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ORIGINAL ARTICLE

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Regulation of calcium release by calcium inside the sarcoplasmic reticulum in ventricular myocytes

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Abstract To study the effects of changes in sarcoplasmic reticulum (SR) intraluminal Ca^{2+} on the Ca^{2+} release mechanism, we correlated the activity of single cardiac ryanodine receptor (RyR) channels, monitored in planar bilayers, with the properties of spontaneous elementary Ca^{2+} release events (sparks) in intact ventricular myocytes, monitored by scanning confocal microfluorimetry. Under both normal conditions and Ca^{2+} overload, induced by elevation of extracellular [Ca^{2+}], Ca^{2+} sparks represented single populations of events. During Ca^{2+} overload, the frequency of sparks increased from 0.8 to 3.1 events per second per 100 μm line scanned, and their amplitude increased from 100 nM to 400 nM. The duration of the Ca^{2+} sparks, however, was not altered. Changes in the properties of Ca^{2+} sparks were accompanied by only an ≈ 30% increase in the SR Ca^{2+} content, as determined by emptying the intracellular Ca^{2+} stores using caffeine. When single Ca^{2+} release channels were incorporated into lipid bilayers and activated by cytoplasmic Ca^{2+} (≈ 100 nM) and ATP (3 mM), elevation of Ca^{2+} on the luminal side from 20 μM to 0.2–20 mM resulted in a 1.2-fold to 7-fold increase, respectively, in open probability (P_o). This potentiation of P_o was due to an increase in mean open time and frequency of events. The relative effect of luminal Ca^{2+} was greater at low levels of cytoplasmic [Ca^{2+}] than at high levels of cytoplasmic [Ca^{2+}], and no effect of luminal Ca^{2+} was observed to occur in channels activated by 0.5–50 μM cytoplasmic Ca^{2+} in the absence of ATP. Our results suggest that SR Ca^{2+} release channels are modulated by SR intraluminal Ca^{2+}. These alterations in properties of release channels may account for, or contribute to, the mechanism of spontaneous Ca^{2+} release in cardiac myocytes

Key words Confocal Ca^{2+} imaging · Ca^{2+} release channels/ryanodine receptors · Intraluminal Ca^{2+} · Ventricular cardiac myocytes

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Introduction

In cardiac muscle, Ca^{2+} release from the sarcoplasmic reticulum (SR) is mediated by a specialized Ca^{2+} release channel called the ryanodine receptor (RyR) [1, 19]. The RyR is activated by Ca^{2+} influx through L-type Ca^{2+} channels in the sarcolemma. This process, termed Ca^{2+}-induced Ca^{2+} release (CICR), underlies the process of excitation–contraction (E–C) coupling in the heart [18, 24] and might be involved in Ca^{2+} signaling in other cell types. The activity of the cardiac RyR is modulated by a number of cytoplasmic ligands, besides Ca^{2+}, including ATP, Mg^{2+} and calmodulin [17]. Consequently, the cytoplasmic side of the RyR is thought to have the corresponding ligand-binding sites. A growing body of evidence suggests that RyR might also be regulated by SR intraluminal Ca^{2+}. It has been reported that the efficacy of the inward Ca^{2+} current, in triggering release, depends on the Ca^{2+} content within the SR [2, 15, 16]. The probability of observing local spontaneous Ca^{2+} release events (Ca^{2+} sparks) is increased in myocytes incubated in solutions containing elevated Ca^{2+} [5, 7]. Furthermore, when the Ca^{2+} content of the cell is sufficiently high ("Ca^{2+} overload"), spontaneous release of Ca^{2+} from the SR results in local elevations of Ca^{2+} that can propagate across the cell as regenerative Ca^{2+} waves [18, 22]. It has also been reported that the activity of single cardiac RyR channels incorporated into lipid bilayers increases when Ca^{2+} on the luminal side of the channel is elevated [21]. The activation of single cardiac RyR channels by luminal Ca^{2+}, however, was dependent on the presence of a non-physiological activating ligand, sulmazole [21].

In the present study, we correlated the effect of SR Ca^{2+} load on the properties of Ca^{2+} sparks, monitored by scanning confocal microfluorimetry, with the effect of luminal Ca^{2+} on the activity of single cardiac RyR channels, monitored in planar bilayers. A relatively small elevation (30%) in SR Ca^{2+} content dramatically increased the frequency (400%) and amplitude (400%) of spontaneous Ca^{2+} sparks. In the presence of physiological resting levels of cytoplasmic ATP and Ca^{2+}, millimolar lev-

els of Ca^{2+} on the luminal side of the channel increased the mean open time and open probability (P_o) of single cardiac RyR channels. These results suggest that SR Ca^{2+} release channels are modulated by SR intraluminal Ca^{2+}.

Materials and methods

Confocal imaging of Ca^{2+} in rat ventricular myocytes

Cells

Single ventricular myocytes were obtained from adult Sprague-Dawley rat hearts by enzymatic dissociation [28]. The cells were loaded with Fluo-3 via a 20-min incubation with 5 μM Fluo-3 (acetoxymethyl ester form, Molecular Probes, Eugene, Ore., USA) at room temperature.

Solutions

The standard bathing solution contained (in mM): 140 NaCl, 2 KCl, 0.5 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, 0.25 NaH_2PO_4, 5.6 glucose, pH 7.3. Tetrodotoxin at 10 μM and verapamil at 50 μM were added to the bathing solution to avoid depolarization-induced Ca^{2+} release due to spontaneous generation of action potentials.

Confocal microscope

Experiments were performed using an Olympus Laser Scanning Confocal Microscope (LSM-GB200) equipped with an Olympus 60 × 1.4 N.A. objective. For imaging intracellular $[Ca^{2+}]$ the system was operated in the line scan mode. Fluo-3 was excited by light at 488 nm (25 mW argon laser, intensity attenuated to 1%) and fluorescence was measured at wavelengths of >515 nm. An analog recording of fluorescence intensity during each scan was digitized into 640 pixels, giving a nominal pixel dimension of 0.41 μm. Images were acquired at a rate of 2.1 or 8.3 ms per scan with the confocal detector aperture set to 10–25% of maximum. As measured with 0.1-μm fluorescent beads (Molecular Probes), the system provided an axial resolution of 0.4 μm and a depth of field of about 0.7 μm. Ca^{2+} measurements under normal Ca^{2+} load versus Ca^{2+} overload conditions were performed using the same individual cells by acquiring $[Ca^{2+}]$ images before and after changing extracellular $[Ca^{2+}]$ ($[Ca^{2+}]_o$)from 1 to 10 mM. For calibration puposes, the total line scan $[Ca^{2+}]$ in 1 mM $[Ca^{2+}]_o$ (normal load) was assumed to be 100 nM and it served as a reference point for the determination of background and peak $[Ca^{2+}]$ for each spark measured before and after changing the loading conditions. $[Ca^{2+}]$ was calculated from Fluo-3 fluorescence using an equation and calibration parameters described previously [4, 5]. Image processing and analysis was performed using a 6100/60 Power MacIntosh and an image-processing software package (NIH Image).

Lipid bilayer experiments

Heavy SR microsomes were isolated by differential centrifugation from the ventricles of dog hearts by standard procedures [8]. Microsomes were fused into planar lipid bilayers, and single channels were monitored as described previously [13]. Bilayers were composed of 80% phosphatidylethanolamine and 20% phosphatidylcholine dissolved in decane at a final concentration of 50 mg/ml. SR vesicles were added into one side of the bilayer (defined as *cis*) and the other side was defined as *trans* (virtual ground). The orientation of the incorporated RyR channels was such that the cytoplasmic side was in the *cis* compartment [13]. Standard solutions contained 300 mM *cis* $CsCH_3SO_3$, 20 mM *trans* $CsCH_3SO_3$, 20 μM $CaCl_2$, 20 mM Hepes (pH 7.2). After channel incorporation, the *trans* $CsCH_3SO_3$ was adjusted to 300 mM. A custom current/voltage conversion amplifier was used to optimize the single-channel recording [13]. Acquisition software (pClamp, Axon Instruments, Burlingame, Calif., USA), an IBM-compatible 486 computer and a 12 bit A/D–D/A converter (Axon Instruments) were also used. Single-channel records were digitized at 2–5 kHz and filtered at 1 kHz.

Results

Figure 1 shows representative line scan images obtained from a myocyte before and after elevation of $[Ca^{2+}]_o$

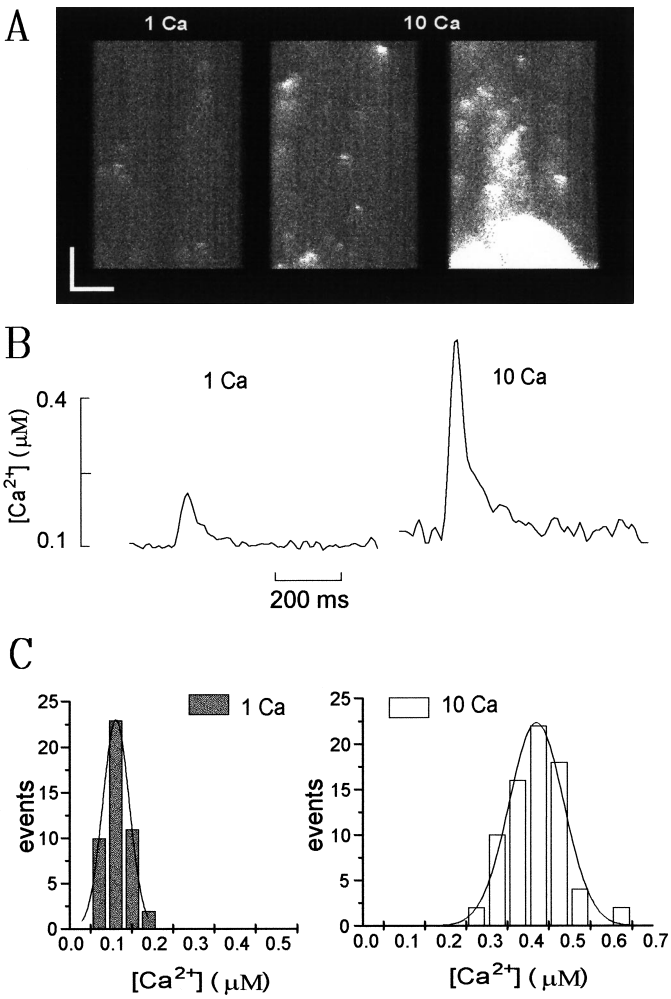

Fig. 1A–C Effect of Ca^{2+} load on spontaneous Ca^{2+} release events in ventricular myocytes. **A** Line scan images of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) changes in a cell before (*left panel*) and after (*middle* and *right panels*) changing extracellular $[Ca^{2+}]$ from 1 mM to 10 mM. *Calibration bars* : horizontal 5 μm, *vertical* 200 ms. **B** Time-dependent changes of $[Ca^{2+}]$ associated with Ca^{2+} sparks in 1 (*left trace*) and 10 (*right trace*) mM $[Ca^{2+}]$. Each *trace* is an average of ten individual line plots of $[Ca^{2+}]_i$ through the peaks of the sparks. **C** Amplitude histograms of sparks recorded in 1 mM (*filled bars*) and 10 mM (*open bars*) $[Ca^{2+}]_o$. The amplitude of each spark was determined as a difference between a peak $[Ca^{2+}]$ during the spark and the average $[Ca^{2+}]$ during the 100-ms period prior to the onset of the spark. The lines are Gaussian fits of the histograms; the center and width at half maximum were 0.11 and 0.06 μM for 1 mM $[Ca^{2+}]_o$ and 0.41 and 0.13 μM for 10 mM $[Ca^{2+}]_o$, respectively

Table 1 Spatiotemporal properties of Ca^{2+} sparks under normal conditions and during Ca^{2+} overload. Ca^{2+} overload was produced by increasing the bathing Ca^{2+} concentration from 1 to 10 mM

	Frequency[a] ($s^{-1}100\ \mu m^{-1}$)		Background[b] (nM Ca^{2+})		Peak (nM Ca^{2+})		Amplitude[c] (nM Ca^{2+})		Duration[d] (ms)		Width[e] (μm)	
$[Ca^{2+}]_o$	1	10	1	10	1	10	1	10	1	10	1	10
Mean	0.8	3.1	98	124	212	543	114	419	37.1	39.4	1.7	2.8
±SE	0.4	0.9	1.7	4.1	4.4	12	4	8	1.5	2.4	0.1	0.2
n	9	5	51	74	51	74	51	74	51	74	51	74
P	<0.0001		<0.0001		<0.0001		<0.0001		<0.4667		<0.0001	

[a] Spark frequency was defined as the number of events per second per 100-μm line scanned
[b] Backgound $[Ca^{2+}]$ was defined as the average $[Ca^{2+}]$ measured during a 100-ms interval prior to the onset of the spark
[c] Amplitude was determined as a difference between the peak $[Ca^{2+}]$ and background $[Ca^{2+}]$ levels
[d] Duration and [e]width were measured at half-maximal amplitude

from 1 to 10 mM. High levels of $[Ca^{2+}]_o$ apparently alter intracellular Ca^{2+} homeostasis and result in an increased SR Ca^{2+} content or Ca^{2+} overload [24]. A line across the cell's width was repeatedly scanned and line intensities were successively placed beneath one another to build a line scan image. In 1 mM $[Ca^{2+}]_o$, the line scan image of the cell reveals sporadic localized increases in fluorescence called Ca^{2+} sparks. In accordance with previous studies [5, 7], in 10 mM $[Ca^{2+}]_o$ there was an increase in the frequency of sparks and propagating Ca^{2+} waves arose regularly (5–20 waves in 1 min). The effect of Ca^{2+} overload on the amplitude and kinetics of the sparks is shown in Fig. 1B. The traces were obtained by averaging ten individual sparks both before and after elevation of $[Ca^{2+}]_o$ from 1 to 10 mM. To avoid ambiguities due to variations in resting cytoplasmic $[Ca^{2+}]$ ($[Ca^{2+}]_i$) and SR Ca^{2+} load in spontaneously Ca^{2+}-oscillating cells (see below), we selected only the sparks recorded during quiescent periods when $[Ca^{2+}]_i$ was close to the baseline. It can be seen from these averaged traces that during Ca^{2+} overload the peak Ca^{2+} attained during the sparks was increased from ≈ 200 nM to ≈ 500 nM, whereas the time course of the sparks remained essentially unchanged. The effects of Ca^{2+} overload on Ca^{2+} sparks are summarized in Table 1. As can be seen, besides the significant increase in frequency and amplitude of the sparks, there was also a substantial increase in their width, although the duration of the sparks did not change significantly. The background $[Ca^{2+}]$ prior to the onset of the sparks increased from 98 nM to 124 nM. The amplitude distribution of sparks in both control and Ca^{2+}-overloaded myocytes (Fig. 1C) could be well described by a single Gaussian distribution, consistent with sparks representing single classes of release events. This result suggests the increase in the magnitude of sparks during Ca^{2+} overload is not due to the summation of individual sparks, but rather an increase in the amount of Ca^{2+} released during the sparks.

The changes in the level of the SR Ca^{2+} content associated with the observed changes in spontaneous Ca^{2+} release events were estimated by application of caffeine. In Ca^{2+}-overloaded cells, cyclical spontaneous releases depleted the SR of Ca^{2+} regularly. Thus, first the stability of the SR Ca^{2+} content at different intervals following a Ca^{2+} wave was determined by caffeine application. Figure 2A shows an example of a line scan image in which the SR was emptied by rapid application of caffeine (20 mM, at the arrow) while the wave propagated along the cell. The wave propagated from the left end of the cell to the right end, resulting in a sloping fluorescence band on the line scan image. The caffeine-induced release occurred synchronously across the cell, and thus appears as a horizontal band. The time interval between the wave and the caffeine-induced release decreases from the left to the right until the two releases intersect at the bottom right-hand corner of the image. Next to the image, five line plots are aligned according to the peak amplitude of the local spontaneous Ca^{2+} transients to illustrate the changes in caffeine-induced Ca^{2+} transients as a function of time following spontaneous Ca^{2+} release. These data demonstrate that: (1) Ca^{2+} release during a Ca^{2+} wave is near maximal; (2) reloading of the Ca^{2+} store following a Ca^{2+} wave can be relatively fast (<1 s), and (3) the peak caffeine-induced Ca^{2+} transient in Ca^{2+}-overloaded myocytes is not altered considerably by the presence of Ca^{2+} waves. Similar results were obtained in four other experiments.

To determine the relative levels of SR Ca^{2+} content in the control ($[Ca^{2+}]_o$ =1 mM) and Ca^{2+}-overloaded myocytes ($[Ca^{2+}]_o$ = 10 mM), caffeine was applied under both experimental conditions. Figure 2B shows representative line scan images of Ca^{2+} transients elicited by 20 mM caffeine before and after elevating $[Ca^{2+}]_o$. Next to the images, the line plots of time-dependent changes in $[Ca^{2+}]$ are also presented. It can be seen that the decay of the caffeine-induced transient is considerably slower ($t_{1/2}$ = 1.6 vs 0.7 s) in 10 mM Ca^{2+} than in 1 mM Ca^{2+}. This result could be ascribed to the decrease in the efficiency of the Ca^{2+} extrusion mechanisms in high external Ca^{2+} (i.e. Na^+/Ca^{2+} exchanger working against a higher Ca^{2+} gradient). In the experiment shown in Fig. 2B, the SR Ca^{2+} load, assessed from the amplitude of the caffeine-induced Ca^{2+} transients, was 27% higher in Ca^{2+}-overloaded myocytes than in control myocytes. The average increase in SR Ca^{2+} content in ten cells was 29 ± 7%. Therefore, a 400% increase in the amplitude of

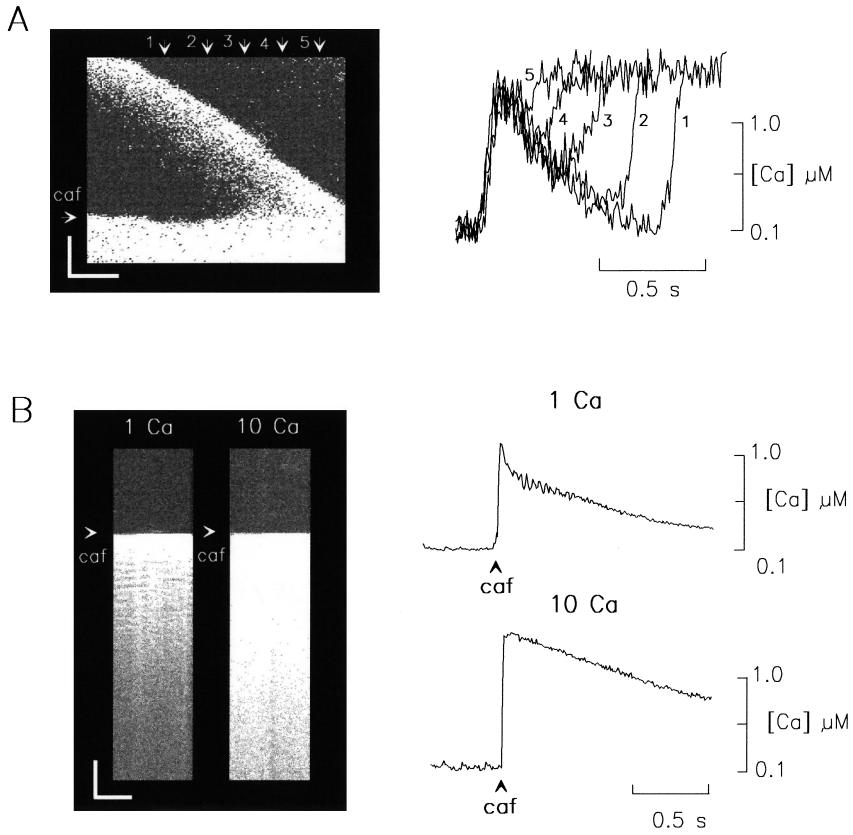

Fig. 2A, B Estimation of the levels of sarcoplasmic reticulum (SR) Ca^{2+} load by caffeine application. **A** On the *left* is a line scan image of a caffeine-induced Ca^{2+} transient (*horizontal band* of *fluorescence*) recorded during propagation of a spontaneous Ca^{2+} wave. The time of application of caffeine (20 mM) was inferred from the onset of the Ca^{2+} transients. *Calibration bars*: horizontal 10 μm, *vertical* 200 ms. On the *right* are plotted time-dependent changes in $[Ca^{2+}]$, recorded by averaging three adjacent pixels of the line scan image at sites indicated by *numbered arrows*. The traces were superimposed with the peaks of the spontaneous Ca^{2+} transients and aligned to illustrate the time course of resequestration of Ca^{2+} into SR. **B** On the *left*, are line scan images of caffeine-induced Ca^{2+} transients recorded in 1 (*left panel*) and 10 (*right panel*) mM Ca^{2+}. The time of application of caffeine (20 mM) was inferred from the onset of the Ca^{2+} transient. *Calibration bars*: *horizontal* 10 μm, *vertical* 250 ms. On the *right* are shown time-dependent changes in $[Ca^{2+}]_i$ associated with the line scan images shown on the *left*

the spark was accompanied by only a 30% increase in the SR Ca^{2+} content in Ca^{2+}-overloaded cells. These results suggest that the increase of the pool of releasable Ca^{2+} alone is not likely to account for the increase in the amplitude of the sparks.

To test the possibility that elevation of intraluminal $[Ca^{2+}]$ alters the gating of the Ca^{2+} release channels, we performed measurements using single Ca^{2+} release channels incorporated into lipid bilayers. The resting levels of ATP and Ca^{2+} in cardiac cells are approximately 3 mM and 0.1 nM (pCa 7), respectively [3]. The ATP and Ca^{2+} concentrations in the solution on the cytoplamic side of the channel were adjusted to mimic physiological levels at rest. To simplify data interpretation, RyR channel activity was monitored using Cs^+ as the charge carrier. All channels were reconstituted with 20 μM Ca^{2+} (pCa 4.8) on both sides of the channel (Fig. 3A). In the presence of 3 mM ATP and pCa 7 on the cytoplasmic side, RyR channel activity was quite low (P_o = 0.010 ± 0.003,

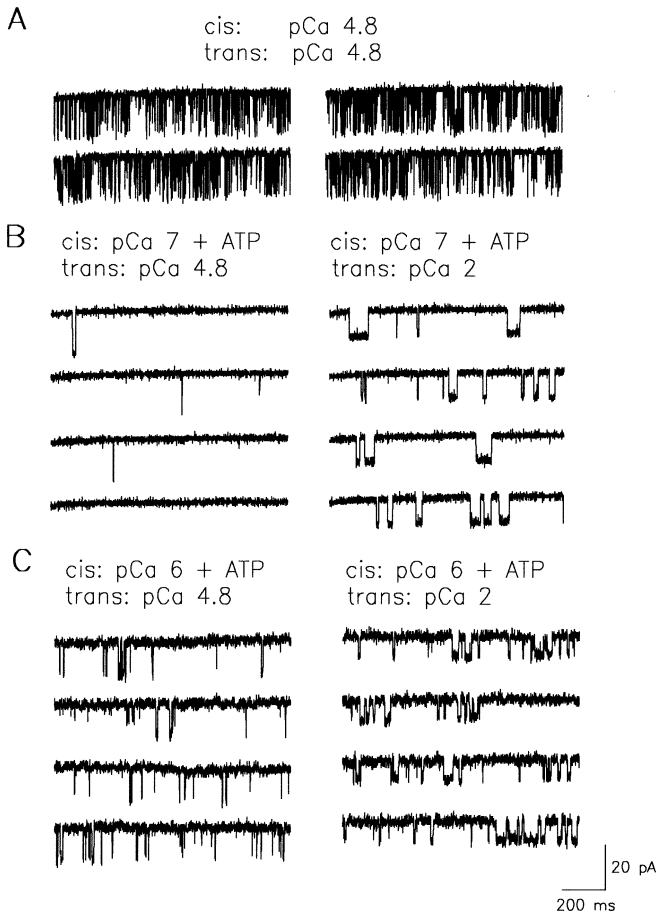

Fig. 3A–C The effect of luminal Ca^{2+}on channel activity in the presence of Ca^{2+} and ATP on the *cis* side of the channel. **A, B** Single-channel current fluctuations under initial conditions (20 μM *cis* and *trans* Ca^{2+}, **A**), following addition of 0.4 mM EGTA and 3 mM ATP (**B**, *left-hand panel*) and after increasing *trans* Ca^{2+} from 20 μM to 10 mM (**B**, *right-hand panel*). Holding potential was –40 mV. **C** Single-channel fluctuations in the presence of 1 μM Ca^{2+} and 3 mM ATP on the *cis* side of the channel before (*left-hand-panel*) and after increasing *trans* $[Ca^{2+}]$ from 20 μM to 10 mM (*right-hand panel*). Holding potential was –30 mV. Single-channel openings are shown as *downward deflections*. Values of *cis* and *trans* $[Ca^{2+}]$ for each condition are indicated above the traces

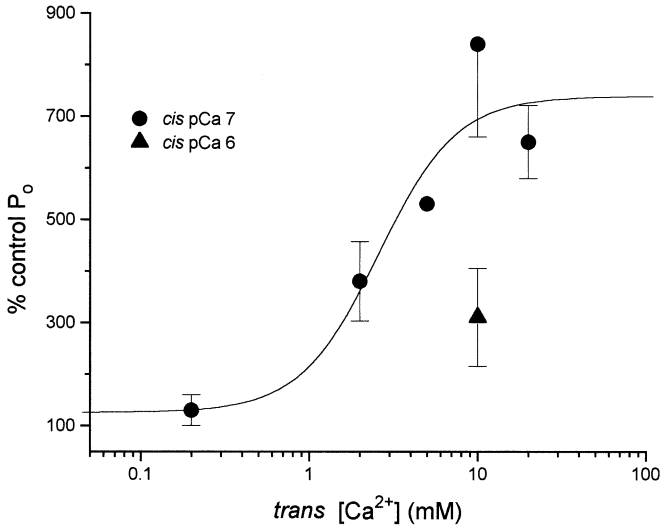

Fig. 4 Relative open probability (P_o) as a function of luminal [Ca^{2+}] in the presence of 0.1 μM Ca^{2+} and 3 mM ATP (*circles*) on the *cis* side of the channel. Also shown are the results obtained with 1 μM Ca^{2+} and 3 mM ATP (*triangle*) on the *cis* side of the channel. The values are means (± SEM) obtained from 1–5 individual experiments. The Hill coefficient and EC_{50} values were 1.9 and 2.6 mM, respectively

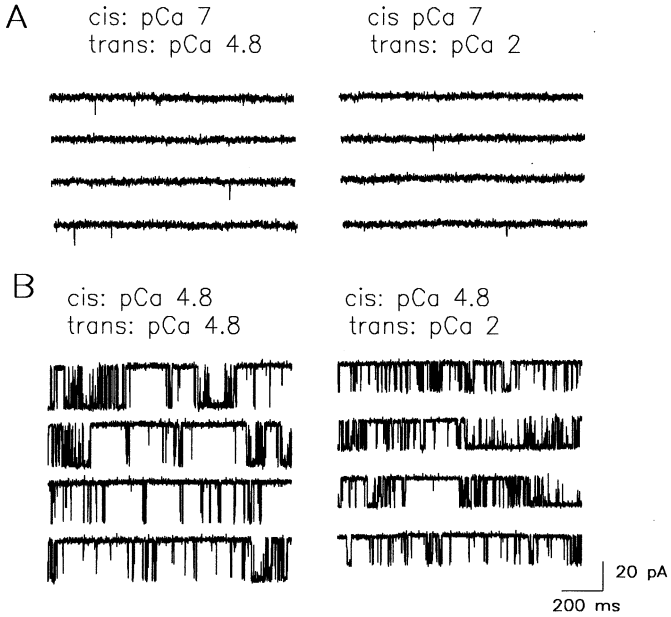

Fig. 5A, B The effect of increasing luminal Ca^{2+} on channel activity in the absence of cytoplasmic ATP at two different levels of *cis* [Ca^{2+}]. **A** Single-channel current fluctuations measured in the presence of 0.1 μM *cis* Ca^{2+} before (*left-hand panel*) and after (*right-hand panel*) increasing *trans* [Ca] from 20 μM to 10 mM at a holding potential of –30 mV. P_o was 0.0005 and 0.0004 before and after changing luminal Ca^{2+}, respectively. **B** Single-channel current fluctuations measured in the presence of 20 μM *cis* [Ca^{2+}] before (*left-hand panel*) and after changing *trans* [Ca^{2+}] from 20 μM to 10 mM (*right-hand panel*) at a holding potential of –40 mV. P_o was 0.2321 and 0.2097 before and after changing *trans* [Ca^{2+}], respectively. Single-channel openings are shown as *downward deflections*. Values of *cis* and *trans* [Ca^{2+}] for each condition are indicated

SEM, n = 7; Fig. 3B, left panel). Elevating the luminal [Ca^{2+}] from pCa 4.8 to pCa 2 (10 mM) simultaneously increased P_o (0.067 ± 0.023, n = 7) and mean open time (5.4 ± 1.7 vs 22.3 ± 5.9 ms, n = 6). In the presence of 10 mM luminal Ca^{2+}, unitary current was reduced as Ca^{2+} competes with the primary charge-carrying ion (Cs^+) in the pore [26]. Thus, elevation of luminal Ca^{2+} increased P_o (seven-fold) and mean open time (fourfold) in the presence of resting cytoplasmic levels of ATP and Ca^{2+}.

Global intracellular $[Ca^{2+}]_i$ measurements indicate that during a [Ca^{2+}] transient induced by an action potential, the cytoplasmic [Ca^{2+}] rises from 100 nM to micromolar levels [3]. Therefore, the free [Ca^{2+}] on the cytoplasmic side of single cardiac RyR channels was adjusted to pCa 6 (1 μM). At this level of cytoplasmic Ca^{2+} and in the presence of 3 mM ATP, P_o was relatively high and increased about twofold (0.128 ± 0.030 vs 0.254 ± 0.063, n = 6) when the luminal [Ca^{2+}] was elevated from pCa 4.8 to pCa 2 (Fig. 3C). The elevation in luminal Ca^{2+} approximately doubled the mean open time (from 7.1 ± 2.1 to 17.4 ± 2.6 ms, n = 6). Thus, luminal Ca^{2+} modulated channel activity in the presence of either low (pCa 7) or high (pCa 6) cytoplasmic [Ca^{2+}], however, the relative efficacy of luminal Ca^{2+} was greater at lower [Ca^{2+}].

The free [Ca^{2+}] inside the SR at rest has not been clearly established. Estimates range from 100 μM to 10 mM [3]; therefore, the efficacy of luminal Ca^{2+} was determined over this range (Fig. 4). At the low cytoplasmic [Ca^{2+}] (pCa 7; circles), the P_o (% control) increased sigmoidally with the increase of luminal (*trans*) [Ca^{2+}] and saturated at [Ca^{2+}] values above 10 mM. At the higher cytoplasmic [Ca^{2+}] (pCa 6, triangles), the efficacy of 10 mM luminal Ca^{2+} was about half that at the lower cytoplasmic [Ca^{2+}].

It has been suggested that the luminal Ca^{2+} dependence of reconstituted skeletal muscle RyR channels depends on the presence of ATP on the cytoplasmic side of the channel [21]. The luminal Ca^{2+} dependence of the cardiac RyR channel was determined in the absence of ATP (Fig. 5). An increase of luminal [Ca^{2+}] from pCa 4.8 to pCa 2 did not alter significantly P_o and mean open time in the presence of resting levels of cytoplasmic Ca^{2+} (pCa 7; Fig. 5A) or in the presence of a high cytoplasmic [Ca^{2+}] (pCa 4.8; Fig. 5B). The presence of 10 mM Ca^{2+} on the luminal side of the channel is evidenced by the reduction in unitary current (Fig. 5B, right panel). Similar results were obtained in seven other experiments with cytoplasmic [Ca^{2+}] levels ranging from 50 nM to 50 μM. Thus, channel gating was not altered by the luminal [Ca^{2+}] change in the absence of cytoplasmic ATP.

Discussion

In this study, we explored the possibility that [Ca^{2+}] inside the SR modulates the activity of the Ca^{2+} release

channels. The impact of increased SR Ca^{2+} content on spontaneous local Ca^{2+} release events (Ca^{2+} sparks) was quantified in intact cardiomyocytes. The luminal Ca^{2+} dependence of single RyR channels was defined in the presence of physiological levels of cytoplasmic ATP and Ca^{2+}. Our results suggest that both in vitro and in situ the activity of the SR Ca^{2+} release channels is modulated by luminal Ca^{2+}.

Effect of SR Ca^{2+} load on Ca^{2+} sparks

In accordance with earlier results obtained by confocal imaging [5, 6], Ca^{2+} overload induced by elevating extracellular Ca^{2+} led to an increase in the frequency and magnitude of elementary release events (sparks) and to the generation of propagating Ca^{2+} waves. Spark amplitude and width were increased fourfold and 1.5-fold, respectively, whereas duration remained practically unchanged (Fig. 1, Table 1). Changes in the properties of Ca^{2+} sparks were accompanied by an ≈ 30% increase in SR Ca^{2+} content. A 400% increase in spark amplitude associated with only a 30% increase in SR Ca^{2+} content suggests that the increase in spark magnitude is not simply due to a larger Ca^{2+} gradient across the SR membrane. However, it should be noted that because of the progressive saturation of intraluminal Ca^{2+} buffers (i.e., calsequestrin) during Ca^{2+} overload, the increase in intraluminal free [Ca^{2+}] (and in the gradient across the SR membrane) may be considerably higher than that suggested by changes in the total SR Ca^{2+} content. On the other hand, currents carried by divalent cations through the Ca^{2+} release channel are known to saturate when the concentration of the cations on the luminal side is raised to between 2 and 5 mM [25, 26]. If the SR free [Ca^{2+}] in normal myocytes is about 1–3 mM, as is estimated for skeletal muscle [27], any further increase in luminal [Ca^{2+}] would not produce a significant change in current amplitude. Hence, it appears the increase in spark magnitude in Ca^{2+}-overloaded cells is due to both a greater pool of Ca^{2+} to be released and to altered properties of the release mechanism.

A fourfold increase in frequency of sparks was accompanied by a significant elevation of background [Ca^{2+}] (from 98 nm to 124 nM; Fig. 1, Table 1). This observation is inconsistent with the existence of high-affinity Ca^{2+} inactivation [10] or adaptation sites [6], occupation of which by Ca^{2} would be expected to reduce the frequency of events. Although, Fabiato [11] has shown that, in skinned cardiac cells, spontaneous Ca^{2+} release caused by an increased SR Ca^{2+} load may occur at high bathing [Ca^{2+}] when the CICR is inactivated. Thus, the activating effect of intraluminal Ca^{2+} may some how overcome inactivation of release by cytoplasmic Ca^{2+}.

The mechanisms underlying termination of Ca^{2+} release during a Ca^{2+} spark have not been defined. One possibility is that sparks simply die off spontaneously [22]. Theoretically, the rate of decay of Ca^{2+} release from a regenerative release unit should be very sensitive to the number of channels composing the unit and to the magnitude of the positive feedback gain that sustains the local Ca^{2+} elevation [22]. Thus, if the decay of local release events is due to a stochastic mechanism, an increase in the magnitude of the local release events should result in an increase in the duration of the events. Alternatively, self-regeneration of CICR at the sparking sites could be limited by a negative feedback mechanism like inactivation [10] or adaptation [12, 29]. We showed that a fourfold increase in the amplitude of the sparks was not accompanied by significant alterations in spark duration (Fig. 1B). Thus, our results are not consistent with a stochastic decay mechanism and rather suggest that termination of elementary release events is determined by an inactivation/adaptation mechanism.

Effect of luminal Ca^{2+} on single channels

At the single-channel level, we found that luminal Ca^{2+} can have profound effects on channel activity. Importantly, the effect of luminal Ca^{2+} depended on the mechanism of cytosolic activation of the channel. Thus, luminal Ca^{2+} exerted no regulatory effect on channels activated by cytoplasmic Ca^{2+} alone. On the other hand, channels activated by ATP and Ca^{2+} showed a clear increase in P_o and open time with increase in luminal [Ca^{2+}]. These results are in agreement with observations made, by Sitsapesan and Williams [20], of sheep cardiac channels activated by sulmazole, and also more recent observations of sheep skeletal release channels activated by ATP and Ca^{2+} [21]. In addition, we showed that the relative efficacy of luminal Ca^{2+} on channel gating was higher at a lower *cis* [Ca^{2+}] than at a higher *cis* [Ca^{2+}], suggesting that the sensitivity of the channel to *cis* (cytoplasmic) Ca^{2+} is decreased as a result of Ca^{2+} acting at an intraluminal site. This result is consistent with a greater caffeine sensitivity of Ca^{2+} release in our SR vesicle experiments under conditions of high Ca^{2+} load (unpublished results). Thus, the effect of luminal Ca^{2+} on the Ca^{2+} release channel may involve complex allosteric interactions between the ATP- and Ca^{2+}-binding sites on both sides of the channel. Alternatively, the observed effects of luminal Ca^{2+} on channel activity could be due to Ca^{2+} passing through the open channel and acting on the cytoplasmic activation site. However, this possibility is not consistent with our results indicating that luminal Ca^{2+} at millimolar concentrations does not exert any effects on channel gating in the absence of cytoplasmic ATP (Fig. 5). In addition, if luminal Ca^{2+} had access to the cytoplasmic activation site, then Mg^{2+} and Ba^{2+}, both of which concurrently inhibit Ca^{2+} activation of the channel and have high relative permeability, would be expected to reduce channel activity when added to the luminal side. Neither Mg^{2+} or Ba^{2+} at high luminal concentrations affect channel gating [25, 26]. Regardless of the exact mechanism, the ability to respond to elevations of luminal [Ca^{2+}] appears to be a fundamental property of the RyR channel, which may account for, or contrib-

ute to, the potentiation of Ca^{2+} release at increased levels of SR Ca^{2+} load.

Correlation between single-channel(s) events and Ca^{2+} sparks

Although it is generally believed that Ca^{2+} sparks arise from SR Ca^{2+} release events, it is not clear whether these events are due to the opening of one (or a few) RyR channels or the concerted opening of many channels. It is also unclear how single RyR gating properties impact the spatial and temporal attributes of the Ca^{2+} spark. Our results provide evidence for how sparking activity may correlate with single-channel gating. Elevating $[Ca^{2+}]$ at the luminal side of the channel increased P_o and mean open time of single RyR channels. Elevating the SR Ca^{2+} content of intact myocytes increased the rate of occurrence and magnitude of Ca^{2+} sparks, however, it did not alter their duration. The increase in P_o of single RyR channels may account for, or contribute to, the increase in spark frequency in myocytes. The increase of mean open time for single channels appears to correlate with spark amplitude but not duration. Longer channel openings should result in translocation of larger amounts of Ca^{2+} into the volume governed by a spark, thus the magnitude of local $[Ca^{2+}]$ elevations logically should become higher. However, it is less evident why the increase in duration of channel openings was not reflected in an increase in spark duration.

One possibility is that the time course of the spark is dominated by the kinetics of Fluo-3 and intracellular Ca^{2+} buffers. Indeed, the maximum kinetic response of Fluo-3 ($k_{off} \approx 6$ ms in vitro; [9]) is comparable to the time constant of the decay in the Ca^{2+} spark ($\tau \approx 20$ ms; [5]; this study, Fig. 1B). If the increase in channel life time is small relative to the dissociation rate constant (k_{off}) of the dye, it may not result in significant changes in spark duration. In our bilayer experiments, performed in the presence of 100 nM Ca^{2+} and 3 mM ATP on the cytoplasmic side, the mean open times were relatively long (about 5 and 20 ms at 50 μM and 10 mM *trans* $[Ca^{2+}]$, respectively). However, under physiological conditions (i.e., in the presence of Mg^{2+}, calmodulin, etc.) the channel openings may be shorter.

Implications for the mechanism of spontaneous Ca^{2+} release

In mammalian cardiac myocytes, the process of E–C coupling is mediated by Ca^{2+} influx from the extracellular space triggering CICR from the SR. Under various conditions known as Ca^{2+} overload, myocytes exhibit another form of Ca^{2+} release which starts spontaneously in a small area and then propagates along the cell as a regenerative Ca^{2+} wave [18, 22]. The mechanism of spontaneous Ca^{2+} release has not been defined. The possibilities include [11, 24]: (1) Ca^{2+} acting as a trigger at the cytoplasmic activation site, (2) Ca^{2+} acting as a trigger from within the SR, and (3) combination of the first and the second mechanisms, when the sensitivity of the channel to external Ca^{2+} is reduced as a consequence of Ca^{2+} acting on an internal site. Our results showed that, in intact cells, increasing the Ca^{2+} of the SR results in an increase in the frequency and amplitude of elementary release events (Figs. 1, 2). In single-channel bilayer experiments, increasing $[Ca^{2+}]$ at the luminal face of the channel led to an increase in probability and duration of channel openings (Fig. 3), and also to a decrease in the apparent sensitivity of the channel to the cytoplasmic $[Ca^{2+}]$ (Fig. 4). Based on these results, we propose that spontaneous Ca^{2+} release involves the following events:.

1. Binding of Ca^{2+} to an intraluminal site, which enhances the sensitivity of the activation site to cytoplasmic Ca^{2+} resulting in more frequent activation of local spontaneous Ca^{2+} release events. The magnitude of the sparks is also increased because of the greater Ca^{2+} gradient across the SR membrane and because of the longer duration of channel openings.

2. Whenever local $[Ca^{2+}]_i$ attains a critical threshold(as a result of spark activation), it triggers CICR from adjacent release sites leading to a self-regenerating CICR. The probability that sparks will trigger CICR in Ca^{2+}-overloaded cells is increased due to (i) their greater frequency, (ii) greater magnitude and (iii) a decrease in sensitivity of the release channels to cytoplasmic Ca^{2+}.

According to this scenario, spontaneous Ca^{2+} release is *initiated* by Ca^{2+} from within the SR and *propagates* due to diffusion-coupled CICR. An alternative mechanism for propagation of spontaneous Ca^{2+} release involves Ca^{2+} uptake into the highly loaded SR and triggering release from inside [11, 22]. Although most studies of Ca^{2+} waves in intact cells favor the CICR mechanism of wave propagation (i.e., [7, 22]), this alternative mechanism has not been ruled out entirely. Our finding that Ca^{2+} release channels can be activated by luminal Ca^{2+} is not inconsistent with this mechanism.

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