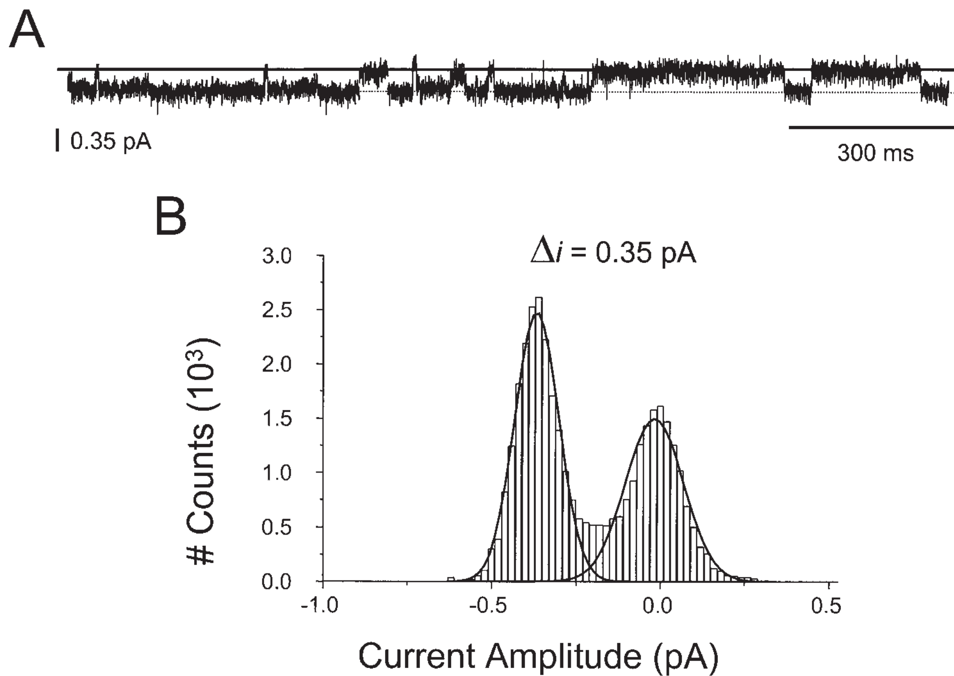

FIGURE 2. Unitary Ca^{2+} current in presence of 150 mM Cs^+ and without Mg^{2+}. Single-channel activity recorded at 0 mV holding potential, using 2 mM Ca^{2+} as current carrier, and in the presence of 150 mM symmetrical Cs^+. (A) Representative trace of the unitary current recorded under these conditions. Opening events are displayed as downward deflections. The continuous line represents the zero current level, while the dotted line indicates the mean opening level. The current signal was filtered at 300 Hz. (B) Total amplitude histogram, fitted by a sum of two gaussian functions, revealing an open channel current of 0.35 pA.

cal Mg^{2+} was also measured here. Sample single-channel records at different lumenal Ca^{2+} concentrations (2, 5, and 10 mM) in the absence (Fig. 5, left) and presence (Fig. 5, right) of 1 mM Mg^{2+} are shown. All these measurements were conducted in the presence of low monovalent cation concentration (<5 mM Cs^+). Single-channel records were selected to illustrate differences in the unitary current amplitude. The action of Mg^{2+} on the open probability of the channel was not evaluated. Nevertheless, the data presented in Fig. 5 clearly illustrates that the amplitude of unitary Ca^{2+} current was significantly reduced in the presence of 1 mM Mg^{2+}. Additionally, an apparent reduction of the noise level was a frequent observation after addition of Mg^{2+}.

Unitary Ca^{2+} Current Attenuation by Simultaneous Presence of Symmetrical Mg^{2+} and Cs^+

At this point, our results indicated that unitary Ca^{2+} current was attenuated by both Cs^+ and Mg^{2+}. In addition, we found that the extent of attenuation depended

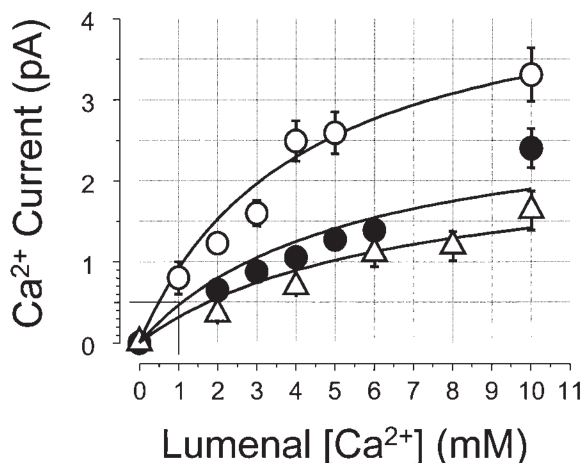

FIGURE 3. Effect of different [Cs^+]'s on unitary Ca^{2+} current amplitude. Unitary Ca^{2+} current amplitude was measured at 0 mV, in the absence of Mg^{2+}, in 5 (○; n = 6), 50 (●; n = 5), and 150 (△; n = 3) mM Cs^+. Experimental data were fitted by single rectangular hyperbolic functions.

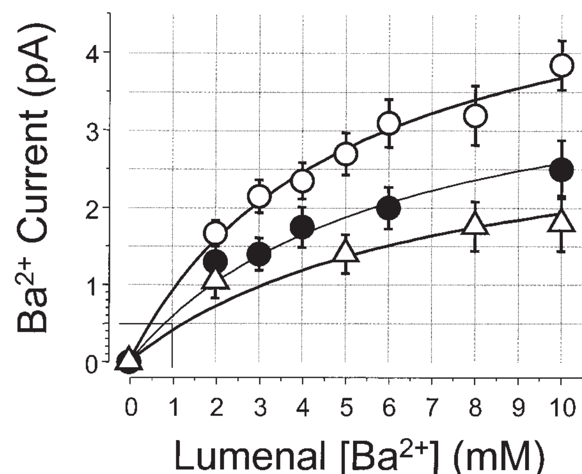

FIGURE 4. Effect of different [Cs^+]'s on unitary Ba^{2+} current amplitude. Unitary Ba^{2+} current amplitude was measured at 0 mV, in 5 (○; n = 6), 50 (●; n = 4), and 150 (△; n = 3) mM Cs^+. Experimental data were fitted by single rectangular hyperbolic functions.

on both the Cs^+ and Mg^{2+} concentrations. We then investigated the effect of those competing ions on the unitary Ca^{2+} current amplitude when they were present simultaneously. The main results of those experiments are illustrated in Fig. 6. Unitary Ca^{2+} current amplitude was plotted as a function of lumenal Ca^{2+} concentration to allow direct extrapolation to lower Ca^{2+} concentrations. In the absence of both competing ions (Mg^{2+} and Cs^+; Fig. 6, ●; $n = 6$), unitary Ca^{2+} current amplitude was described by a single rectangular hyperbolic function of lumenal Ca^{2+} concentration (0–30 mM). When only Mg^{2+} (1 mM) was added to both sides of the channel, the amplitude of the unitary Ca^{2+} current was significantly reduced to ~50% of the original amplitude (Fig. 6, ○; $n = 5$). When Mg^{2+} (1 mM) and Cs^+ (50 mM) were added simultaneously, the attenuation of the unitary Ca^{2+} current appeared additive, as revealed by an ~70% reduction of the current (Fig. 6, shaded triangles; $n = 2$). Due to the large reduction of the current amplitude induced by the presence of both Mg^{2+} and Cs^+, it was impossible to reliably measure the amplitude of unitary Ca^{2+} current when $[Cs^+]$ was larger than 50 mM. As it is shown in Fig. 6 A (shaded triangles), in the presence of 1 mM Mg^{2+} and 50 mM Cs^+, the minimum resolvable current amplitude was 0.8 pA with 10 mM lumenal $[Ca^{2+}]$. Since current amplitude measurements at lower lumenal $[Ca^{2+}]$'s were not reliable, we could not precisely fit a hyperbolic function in this range. Thus, to extrapolate the value of unitary Ca^{2+} current amplitude at lower lumenal $[Ca^{2+}]$'s, we scaled the curve obtained for the no competing ions data set and applied it to the competing ion data set.

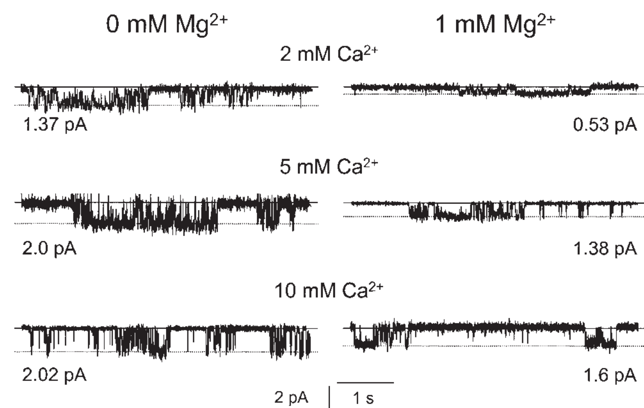

Figure 5. Effect of Mg^{2+} concentration on unitary Ca^{2+} current amplitude. Ca^{2+} current amplitude was measured at 0 mV, in 2 (top), 5 (middle), and 10 (bottom) mM Ca^{2+}, in the absence (left) and presence (right) of 1 mM Mg^{2+}. Current signal was filtered at 1 kHz, except for the trace in 2 mM Ca^{2+} and 1 mM Mg^{2+}, which was filtered at 300 Hz. Continuous lines indicate the zero current level and the dotted lines indicate the mean opening level. The corresponding values of current amplitude obtained from the total amplitude histograms are indicated below each trace.

In addition, no dramatic differences in the apparent gating kinetics were observed when both Mg^{2+} and Cs^+ were present. The single-channel trace shown in Fig. 6 B illustrates such a condition (10 mM lumenal Ca^{2+}, 1 mM symmetrical Mg^{2+} and 50 mM symmetrical Cs^+). As in previous figures, the corresponding total amplitude histogram was fitted with two gaussian functions. The difference of the peaks revealed a mean opening level with an amplitude of ~0.7 pA.

Discussion

The goal of this work was to measure the amplitude of unitary Ca^{2+} current through single cardiac RyR channel, under concentrations of Ca^{2+}, monovalent cations, and Mg^{2+} that are assumed to be present in the intact cell. Our results indicate that physiological levels of monovalent cations and Mg^{2+} significantly attenuate unitary Ca^{2+} current. The unitary Ca^{2+} current carried by 2 mM lumenal Ca^{2+} was <0.6 pA in the presence of either 150 mM Cs^+ or 1 mM Mg^{2+}. Attenuation of the unitary Ca^{2+} current was most likely due to the competition of permeable ions for occupancy of the RyR

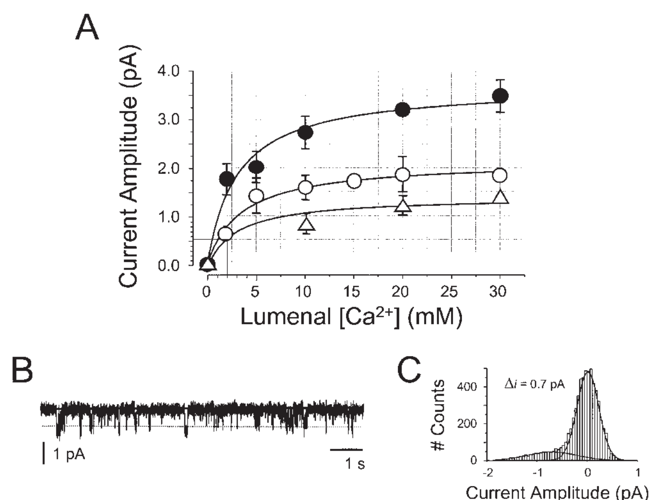

Figure 6. Unitary Ca^{2+} current amplitude at different lumenal $[Ca^{2+}]$'s in the simultaneous presence of Mg^{2+} and Cs^+. (A) Unitary Ca^{2+} current amplitude at 0 mV was measured in the virtual absence of competing ions (0 mM Mg^{2+} and 5 mM Cs^+, ●, $n = 6$), with 1 mM symmetrical Mg^{2+} and 5 mM Cs^+ (○, $n = 5$), and with 1 mM Mg^{2+} plus 50 mM symmetrical Cs^+ (shaded triangles, $n = 2$). The results were plotted as a function of the lumenal Ca^{2+}. For the first two conditions, the experimental data were fitted with a single rectangular hyperbolic function. For comparison, the hyperbolic function fitted to the data obtained in the absence of competing ions was scaled to the multi-ion data. (B) Representative current trace obtained at 0 mV, with 10 mM lumenal Ca^{2+}, 10 μM cytoplasmic Ca^{2+}, 1 mM symmetrical Mg^{2+}, and 50 mM symmetrical Cs^+. (C) The corresponding total amplitude histogram fitted with two gaussian functions reveals a mean opening level with an amplitude of ~0.7 pA.

pore. This assumption was favored by the observation that when Cs^+ and Mg^{2+} were present simultaneously, the attenuating effect on the current amplitude was even larger. These data indicate that unitary Ca^{2+} current through a single RyR channel in a cell (i.e., with both monovalent cations and Mg^{2+} present) would be considerably <0.6 pA. Nevertheless, the physiological relevance of this measurement should be drawn cautiously, since the role of multiple factors existing in the cell that clearly modify the permeation properties of RyR (i.e., polyamines, FKBP12, etc.) were absent in our study. Likewise, the potential impact on the RyR permeation of other intracellular ions such as Na^+, Cl^-, polyamine, etc., was not addressed in this work.

Significance of Using Cs^+ as Representative Monovalent Cation

Single RyR channels were incorporated into planar lipid bilayers by fusion of heavy SR microsomes that contained the RyR channel. The SR microsomes are known to also contain Cl^- and K^+ channels (Cukierman et al., 1985; Hamilton et al., 1989). Therefore, the use of Cs^+ as a charge carrier allowed us to identify the bilayers with RyR channel before the addition of divalent charge carriers, without the interference of K^+ channels. An impermeable anion substitute ($CH_3SO_3^-$) was used to avoid currents due to the presence of Cl^- channels. This strategy for recording single RyR channels is commonly employed. The results presented here can therefore be directly compared with other studies using this common RyR channel fusion method. Additionally, studies on purified RyR channels show that P_{Ca}/P_{Cs} and P_{Ca}/P_K ratios are almost identical. This implies that Cs^+ and K^+ compete with Ca^{2+} almost equally for occupancy of the RyR pore. Thus, few disadvantages of using Cs^+ instead of K^+ as the competing monovalent ion in this study were outweighed by the ability to prescreen bilayers for RyR channels.

Unitary Ca^{2+} Current Amplitude under Simple Ionic Conditions

Unitary Ca^{2+} current in most single RyR studies has been defined in the presence of large lumenal Ca^{2+} concentrations and/or in the absence of permeable competing ions. In our study, unitary Ca^{2+} current (at 0 mV) in 30 mM lumenal Ca^{2+} was 3.5 ± 0.4 pA (see Fig. 6, ●). For comparison, Smith et al. (1988) reported that unitary Ca^{2+} current (at 0 mV) in 54 mM lumenal Ca^{2+} was ~4.2 pA. Tinker et al. (1992) reported that unitary Ca^{2+} current (at 0 mV) in 210 mM lumenal Ca^{2+} was ~5.5 pA. Thus, the unitary Ca^{2+} currents observed here in the absence of permeable competing ions are consistent with previously published values. Therefore, we conclude that the small amplitudes measured in the presence of competing ions reported here are not due to selection of RyR channels with unusually small unitary conductance. The characteristic behavior of the channels used in this study was further confirmed by their ryanodine sensitivity when conducting 2 mM Ca^{2+} (Fig. 1 B).

Unitary Ca^{2+} Current Amplitude in the Presence of Competing Ions

The monovalent cation and Mg^{2+} concentrations on both sides of the channel were equal in this study. Thus, there was no net monovalent or Mg^{2+} current at 0 mV where all our single-channel measurements were made. On the other hand, the free Ca^{2+} concentration was asymmetric across the bilayer (2–30 mM trans; 0.01 mM cis), creating a 100-fold Ca^{2+} gradient across the channel. Thus, the net current (inward by convention) through the RyR channel at 0 mV was carried by Ca^{2+}. Experiments were done at 0 mV to mimic what is thought to represent the physiological potential across the SR (García and Miller, 1984). The interpretation of our data is based on the assumption that, even in the absence of a net monovalent or Mg^{2+} current, these permeant ions will effectively compete for occupancy of the pore. Thus, attenuation of net Ca^{2+} current would be expected in the presence of other permeant ions as those ions compete with Ca^{2+} for occupancy of the conduction pore. This interpretation is consistent with the relatively low selectivity of the RyR channel (Smith et al., 1988).

Our results show that physiologically relevant concentrations of monovalent cation (150 mM Cs^+) or Mg^{2+} (1 mM) significantly attenuate Ca^{2+} current through single RyR channels. Assuming that lumenal free Ca^{2+} inside the SR is between 1 and 2 mM (Bers, 1991; Chen et al., 1996; Shannon and Bers, 1997), our data indicate that unitary Ca^{2+} current will be <0.6 pA in the presence of a competing monovalent cation (Cs^+). The data also show that the attenuation of the current due to 1 mM Mg^{2+} is close to that induced by 150 mM Cs^+. The large noise inherent to planar bilayer studies made it technically impossible to directly measure the unitary Ca^{2+} current in the simultaneous presence of high concentrations of both competing ions (Cs^+ and Mg^{2+}). However, with high Ca^{2+} concentrations (10 mM, lumenal), we were able to evaluate the effect of these competing ions (50 mM Cs^+ and 1 mM Mg^{2+}) when added together (Fig. 6, shaded triangles). As expected, under these conditions the current attenuation was greater than the effect induced separately by each ion. Thus, we conclude that unitary Ca^{2+} current in the intact cell in the presence of physiological salts must be considerably <0.6 pA.

This conclusion has two interesting implications. First, the value we observed is about fourfold smaller than a previously published estimate of the unitary

Ca^{2+} current through the cardiac RyR channel under quasi-physiological conditions (~1.4 pA in 1.2 mM lumenal Ca^{2+}; Tinker et al., 1993). Second, the amplitude of the unitary current through the RyR is critical to the interpretation of the "Ca^{2+} spark" observed in scanning confocal imaging studies exploring local control of SR Ca^{2+} release.

Comparison with Previous Estimations

Tinker et al. (1993) first explored how unitary Ca^{2+} current through single RyR channels may be impacted by the presence of other permeant ions. They measured net currents in a quasi-physiological mixture of ions (symmetrical 120 mM K^+, 0.5 mM Mg^{2+}, 10 mM lumenal Ca^{2+}) and fit those data using an Eyring-rate model for RyR permeation (Tinker et al., 1992). Their model predicted that unitary Ca^{2+} current through RyR (at 0 mV) would be ~1.4 pA at a more physiological lumenal Ca^{2+} concentration (1.2 mM). There is a fourfold discrepancy between this model prediction (1.4 pA) and our experimental measurements (0.35 pA, measured with 2 mM lumenal Ca^{2+}, 150 mM symmetrical Cs^+, and no Mg^{2+}).

A very simple argument can be made suggesting that the single-channel current must be <1.4 pA. It is well established that $[Ca^{2+}]$ in the lumen of the SR does not exceed 2 mM and is probably in the range of 1–1.5 mM (Chen et al., 1996). Since the channel exhibits ion selectivity and discrete gating, the permeation path must pass through a true pore with a radius r_0 well under 1 nm. If we assume that Ca^{2+} ions entering the pore on the lumenal side converge by spherically symmetrical diffusion down to a radius of r_0, and diverges similarly from a radius r_0 on the cytosolic side, then the minimum possible transmembrane $[Ca^{2+}]$ gradient when passing a unitary current i, at zero transmembrane potential, is $i/(DF\,r_0)$, where D is the diffusion coefficient of Ca^{2+} and F is the faraday constant. If we use the liberal values $r_0 = 1$ nm and $D = 0.78 \times 10^{-5}$ cm^2/s (the value in free aqueous solution), this minimum gradient is 3 mM, even without considering the resistance of the true pore. Therefore, our measurements of unitary current amplitude are much more consistent with diffusion theory than previous estimates. This was also pointed out by Tinker et al. (1992), who found that their Eyring-rate permeation model required that the association rate of Ca^{2+} to the potential wells at the mouth of the pore exceed the diffusion limit by about one order of magnitude.

To clearly understand these estimates of physiologic unitary current, we should consider possible mechanisms that might enhance the diffusion-limited permeability. Two such mechanisms, not mutually exclusive, are (a) electrostatic focussing and (b) the existence of a multibarrel channel. The electrostatic focussing mechanism is based on an electric field produced by fixed negative charges in the lumenal vestibule of the channel (Tu et al., 1994). This electric field would increase the local $[Ca^{2+}]$, resulting in a larger transmembrane driving force. Fields of this nature only exist within a Debye length of the fixed charges (~1 nm in physiological ionic strength). The RyR channel contains many anionic amino acid residues (Takeshima et al., 1989). Thus, specific structural arrays may exist that act as a negatively charged, Ca^{2+}-permeable "sponge", which would function as an "electrostatic funnel" to enhance diffusion-limited permeability. In the APPENDIX, we present the simplest possible model of this effect. In this case, the sponge is modeled as a homogeneously charged sphere that creates a Donnan equilibrium potential. By making extreme assumptions (i.e., $r_0 = 1$ nm, free diffusion of Ca^{2+} within the sponge, ionization of all acidic residues, effective radius of the sponge set at an optimum) it is possible to reduce the diffusion gradient to 0.33 mM, again without including the resistance of the true pore. This suggests that, while electrostatic focussing might assist permeation, it probably could not fully account for a current of 1.4 pA under more realistic assumptions. The second enhancing mechanism, a multibarrel channel, has been suggested by Ondrias et al. (1996). This mechanism is based on their observation of 1/4 conductance states when FKBP12 is removed from the channel, as well as single openings of multiple conductance (up to six times normal) when several channels are in the bilayer in the presence of FKBP12. On this model, each RyR monomer would have its own permeation path. In the presence of FKBP12, the possibility of synchronous gating of the different pores (within and among tetramers) is somehow favored. Given the large size of the tetramer, this could reduce the diffusion gradient by nearly a factor of 4. Our estimate of unitary current of <0.6 pA substantially reduces the need to include these enhancing mechanisms in our model. Assuming a diffusion coefficient of 0.39×10^{-5} cm^2/s and a true pore capture radius of 1 nm, the unassisted diffusion gradient for a unitary current of 0.4 pA is 1.7 mM. Therefore, the existence of any permeability-enhancing mechanisms would make the necessary Ca^{2+} gradient across the SR considerably <1.7 mM.

Physiologic Release Flux

Taking advantage of the detailed morphometry that has been conducted on skeletal muscle membranes, we tested whether our estimates of single-channel current could account for the release flux measured in whole-cell experiments. According to Eisenberg and Peachey (1975), the lumenal length of T-tube per unit fiber volume is 0.82 μm/μm^3. If 80% of that is junctional and there are 60 channels per micrometer on each side of

the junction, then there are 76 channels/μm^3. Assuming that our estimate is valid for skeletal muscle (where these geometric relationships have been better defined), a unitary current of 0.35 pA per channel would yield 27 pA/μm^3, or 135 mM/s when all channels are open. If we consider an accessible aqueous volume of 70%, the effective release flux density would be 193 mM/s. This flux would provide enough Ca^{2+} to saturate troponin (240 μM; Baylor et al., 1983) in <2 ms, and would be approximately equal to the largest estimates from cut fiber experiments in frog (180 mM/s with combined voltage and caffeine stimulation; Shirokova and Ríos, 1997; 200 mM/s with action potential stimulation; Pape et al., 1995). The present estimate is therefore generally consistent with the work with cell segments, under the assumption that it is possible to have all channels open in a maximally activated fiber.

Consequences for Interpretation of the Ca^{2+} Spark

Cheng et al. (1993) estimated the release current underlying a spark at 4 pA. Based on available measurements of unitary Ca^{2+} current, which at the time were ~3 pA (Rousseau et al., 1987; Rousseau and Meissner, 1989), they suggested that a single Ca^{2+} spark could arise from the opening of individual SR Ca^{2+} release channels. As discussed above, the 3-pA value was obtained in the presence of high lumenal Ca^{2+} (50 mM) and in the absence of both monovalent cations and Mg^{2+}. Here, we demonstrate that the unitary Ca^{2+} current through a single cardiac RyR in the cell is probably <0.5 pA. Given the estimate of 4 pA for the SR Ca^{2+} flux underlying a Ca^{2+} spark (Cheng et al. 1993; Blatter et al., 1997), ~10 RyR channels with a unitary Ca^{2+} current of 0.35 pA would be required. Our single-channel data are, therefore, more consistent with the idea that individual Ca^{2+} sparks arise from the concerted opening of a cluster of RyR channels (Lipp and Niggli, 1996; Parker et al., 1996; Blatter et al., 1997). The agreement is not quantitative, however, because morphological studies indicate that cardiac RyRs are arranged in junctional arrays of several tens of channels (Sun et al., 1995; Franzini-Armstrong and Protasi, 1997).

A more quantitative agreement between spark amplitude and the morphology of channel clusters is found in skeletal muscle. Recent estimates place the current underlying large skeletal muscle sparks at between 12 and 15 pA (Ríos et al., 1998; Ríos, E., M.D. Stern, A. Gonzalez, G. Pizarro, and N. Shirokova, manuscript submitted for publication). Therefore, between 35 and 43 fully open channels of 0.35 pA would be required to generate such current. In skeletal muscle, the arrays of channels on either side of a junctional transverse tubule segment, or couplon, are 200–1,000-nm long and have between 20 and 70 channels. Our estimate of unitary current (extrapolated to skeletal muscle) is, therefore, consistent with the idea that the activation of all or some of the channels in such couplons constitutes a spark (Stern et al., 1997). Again, this is consistent with estimates of maximal flux density obtained in cut fiber experiments. Indeed, there should be two or three couplons per cubic micrometer, providing 20–50 pA release current/μm^3, or ~200 mM/s of Ca^{2+} flux density, when fully activated.

APPENDIX

Electrostatic Focussing of Ca^{2+} Diffusion

We modeled diffusional access of Ca^{2+} to the channel pore as spherically symmetrical diffusion converging to a radius r_0 on the luminal side of the membrane, and diverging from r_0 on the cytosolic side (Fig. 7). The [Ca^{2+}] at r_0 is assumed to be the same on both sides of the membrane; i.e., the resistance across the true pore is neglected. A uniform distribution of fixed negative charge is assumed to be present out to a radius r_1. Within the sphere, the Ca^{2+} diffusion coefficient is D_i,

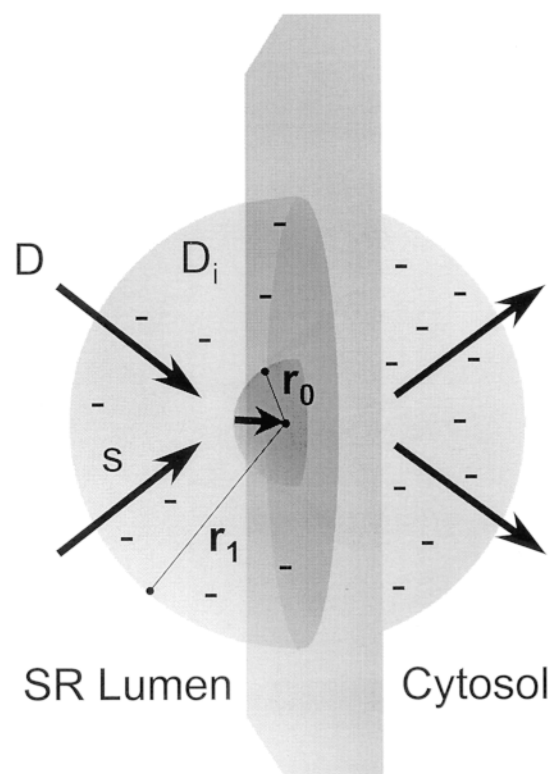

FIGURE 7. Schematic representation of the electrostatic focussing diffusion model. Ca^{2+} ions diffuse in a spherically symmetrical manner, passing through a permeable, negatively charged sphere of radius r_1 to an inner sphere of radius r_0 (which represents the capture radius of the true pore). The ions reappear at r_0 (having passed through the pore without resistance) and diffuse spherically into the cytosolic space, passing again through the charged sphere. A Donnan equilibrium potential difference exists across the outer boundary of the charged sphere, increasing [Ca^{2+}] by a Nernst factor upon entering the sphere.

while in the outside it is D. The spherical sponge is assumed to be permeable to small ions. There will be a Donnan equilibrium potential difference across the boundary of the sponge; this causes a jump in $[Ca^{2+}]$ by a Nernst factor. Outside and within the sponge, $[Ca^{2+}]$ is assumed to diffuse in the absence of electric fields.

Donnan Equilibrium

The cytosol contains monovalent cations (activity k) and an equal concentration of mobile anions. The channel sponge has a concentration a of fixed negative charges. Inside the sponge, diffusible cations are enhanced by a Nernst factor $q = \exp(eV/kT)$ where V, the Donnan potential, is to be determined. Mobile anions are reduced by the same factor. Electroneutrality within the sponge requires:

$$kq = \frac{k}{q} + a, \tag{1}$$

whose solution is:

$$q = \frac{\sqrt{4k^2 + a^2 + a}}{2k}. \tag{2}$$

Inclusion of 2 mM divalent cations would make Eq. 1 a cubic equation, and reduce the Donnan factor by only 3%, so it has been omitted for simplicity.

Diffusion Calculation

In the cytosol, the diffusional free $[Ca^{2+}]$ produced by a source flux s is given by:

$$[Ca^{2+}]_{Cyto} = \frac{s}{2\pi D_i r}. \tag{3}$$

Within the cytosolic hemisphere (CS) of the sponge, the $[Ca^{2+}]$ is:

$$[Ca^{2+}]_{CS} = \frac{s}{2\pi D_i r} + b_1, \tag{4}$$

where the constant b_1 is to be determined by the boundary condition that $[Ca^{2+}]$ must jump by the factor q^2 at the boundary of the sponge, $r = r_1$. Solving for b_1 and replacing it in the previous equation gives:

$$[Ca^{2+}]_{CS} = \frac{(D_i q^2 - D)_s}{2\pi D D_i r_1} + \frac{s}{2\pi D_i r}. \tag{5}$$

In the luminal hemisphere (LS) of the sponge, the solution of the diffusion equation is:

$$[Ca^{2+}]_{LS} = b_2 - \left(\frac{s}{2\pi D_i r}\right), \tag{6}$$

where the constant b_2 is to be determined by requiring that the concentration at the true pore radius r_0 be equal on both sides of the membrane (i.e., leaving out the diffusion resistance of the true pore). Solving for b_2 then gives:

$$[Ca^{2+}]_{LS} = \frac{(D r_0 r_1 - 2 D r r_1 - D_i q^2 r r_0 + D r r_0)_s}{2\pi D D_i r r_0 r_1}, \tag{7}$$

while in the lumen the solution is given by:

$$[Ca^{2+}]_{lum} = b_3 - \left(\frac{s}{2\pi D r}\right), \tag{8}$$

where the constant b_3 is again determined by the Donnan factor boundary condition at r_1. Solving for b_3, replacing it, and taking the limit as $r \to$ infinity, we find the free Ca^{2+} in the SR lumen:

$$[Ca^{2+}]_{lum} = \frac{[D\ r_1 + (D_i q^2 - D) r_0] s}{\pi D\ D_i q^2 r_0 r_1}. \tag{9}$$

Substituting the value of q from Eq. 2, and noting that the anion concentration is given by:

$$a = \frac{3n}{4\pi N_a r_1^3}, \tag{10}$$

where n is the number of negative charges, N_a is Avogadro's number, and r_1 is the radius of the sphere within which the charges are confined, and the fact that $s = i/F$, where i is the unitary current, gives:

$$[Ca^{2+}]_{lumen} =$$

$$\frac{2i(3D_i n r_0 \sqrt{64\pi^2 k^2 N_a^2 r_1^6 + 9n^2} + 32\pi^2 D k^2 N_a^2 r_1^7 +}{\pi D\ D_i F r_0 r_1 (\sqrt{64\pi^2 k^2 N_a^2 r_1^6 + 9n^2} + 3n)^2}$$

$$\frac{32\pi^2 D_i k^2 N_a^2 r_0 r_1^6 - 32\pi^2 D k^2 N_a^2 r_0 r_1^6 + 9 D_i n^2 r_0)}{\pi D\ D_i F r_0 r_1 (\sqrt{64\pi^2 k^2 N_a^2 r_1^6 + 9n^2} + 3n)^2} \tag{11}$$

Eq. 11 has a minimum as a function of r_1, located, in general, around 5–10 nm. Evaluating Eq. 11 at this optimum radius of the charge sphere gives a lower limit to the lumenal Ca^{2+} for a given unitary current. By assuming that all 2,768 acidic residues of the tetramer are ionized, $r_0 = 1$ nm, and $D_i = D = 0.78 \times 10^{-5}$ cm^2/s, we obtain the very liberal estimate of the minimum lumenal free Ca^{2+} required to drive a unitary current of 3 pA at the zero transmembrane potential that was quoted in the text.

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