# Dynamic Regulation of Sarcoplasmic Reticulum $Ca^{2+}$ Content and Release by Luminal $Ca^{2+}$-Sensitive Leak in Rat Ventricular Myocytes

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ABSTRACT In cardiac muscle, excitation-contraction (E-C) coupling is determined by the ability of the sarcoplasmic reticulum (SR) to store and release $Ca^{2+}$. It has been hypothesized that the $Ca^{2+}$ sequestration and release mechanisms might be functionally linked to optimize the E-C coupling process. To explore the relationships between the loading status of the SR and functional state of the $Ca^{2+}$ release mechanism, we examined the effects of changes in SR $Ca^{2+}$ content on spontaneous $Ca^{2+}$ sparks in saponin-permeabilized and patch-clamped rat ventricular myocytes. SR $Ca^{2+}$ content was manipulated by pharmacologically altering the capacities of either $Ca^{2+}$ uptake or leak. $Ca^{2+}$ sparks were recorded using a confocal microscope and Fluo-3 and were quantified considering missed events. SR $Ca^{2+}$ content was assessed by application of caffeine. Exposure of permeabilized cells to anti-phospholamban antibodies elevated the SR $Ca^{2+}$ content and increased the frequency of sparks. Suppression of the SR $Ca^{2+}$ pump by thapsigargin lowered $[Ca^{2+}]_{SR}$ and reduced the frequency of sparks. The ryanodine receptor (RyR) blockers tetracaine and $Mg^{2+}$ transiently suppressed the frequency of sparks. Upon washout of the drugs, sparking activity transiently overshot control levels. Low doses of caffeine transiently potentiated sparking activity upon application and transiently depressed the sparks upon removal. In patch-clamped cardiac myocytes, exposure to caffeine produced only a transient increase in the probability of sparks induced by depolarization. We interpret these results in terms of a novel dynamic control scheme for SR $Ca^{2+}$ cycling. A central element of this scheme is a luminal $Ca^{2+}$ sensor that links the functional activity of RyRs to the loading state of the SR, allowing cells to auto-regulate the size and functional state of their SR $Ca^{2+}$ pool. These results are important for understanding the regulation of intracellular $Ca^{2+}$ release and contractility in cardiac muscle.

## INTRODUCTION

In mammalian cardiac muscle, the major source of $Ca^{2+}$ required for contractile activation is the sarcoplasmic reticulum (SR). $Ca^{2+}$ sequestered in the SR can be rapidly released in response to a small entry of $Ca^{2+}$ during the action potential. This mechanism is known as $Ca^{2+}$-induced $Ca^{2+}$ release CICR (Fabiato, 1985). Recently, it has been shown that the global $Ca^{2+}$ release is a result of spatial and temporal summation of elementary $Ca^{2+}$ release events called $Ca^{2+}$ sparks (Cheng et al., 1993; Cheng et al., 1996; Wier and Balke, 1999). It appears these local $Ca^{2+}$ release events are generated by concerted openings of ~10–30 single SR $Ca^{2+}$ release channels (ryanodine receptors, RyRs) (Bridge et al., 1999, Lukyanenko et al., 2000). During the E-C coupling process, $Ca^{2+}$ that enters through sarcolemmal voltage-activated $Ca^{2+}$ channels results in local activation of adjacent RyR clusters (Lopez-Lopez et al., 1995; Santana et al., 1996; Shorofsky et al., 1998).

It is becoming increasingly evident that the SR $Ca^{2+}$ release process is regulated not only by cytosolic $Ca^{2+}$ but also by $Ca^{2+}$ inside the lumen of the SR. RyR modulation by luminal $Ca^{2+}$ is important because it could provide a control loop linking activity of the RyRs to the loading state of the SR, thus providing a means for tuning the gain of calcium-induced calcium release (CICR) (Györke and Györke, 1998). The role of luminal $Ca^{2+}$ in modulating the release mechanism has also been implicated in the generation of spontaneous $Ca^{2+}$ waves under conditions of $Ca^{2+}$ overload in cardiac myocytes (Lukyanenko et al., 1999). Fabiato (1992) presented the first evidence for luminal $Ca^{2+}$ regulation of $Ca^{2+}$ release. He showed that, in skinned cardiac myocytes, $Ca^{2+}$release could occur in the presence of elevated cytosolic $[Ca^{2+}]$ when CICR is inactivated. He attributed this release to a mechanism different from the CICR mechanism, one that is determined by the SR $Ca^{2+}$ load. RyR bilayer reconstitution studies have provided direct support for this hypothesis. In particular, elevated luminal $Ca^{2+}$ increased the open probability of native canine RyR channels under conditions when there was no net flow of $Ca^{2+}$ from the luminal to the cytosolic side of the channel (Györke and Györke, 1998). In addition, luminal $Ca^{2+}$-mediated increase in RyR activity could be prevented by tryptic digestion of the luminal portion of the RyR channel (Ching et al., 2000). These findings suggest that modulation of RyR channels by luminal $Ca^{2+}$ involves $Ca^{2+}$-sensing sites on the luminal side of the RyR complex.

In intact cardiac myocytes, many investigators have shown that increasing the SR $Ca^{2+}$ load enhances cell-averaged (i.e., global) $Ca^{2+}$ release (Isenberg and Han, 1994; Bassani et al., 1995; Spencer and Berlin, 1995; Shannon et al., 2000). The highly nonlinear relationship between $Ca^{2+}$ release and SR

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$Ca^{2+}$ content, revealed in these studies, indicates that the effects of load may not be simply due to the increased $Ca^{2+}$ gradient across the SR membrane; rather, they likely involve alterations of RyR channel gating. However, such measurements of global $Ca^{2+}$ could not readily distinguish between intra- and extra-SR effects of $Ca^{2+}$. Increased SR $Ca^{2+}$ load has also been shown to increase the frequency of $Ca^{2+}$ sparks (Cheng et al., 1996; Lukyanenko et al., 1996; Satoh et al., 1997). However, Song et al. (1997) reported no significant change in the frequency of sparks when SR $Ca^{2+}$ load was reduced by thapsigargin and spark statistics were corrected for changes in detectability of the events.

In cardiac muscle, $Ca^{2+}$ release by the SR occurs not only during E-C coupling but also at rest. Although the resting SR $Ca^{2+}$ leak helps explain certain whole-cell phenomena such as the rest-dependent decline of $Ca^{2+}$ transients (Bridge, 1986; Bassani and Bers, 1994), this leak has not been assigned any physiologically useful role. Bassani and Bers (1995) estimated the rate of diastolic $Ca^{2+}$ leak by measuring unidirectional $Ca^{2+}$ loss from the SR in the presence of thapsigargin. They found the leak rate to be insignificant when compared with $Ca^{2+}$ fluxes mediated by other $Ca^{2+}$ transport mechanisms in cardiac myocytes. Subsequently, Ginsburg et al. (1998) suggested that dissipative losses have only a minor role in determining the SR $Ca^{2+}$ content and that the SR $Ca^{2+}$ load is set by the thermodynamic limit of the SR $Ca^{2+}$ pump. On the other hand, it has been demonstrated that inhibition of $Ca^{2+}$ efflux from the SR by the RyR channel blockers tetracaine and ruthenium red leads to a dramatic increase in the SR $Ca^{2+}$ content of rat ventricular myocytes (Györke et al., 1997; Díaz et al., 1997; Lukyanenko et al., 2000). These findings indicate that the SR $Ca^{2+}$ content is limited not by the ability of the $Ca^{2+}$ pump to move $Ca^{2+}$ against its gradient, but rather by the leak of $Ca^{2+}$ through RyR channels. Therefore, at this point, there is little agreement on the rate and role of the SR $Ca^{2+}$ leak in cardiac $Ca^{2+}$ cycling.

In the present study we sought to investigate 1) how SR $Ca^{2+}$ content influences the activity of SR $Ca^{2+}$ release sites and 2) how the activity of RyRs influences the $Ca^{2+}$ storage and release functions of the SR in cardiac muscle. To this end we used confocal $Ca^{2+}$ imaging and Fluo-3 to investigate the effects upon $Ca^{2+}$ sparks and SR $Ca^{2+}$ load by altering either SR $Ca^{2+}$ uptake or leak in permeabilized and patch-clamped cardiac myocytes. Our results indicate that openings of RyR channels, manifested as spontaneous $Ca^{2+}$ sparks, mediate a substantial diastolic release, which is controlled by the level of $Ca^{2+}$ within the SR. This luminal $Ca^{2+}$-sensitive release appears to be an essential part of a dynamic regulatory system that allows cells to auto-regulate the size and functional state of their SR $Ca^{2+}$ pool. The subsequent implication is that the myocyte controls calcium cycling in a more robust manner than previously thought.

## MATERIALS AND METHODS

### Cell isolation

Single ventricular myocytes were obtained from adult male Sprague-Dawley rat hearts by enzymatic dissociation as described previously (Györke et al. 1997). The animals were killed by lethal injection of Nembutal (100 mg/kg intraperitoneally). The standard Tyrode solution contained (in mM) 140 NaCl, 5.4 KCl, 0.5 $MgCl_2$, 1 $CaCl_2$, 10 Hepes, 0.25 $NaH_2PO_4$, 5.6 glucose, pH 7.3. Unless specified otherwise, all chemicals were from Sigma (St. Louis, MO). The experiments were performed at room temperature (21–23°C).

### Experiments in permeabilized myocytes

The cells were permeabilized by exposure to saponin (0.01% for 45–60 s, after Lukyanenko and Györke, 1999). The permeabilization solution contained 100 mM K aspartate, 20 mM KCl, 3 mM MgATP 0.5 mM EGTA, 0.114 mM $CaCl_2$ (free [$Ca^{2+}$], ~100 nM), 0.81 mM $MgCl_2$ (free [$Mg^{2+}$], ~ mM), 10 mM phosphocreatine, 10 mM Hepes, 5 U/ml creatine phosphokinase, and 8% dextran (40,000), pH 7.2. The control experimental solution contained 100 mM K aspartate, 20 mM KCl, 3 mM MgATP, 0.5 mM EGTA, 0.114 mM $CaCl_2$ (free [$Ca^{2+}$], ~100 nM), 0.81 mM $MgCl_2$ (free [$Mg^{2+}$], ~1 mM), 10 mM phosphocreatine, 10 mM Hepes, 0.03 mM Fluo-3 potassium salt (TefLabs, Austin, TX), and 5 U/ml creatine phosphokinase, pH 7.2. The free [$Ca^{2+}$] and [$Mg^{2+}$] at given total $Ca^{2+}$, $Mg^{2+}$, ATP, and EGTA concentrations were calculated using WinMAXC 1.80 (Stanford University, Stanford, CA).

### Patch-clamp measurements

Whole-cell patch-clamp recordings of transmembrane currents were performed using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Micropipettes made from borosilicate glass (Sutter Instrument Co., Novato, CA, 1–3 MΩ resistance) were filled with a solution that contained 120 mM cesium aspartate, 20 mM CsCl, 3 mM $Na_2ATP$, 3.5 mM $MgCl_2$, 5 mM Hepes, 50 $\mu$M Fluo-3, pH 7.3. The external solution contained 140 mM NaCl, 5.4 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM Hepes, 5.6 mM glucose, pH 7.3. Voltage pulses were applied from a holding potential of −50 mV. Current signals were sampled at 5 kHz and filtered at 2 kHz. To inhibit the voltage-dependent $Na^+$ current the external solutions contained 20 $\mu$M tetrodotoxin.

### Confocal $Ca^{2+}$ imaging

$Ca^{2+}$ sparks and caffeine-induced $Ca^{2+}$ transients were recorded with a Bio-Rad laser scanning confocal system (MRC 1024ES, Bio-Rad Laboratories, Hercules, CA) equipped with an Olympus 60× 1.4 N.A. objective (Lukyanenko and Györke 1999; Lukyanenko et al., 2000). Fluo-3 was excited by light at 488 nm (25-mW argon laser, intensity attenuated to 0.3%), and fluorescence was measured at wavelengths of >515 nm. An analog recording of fluorescence intensity during each scan was digitized into 768 pixels, giving a nominal pixel dimension of 0.33 $\mu$m. Line scan images were acquired at a rate of 166 Hz (6.0 ms per line scan) and stored in *tiff* format. The amplitude ($F/F_{o,}$ where $F$ is recorded fluorescence intensity and $F_o$ is background fluorescence) and frequency of sparks were determined from the line scan images by using a computer algorithm similar to that described previously (Song et al., 1997; Cheng et al., 1999; Lukyanenko et al., 2000). The frequency and amplitude of events were corrected for detectability and amplitude distortions introduced by instrument noise as described elsewhere (Song et al., 1997; Lukyanenko et al., 2000). Data were expressed as means ± SE. Comparisons were performed by using the paired $t$-test, and differences were considered significant when $p < 0.05$.

## RESULTS

### Modification of SR $Ca^{2+}$ content by selective modulation of the SR $Ca^{2+}$ATPase

We first manipulated the SR calcium content by altering the function of the SR $Ca^{2+}$ ATPase. To selectively enhance SR $Ca^{2+}$ accumulation, we used a monoclonal antibody raised against phospholamban (APL; anti-phospholamban, mouse monoclonal $IgG_1$, clone A1, Upstate, Lake Placid, NY). This antibody has been shown to enhance the $Ca^{2+}$ transport efficiency of the SR $Ca^{2+}$ ATPase by reducing the inhibition by dephosphorylated phospholamban of the pump (Morris et al., 1991; Sham et al., 1991; Mayer et al., 1996). The cells were permeabilized with saponin in internal solutions containing 0.5 mM EGTA and 30 $\mu$M Fluo-3 (free $[Ca^{2+}]$ = 100 nM). Under these conditions they exhibit $Ca^{2+}$ sparks with properties similar to those observed in intact myocytes (Lukyanenko and Györke, 1999). Fig. 1 *A* shows representative line scan images of a cell under control conditions and at various times after addition of 5 $\mu$g/ml APL. Fig. 1 *B* documents the changes in frequency and amplitude of events along with values of basal fluorescence in the same experiment. The rate of occurrence of sparks under control conditions was ~4 events/s/100 $\mu$m. After addition of the antibody, the frequency of events gradually increased to ~7 events/s/100 $\mu$m and remained at this level for the duration of the experiment (10 min). The basal fluorescence was relatively constant, suggesting that laser illumination did not cause significant dye bleaching or photo-damage to the cell in the course of the experiment. The increase in the frequency of events was accompanied by only a small (~6%, not significant) elevation in the average amplitude of $Ca^{2+}$ sparks, although the magnitude of the brightest sparks in each of the consecutive images increased significantly (33 ± 7%, $p < 0.01$, $n = 5$), consistent with an increase in the SR $Ca^{2+}$ content due to APL. This divergence is understandable considering that the amplitude distributions of $Ca^{2+}$ sparks are dominated by small out-of-focus events, the amplitude of which is more a function of proximity of the event to the line scan than the strengths of the source (Izu et al., 1998; Cheng et al., 1999). Although the overall increase in spark brightness by the antibody was small, it potentially could alter our ability to detect sparks against the background noise and influence the quantification of spark frequency. Therefore, to reveal the true changes in the frequency of events we corrected our data for alterations in detectability. Based on data pulled from g experiments, Fig. 1 *C* illustrates the frequencies of sparks before correction (*dark red bars*) and after correction (*red bars*) for missed events. APL increased the frequency by ~60% (62 ± 11%, $p < 0.001$, $n = 9$) and tended to increase the amplitude of events by 6.1% ($p > 0.05$). After correction for missed events, the APL-induced increase in spark frequency remained highly significant (59 ± 11%, $p < 0.001$, $n = 9$).

To verify that APL indeed enhanced the SR $Ca^{2+}$ accumulation, we assessed the SR $Ca^{2+}$content with caffeine applications. The magnitude of the caffeine-induced $Ca^{2+}$ transients increased by ~30% in the presence of APL (Fig. 1 *D*). Similar results were obtained in four other experiments (31 ± 5.5% increase, $p < 0.05$, $n = 5$). Thus, increases in spark frequency induced by APL were indeed accompanied by increases in SR $Ca^{2+}$ load.

To selectively reduce SR $Ca^{2+}$ accumulation, we used the SR $Ca^{2+}$ ATPase inhibitor thapsigargin (Lytton et al., 1991). Because the effects of luminal $Ca^{2+}$ on RyR gating are more prominent at higher levels of $[Ca^{2+}]_{SR}$ than at lower levels of $[Ca^{2+}]_{SR}$ (e.g., Lukyanenko et al., 1996), we optimized the conditions for revealing the potential effects of reduced load on spark frequency by elevating the initial SR $Ca^{2+}$ load. This was achieved by increasing the bathing calcium concentration from ~100 nM to ~150 nM (Lukyanenko and Györke, 1999). In the experiment illustrated in Fig. 2, application of 0.1 $\mu$M thapsigargin caused a gradual decrease in frequency of sparks. At this dose, thapsigargin has been shown to cause a partial depletion of the SR (Song et al., 1997) Again, the effects on frequency occurred without a significant change in the average amplitude of sparks (−10 ± 4%, $p > 0.05$, $n = 5$), although the amplitude of the brightest events in each image decreased considerably (−17 ± 3%, $p < 0.01$, $n = 5$). On average, thapsigargin caused an ~60% decrease (56 ± 9%, $p < 0.01$, $n = 5$) in the frequency of events but only an ~10% decrease (not significant) in their amplitude (Fig. 2 *C*). After correction for missed events the decrease in frequency of events was still highly significant (51 ± 11%, $p < 0.01$). Thapsigargin at 0.1 $\mu$M reduced the amplitude of the $Ca^{2+}$ caffeine-induced $Ca^{2+}$ transients by 35% (Fig. 2 *D*). Taken together these experiments indicate that the $Ca^{2+}$ content of the SR modulates spark frequency.

### Modification of SR $Ca^{2+}$ content by RyR inhibition

As illustrated in Fig. 3, 0.1 mM tetracaine, a RyR channel inhibitor, caused only a transient decrease in the frequency and amplitude of $Ca^{2+}$ sparks. On removal of tetracaine, their frequency and magnitude transiently increased above the control levels. Specifically, within 1 min after application of the drug, the frequency had decreased by ~80% (84 ± 6%, $p < 0.01$, $n = 5$); after 3–5 min, the frequency had returned to the control level (Fig. 3 *C*, *red bars*). On washout, the frequency increased by ~90% (90 ± 17%, $p < 0.01$, $n = 5$) within the first minute and returned to the control level within 2–3 min. These effects on $Ca^{2+}$ spark frequency occurred without significant changes in the mean amplitude of the events (Fig. 3 *C*, *gray bars*).

Exposure of the cells to 0.1 mM tetracaine (for 5 min) caused an ~40% (38 ± 5%, $p < 0.01$, $n = 5$) increase in the peak amplitude of caffeine-induced $Ca^{2+}$ transients (Fig. 3

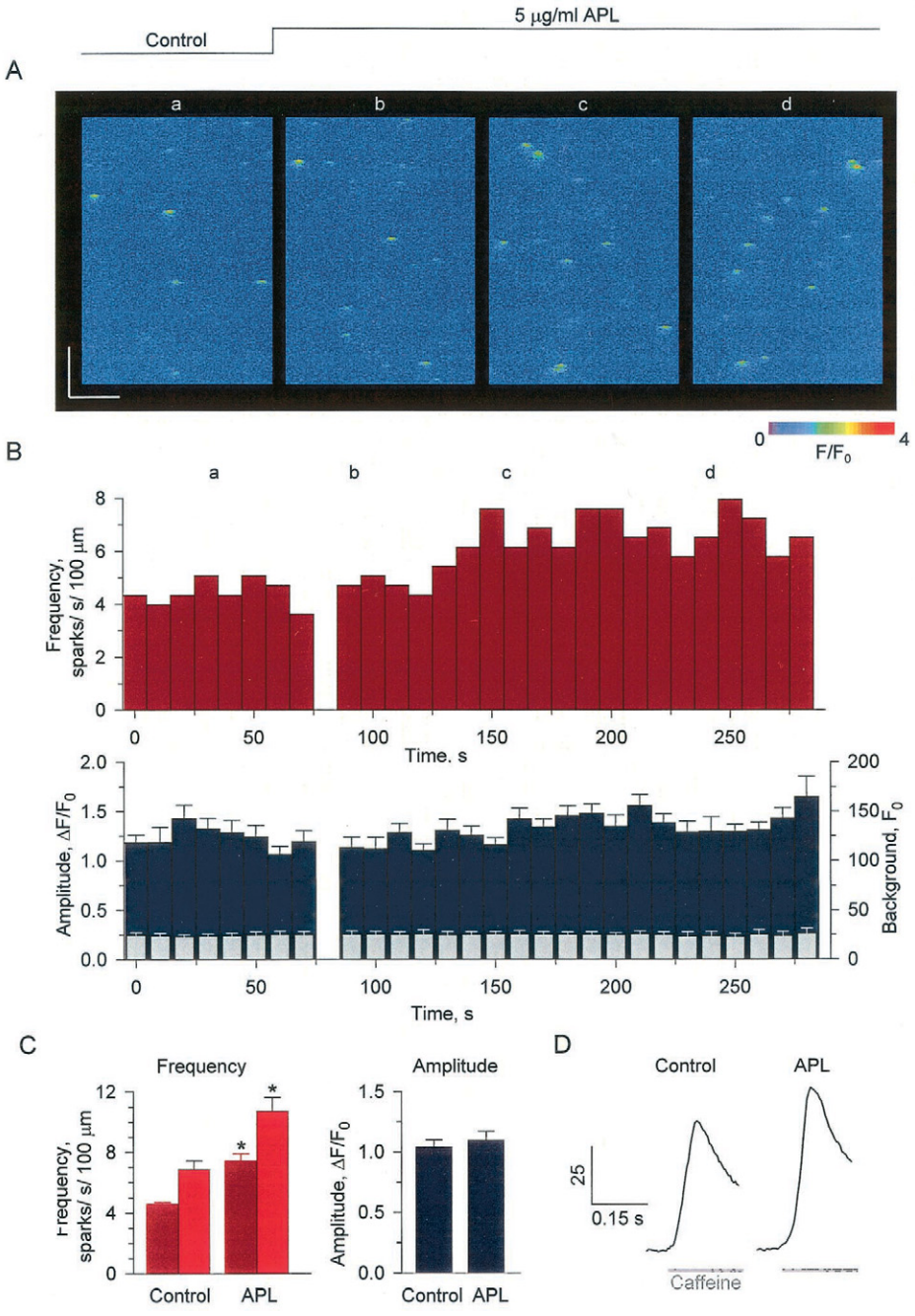


FIGURE 1 The effects of monoclonal antibody raised against phospholamban (APL) on $Ca^{2+}$ sparks and SR $Ca^{2+}$ load. (*A*) Representative line scan fluorescence images under control conditions (*a*) and at different times (*b–d*) after exposure of the cell to 5 μg/ml APL (the experimental protocol is presented schematically at the top of the panel). Calibration bars: horizontal, 0.4 s; vertical, 20 μm. (*B*) $Ca^{2+}$ spark frequency (*dark red*) and amplitude (*blue*) and $F_0$ (*gray*) from corresponding images as a function of time before and after the addition of APL into the bathing solution in the same experiment. Stages *a-d* correspond to images represented in *A*. (*C*) Averaged spark frequency before (*dark red*) and after (*red*) correction for missing events, and amplitude (*blue*) in control solution and 10 min after application of APL; $*p < 0.01$. (*D*) Representative $Ca^{2+}$ transients induced by application of 20 mM caffeine to the whole bath under control conditions and in the presence of APL (10 min).

*D*). These results are consistent with the idea that the transient changes in sparking activity are caused by alterations in the SR $Ca^{2+}$ content, which compensate for the primary changes in the availability of the RyR channels upon addition or removal of tetracaine. We propose the following scenario. Tetracaine inhibits $Ca^{2+}$ efflux through RyRs, resulting in an enhanced accumulation of $Ca^{2+}$ in the SR. The elevated luminal $Ca^{2+}$ concentration increases the probability of RyR activation and $Ca^{2+}$ efflux through the RyR channels, until the enhanced efflux at the new load

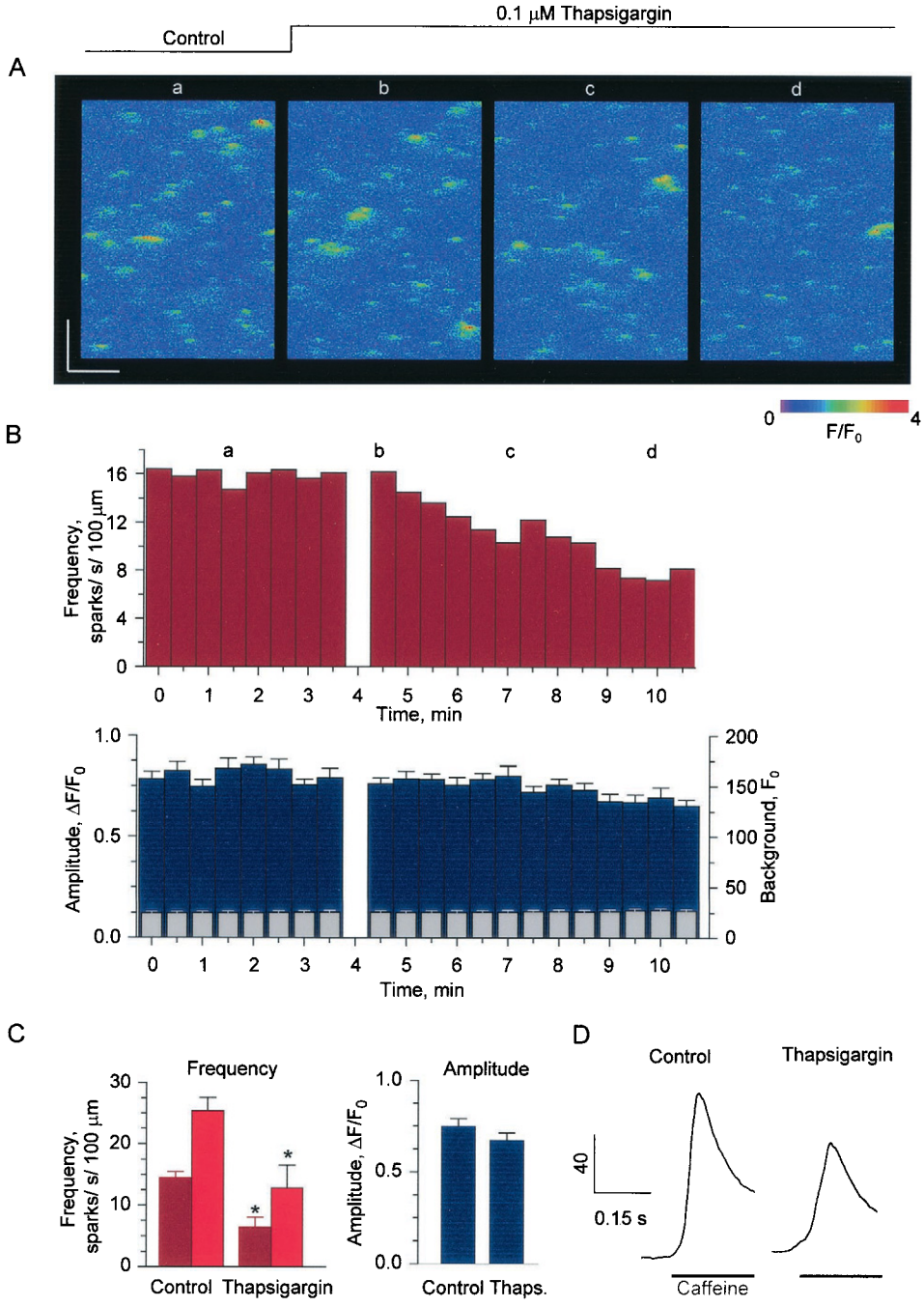


FIGURE 2 The effects of thapsigargin on $Ca^{2+}$ sparks and SR $Ca^{2+}$ load. (*A*) Representative line scan fluorescence images under control conditions (*a*) and at different times (*b–d*) after exposure of the cell to 0.1 μM thapsigargin (the experimental protocol is presented schematically at the top of the panel). Calibration bars: horizontal, 0.4 s; vertical, 15 μm. (*B*) $Ca^{2+}$ spark frequency (*dark red*) and amplitude (*blue*) and $F_0$ (*gray*) from corresponding images as a function of time before and after the addition of thapsigargin into the bathing solution in the same experiment. Stages *a–d* correspond to images represented in *A*. (*C*) Averaged spark frequency before (*dark red*) and after (*red*) correction for missing events, and amplitude (*blue*) in control solution and in 5 min after application of thapsigargin; $*p < 0.01$. (*D*) Representative $Ca^{2+}$ transients induced by application of 20 mM caffeine to the whole bath under control conditions and in the presence of thapsigargin (5 min).

balances the uptake. The transient overshoot of spark frequency upon removal of tetracaine results from the withdrawal of tetracaine inhibition when the SR $Ca^{2+}$ level is still elevated.

To test whether other RyR channel inhibitors can cause similar temporary effects on $Ca^{2+}$ sparks, we explored changes in sparking activity upon exposure of the cells to $Mg^{2+}$. Similar to the results observed with tetracaine, ele-

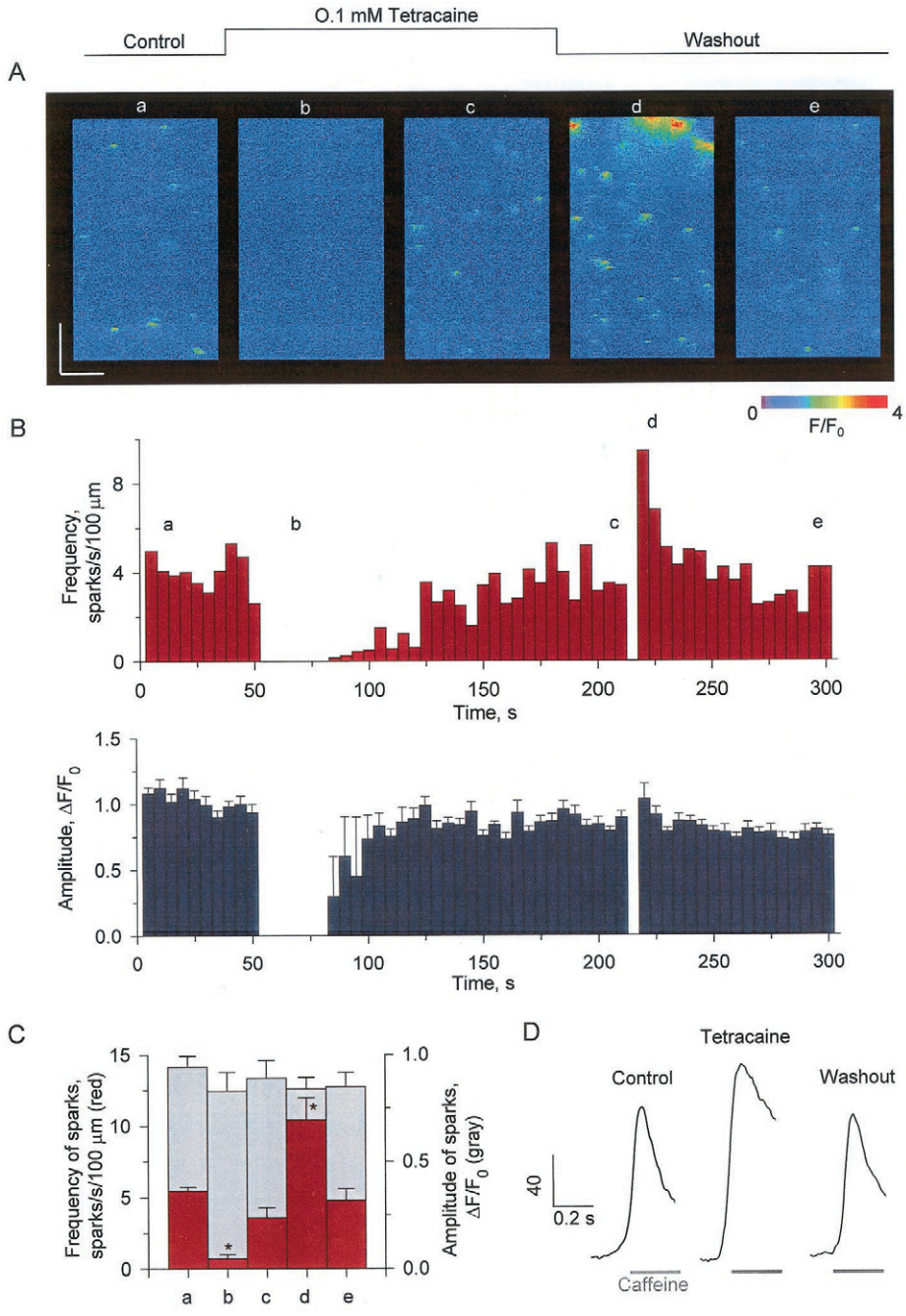


FIGURE 3 The effects of tetracaine on $Ca^{2+}$ sparks and SR $Ca^{2+}$ load. (*A*) Representative line scan fluorescence images of $Ca^{2+}$ release events under control conditions (*a*), and at different times (*b–e*) after exposure of the cell to 0.1 mM tetracaine (the experimental protocol is presented schematically at the top of the panel). Calibration bars: horizontal, 0.4 s; vertical, 20 $\mu$m. (*B*) $Ca^{2+}$ spark frequency (*dark red*) and amplitude (*blue*) as a function of time before and after the addition of tetracaine into the bathing solution in the same experiment. Stages *a–e* correspond to images represented in *A*. (*C*) Averaged spark frequency (*blue*) and amplitude (*gray*) for different stages before (*a*) and after (*b–e*) application of 0.1 mM tetracaine; $*p < 0.01$. (*D*) Representative $Ca^{2+}$ transients induced by application of 20 mM caffeine to the whole bath under control conditions and in the presence of tetracaine (5 min).

vation of $[Mg^{2+}]$ in the bathing solution (from 1 to 5 mM) resulted in a transient inhibition in sparking activity; reverting to normal $[Mg^{2+}]$ resulted in a transient overshoot in frequency of events (Fig. 4). Again, these effects on dynamics of $Ca^{2+}$ sparks were paralleled by an increase (when applying elevated $[Mg^{2+}]$) and a decrease (on washout of elevated $[Mg^{2+}]$) of the SR $Ca^{2+}$ content (Fig. 4 *D*). Thus, the ability of different RyR inhibitors to exert similar effects further supports the hypothesis that these effects are caused by reductions of the $Ca^{2+}$ leak through RyRs and subse-

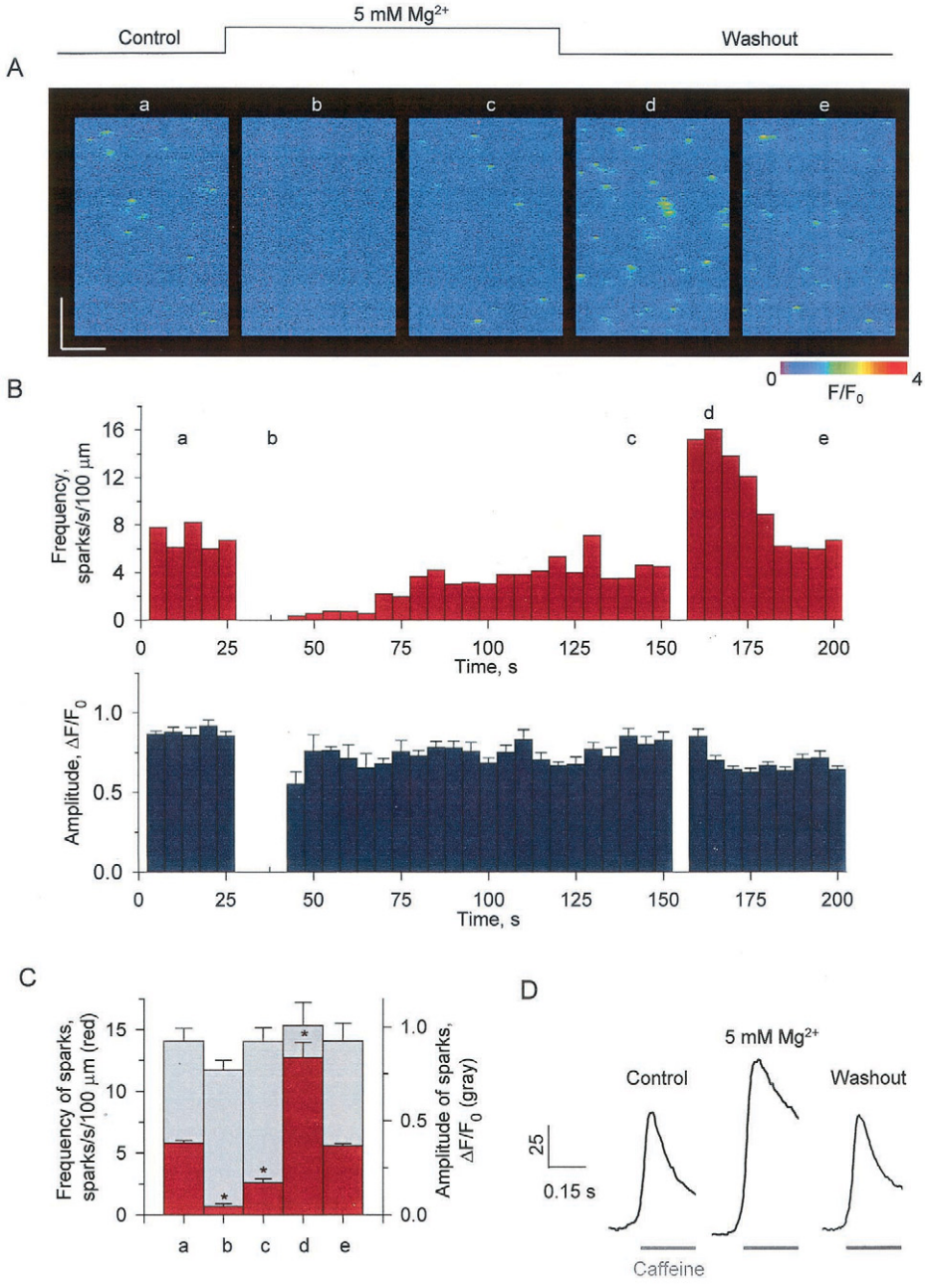


FIGURE 4 The effects of magnesium on $Ca^{2+}$ sparks and SR $Ca^{2+}$ load. (*A*) Representative line scan fluorescence images under control conditions (*a*) and at different times (*b–e*) after exposure of the cell to 5 mM $Mg^{2+}$ (the experimental protocol is presented schematically at the top of the panel). Calibration bars: horizontal, 0.4 s; vertical, 20 $\mu$m. (*B*) $Ca^{2+}$ spark frequency (*dark red*) and amplitude (*blue*) as a function of time before and after the addition of $Mg^{2+}$ into the bathing solution in the same experiment. Stages *a–e* correspond to images represented in *A*. (*C*) Averaged spark frequency (*blue*) and amplitude (*gray*) for different stages before (*a*) and after (*b–e*) application of 5 mM $Mg^{2+}$; $*p < 0.01$. (*D*) Representative $Ca^{2+}$ transients induced by application of 20 mM caffeine to the whole bath under control conditions and in the presence of $Mg^{2+}$ (5 min).

quent compensatory enhancement of RyR activity induced by increased luminal $Ca^{2+}$.

To test directly whether a real cause-and-effect relationship exists between changes in the SR $Ca^{2+}$ content and increased probability of release site activation, we used thapsigargin to prevent the accumulation of extra $Ca^{2+}$ upon exposure of the cell to tetracaine. If the recovery of sparking activity in the presence of tetracaine is due to increased $[Ca^{2+}]_{SR}$, inhibition of $Ca^{2+}$ uptake by the $Ca^{2+}$ ATPase should prevent such potentiation of $Ca^{2+}$ sparks. This hypothesis is supported by the data in Fig. 5. When the cells were pretreated with 0.1 $\mu$M thapsigargin, tetracaine caused a steady inhibition of $Ca^{2+}$ sparks with very little sign of recovery within the time frame of the experiments.

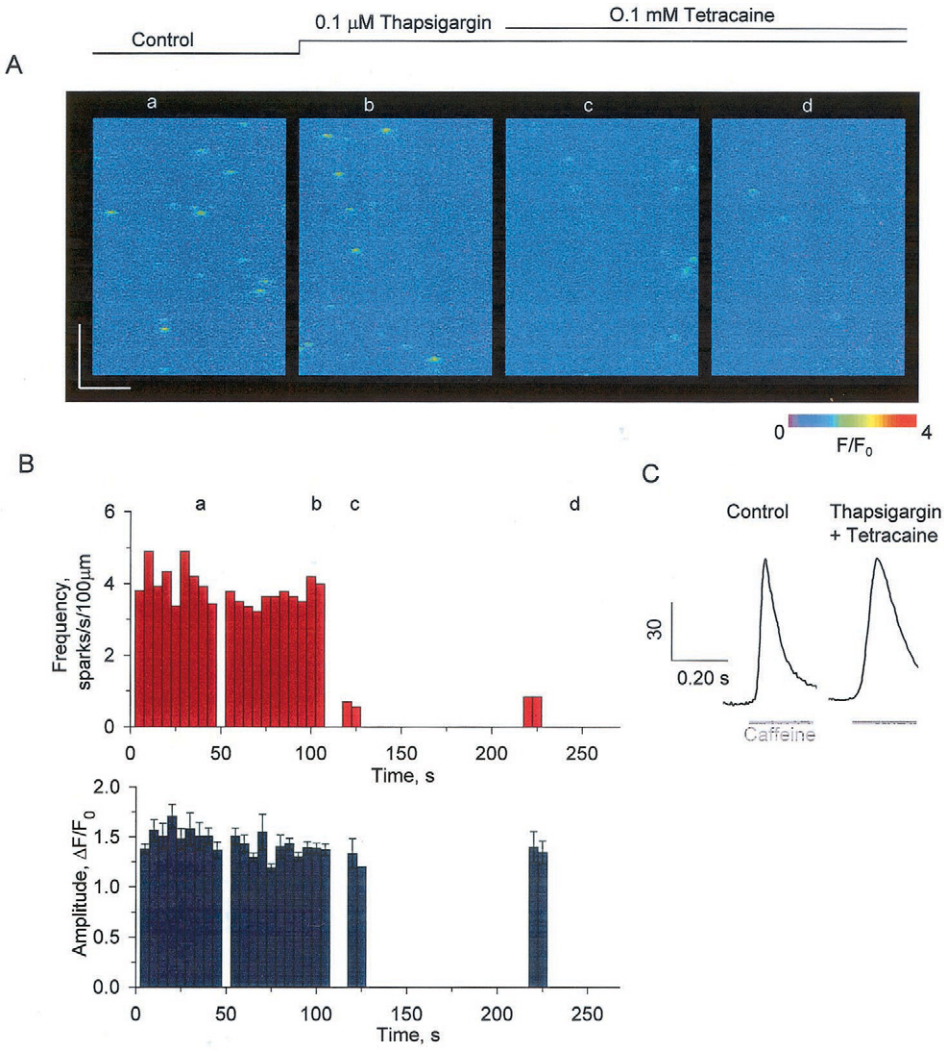


FIGURE 5 Effect of tetracaine in the presence of 0.1 $\mu$M thapsigargin on $Ca^{2+}$ sparks and SR $Ca^{2+}$ load. (*A*) Representative line scan fluorescence images under control conditions (*a*), in the presence of 0.1 $\mu$M thapsigargin (*b*), and at different times (*c* and *d*) after exposure of the cell to 0.1 mM tetracaine (the experimental protocol is presented schematically at the top of the panel). Calibration bars: horizontal, 0.4 s; vertical, 15 $\mu$m. (*B*) $Ca^{2+}$ spark frequency (*dark red*) and amplitude (*blue*) as a function of time before and after the addition of thapsigargin and tetracaine into the bathing solution in the same experiment. Stages *a–d* correspond to images represented in *A*. (*C*) Representative $Ca^{2+}$ transients induced by application of 20 mM caffeine to the whole bath under control conditions and in the presence of tetracaine (5 min).

Similar results were obtained in 15 different myocytes. Importantly, in these experiments the combined application of tetracaine and thapsigargin caused no significant change in the SR $Ca^{2+}$ load (Fig. 5 *C*).

## Modification of SR $Ca^{2+}$ content by RyR activation

Application of 0.2 mM caffeine resulted in a transient increase in the frequency of sparks (Fig. 6). Within 5 s after addition of caffeine, the frequency had increased by ~65% (65 ± 9%, $p < 0.01$, $n = 10$). When measured 1.5–2 min after addition of the drug, the frequency had returned to the control level. Upon washout, the frequency of events had decreased by ~60% (61 ± 12%, $p < 0.01$, $n = 9$) during the first 10–15 s; after 1.5–2 min it was not different from the control (*dark red bars*). These changes were not accompanied by significant alterations in the mean amplitude of events (*gray bars*). The decay of sparking activity in the presence of caffeine was associated with an ~45% reduction of SR $Ca^{2+}$ content (46 ± 11%, $p < 0.05$, $n = 5$), whereas the recovery of spark frequency after washout of caffeine was accompanied by a return of the SR load to the control level (Fig. 6 *D*). Therefore, caffeine induced a behavior that was exactly opposite to that seen with tetracaine and $Mg^{2+}$. These results support the hypothesis that enhancement of $Ca^{2+}$ release through RyRs leads to a net loss of

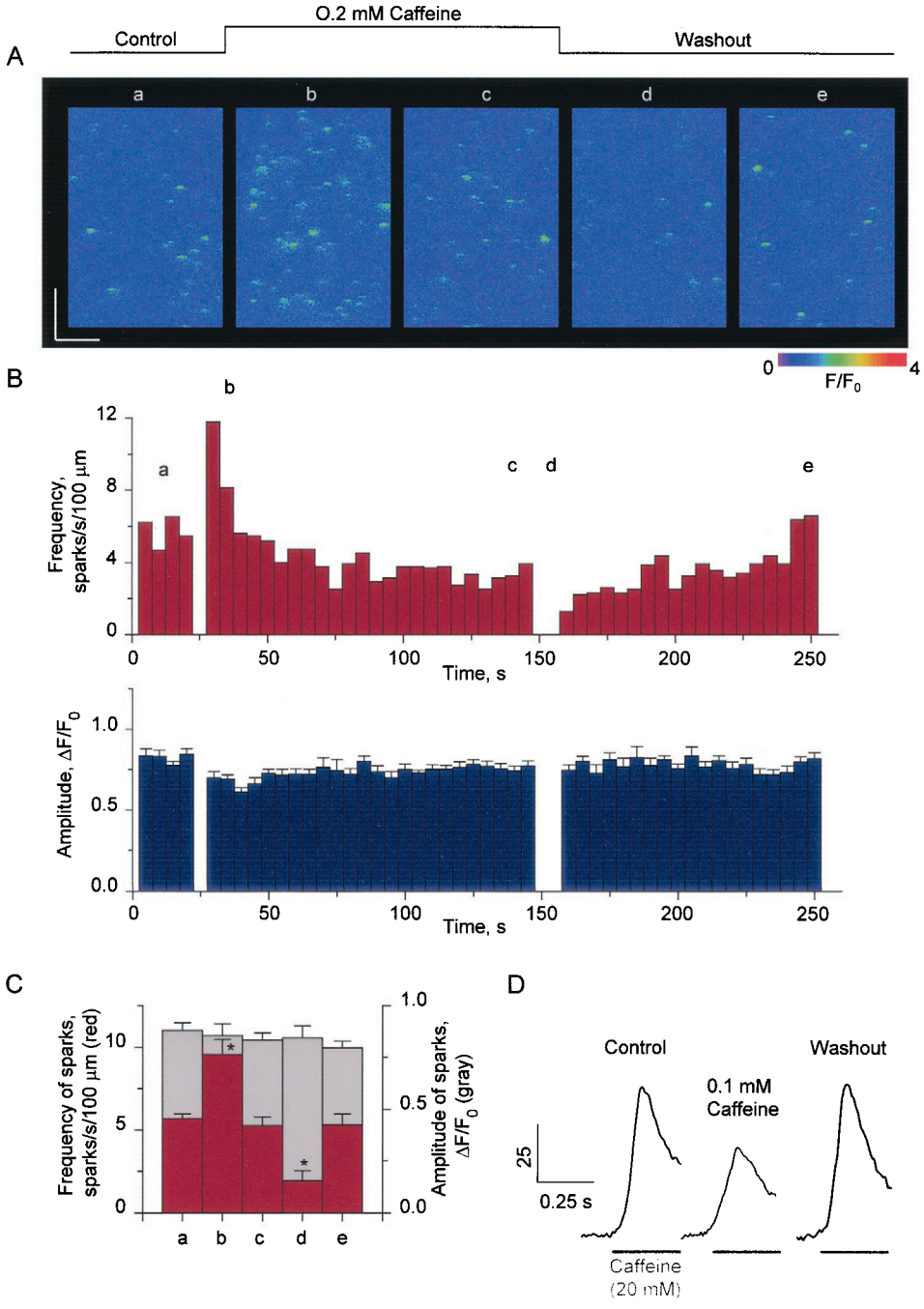


FIGURE 6 The effects of caffeine on $Ca^{2+}$ sparks and SR $Ca^{2+}$ load. (*A*) Representative line scan fluorescence images under control conditions (*a*) and at different times (*b–e*) after exposure of the cell to 0.2 mM caffeine (the experimental protocol is presented schematically at the top of the panel). Calibration bars: horizontal, 0.4 s; vertical, 20 $\mu$m. (*B*) $Ca^{2+}$ spark frequency (*dark red*) and amplitude (*blue*) as a function of time before and after the addition of caffeine into the bathing solution in the same experiment. Stages *a–e* correspond to images represented in *A*. (*C*) Averaged spark frequency (*blue*) and amplitude (*gray*) for different stages before (*a*) and after (*b–e*) application of 0.2 mM caffeine; $*p < 0.01$. (*D*) Representative $Ca^{2+}$ transients induced by application of 20 mM caffeine to the whole bath under control conditions and in the presence of 0.2 mM caffeine (5 min).

the SR $Ca^{2+}$ content, which, in turn, decreases RyR activation until the reduced $Ca^{2+}$ efflux at the new steady SR load balances the $Ca^{2+}$ uptake. The transient undershoot of spark frequency on removal of caffeine could be attributed to the combination of decreased SR $Ca^{2+}$ content and lower RyR activity.

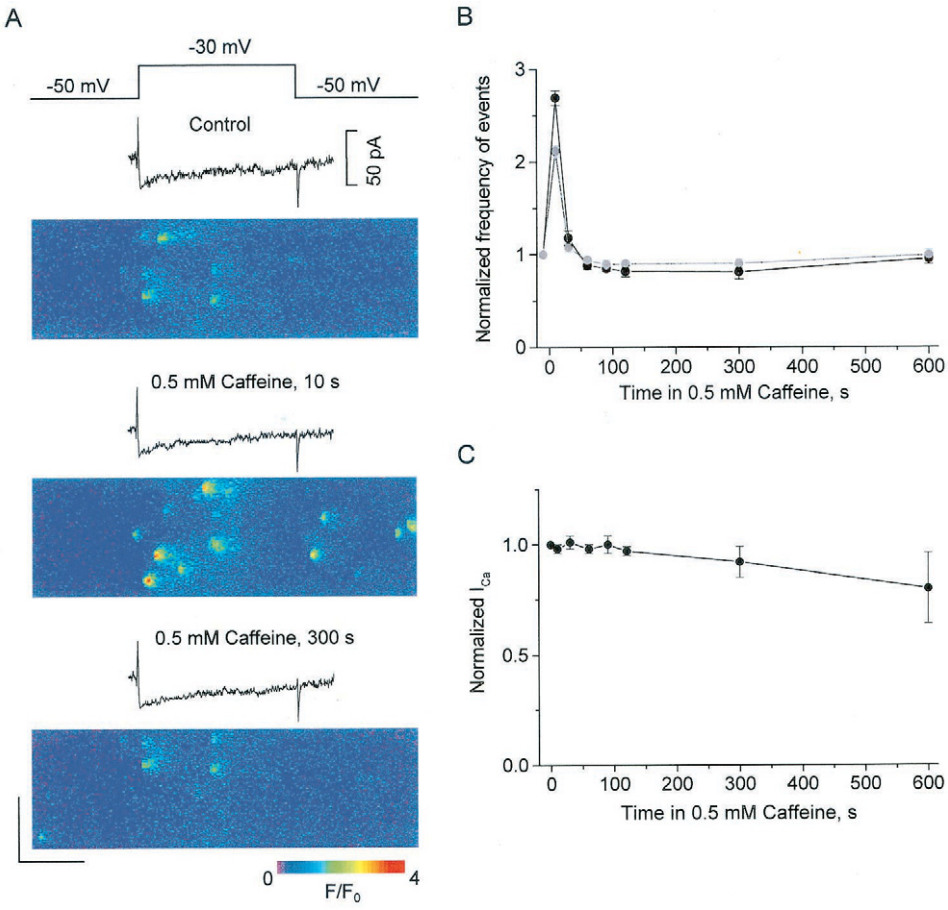


FIGURE 7 Effects of caffeine on inward $Ca^{2+}$ currents and $Ca^{2+}$ sparks in patch-clamped cardiac myocytes. (*A*) Representative traces of $I_{Ca}$ and images of $Ca^{2+}$ sparks elicited by depolarizing steps from −50 to −30 mM before (*top*) and 10 and 300 s after application of 0.5 mM caffeine to the bathing solution (*middle* and *bottom panels*, respectively). The external solution contained 0.5 mM [$Ca^{2+}$]. (*B*) Relative changes in frequency of depolarization-induced (●, acquired during the depolarizing steps) and spontaneous events (○) following application of caffeine (at 0 time). (*C*) Relative changes in peak calcium current upon exposure to caffeine. Data are presented as means ± SEM of three experiments in different cells.

### Role of luminal $Ca^{2+}$ in intact cardiac myocytes

To investigate whether luminal $Ca^{2+}$ modulates $Ca^{2+}$ sparks in intact cardiac myocytes, we carried out $Ca^{2+}$ imaging along with $I_{Ca}$ recordings in patch-clamped cardiac myocytes. $Ca^{2+}$ sparks were induced by depolarizing pulses to −30 mV from a holding potential of −50 mV (Fig. 7 *A*). Under control conditions, the depolarizing step induced $I_{Ca}$ and occasional sparks. Application of 0.5 mM caffeine dramatically increased the number of release events during the depolarizing pulse; however, the increased probability of spark activation was only transient. One minute after caffeine application, the number of events elicited by depolarization returned to levels close to the control. These dynamic changes in $Ca^{2+}$ release were not accompanied by changes in the magnitude or time course of $I_{Ca}$. As can be seen in Fig. 7, *A* and *B*, the frequencies of both spontaneous and evoked $Ca^{2+}$ sparks increased only transiently upon addition of caffeine. These results indicate that transient potentiation of sparks by caffeine occurs in both skinned and intact cells, and changes in the SR $Ca^{2+}$ load modulate the ability of $I_{Ca}$ to trigger $Ca^{2+}$ sparks. The initial enhancement of spark activity is balanced by a loss of SR $Ca^{2+}$ content, with a subsequent reduction of the sensitivity of the release sites to the $Ca^{2+}$ trigger.

## DISCUSSION

### A novel dynamic control mechanism regulating SR $Ca^{2+}$ content and release

In the present study we have shown that spontaneous $Ca^{2+}$ sparks in resting myocytes represent a substantial $Ca^{2+}$ efflux, which plays an important role in determining the SR $Ca^{2+}$ content. The frequency of sparks (and the amount of leak) increased at elevated SR $Ca^{2+}$ loads and decreased at reduced loads, thereby stabilizing SR $Ca^{2+}$ content in the face of alterations of uptake. Because of a rapid and continuous cycling of $Ca^{2+}$ between the SR and cytosolic

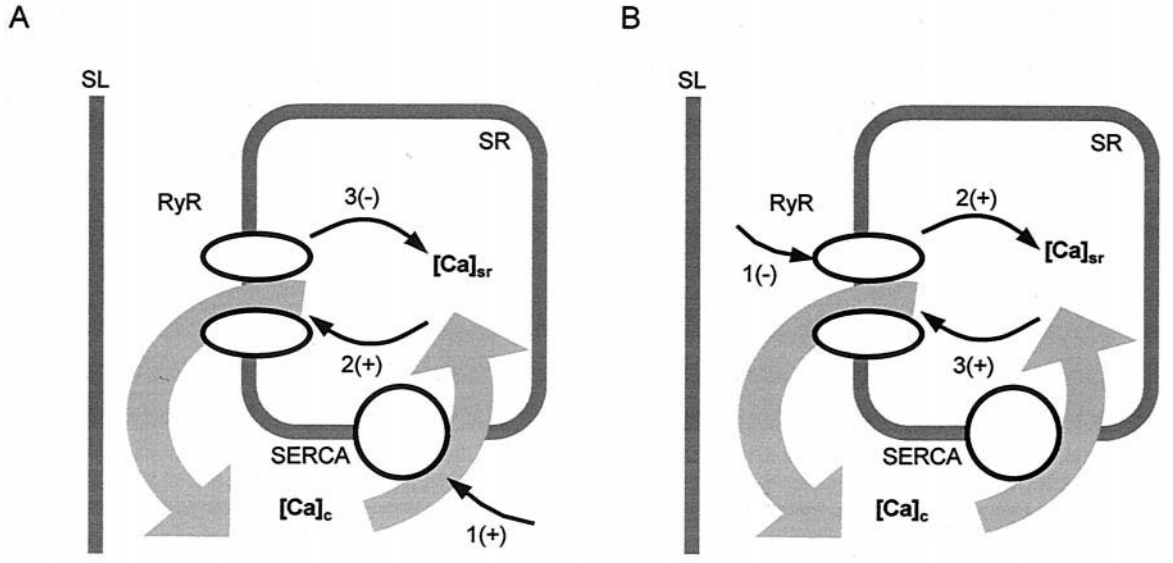


FIGURE 8 Simplified schematic of dynamic control of $Ca^{2+}$ in the SR luminal and cytosolic compartments of the cell during pharmacological interventions either enhancing the activity of the SERCAII pump (*A*) or reducing the activity of the RyR (*B*). In *A*, stimulation of the SERCA pump increases $[Ca]_{SR}$ (1) which, in turn, stimulates more $Ca^{2+}$ efflux through RyR channels (2). This enhanced $Ca^{2+}$ efflux reduces $[Ca]_{SR}$ (3). In *B*, inhibition of RyRs (1) decreases $Ca^{2+}$ efflux through RyR channels. This reduced $Ca^{2+}$ efflux increases $[Ca]_{SR}$ (2) which, in turn, stimulates the opening of more RyRs (3).

compartments, all modulatory influences selectively affecting the activity of RyRs were promptly compensated by alterations in load and resultant luminal-$Ca^{2+}$-dependent changes in release site activity. We interpret these results in terms of a new dynamic control mechanism that regulates the size and functional state of the SR $Ca^{2+}$ pool. A key element of this mechanism is a luminal $Ca^{2+}$ sensor that modulates the functional activity of the RyR by tuning its responsiveness to cytosolic $Ca^{2+}$.

Fig. 8 schematically illustrates the hypothesized control actions of a SR luminal $Ca^{2+}$ sensor to the pharmacological treatments described in the Results. When the SERCA pump is potentiated by a phospholamban antibody, the $Ca^{2+}$ level within the SR increases (Fig. 8 *A*). A luminal $Ca^{2+}$ sensor detects this elevation of $[Ca]_{SR}$ and increases the open probability of the RyR. This, in turn, increases $Ca^{2+}$ release, which reduces $[Ca]_{SR}$ and counterbalances pump potentiation by the antibody. Inhibition of the SERCA pump by thapsigargin leads to the opposite sequence of events and reduction of the open probability of the RyR. When the RyR channel blocker tetracaine is applied, $Ca^{2+}$ release through the RyR channels is decreased, which increases $[Ca]_{SR}$ (Fig. 8 *B*). A luminal $Ca^{2+}$ sensor detects this increase and increases the sensitivity of RyRs to cytosolic $Ca^{2+}$, thereby enhancing the probability of their activation. This, in turn, increases release, which counterbalances the inhibition by tetracaine. The sequence of events is the opposite when the RyR agonist caffeine was applied. Thus, our results support the hypothesis that there is an intrinsic auto-regulation at the level of RyRs in which the functional activity of the release channels and the loading state of the SR are coupled to stabilize the SR $Ca^{2+}$ content and its release. The relationship of these findings to previous work in the literature, as well as their implications regarding certain aspects of SR $Ca^{2+}$ release regulation, are discussed below.

## Modulation of RyR activity by luminal $Ca^{2+}$

Our results extend those of previous studies that focused on the effects of SR $Ca^{2+}$ load on activity of SR $Ca^{2+}$ release sites (Cheng et al., 1996; Lukyanenko et al., 1996; Satoh et al., 1997). Previously, the SR $Ca^{2+}$ load was altered by exposing the cells to elevated extracellular $[Ca^{2+}]$ (Cheng et al., 1996; Lukyanenko et al., 1996) or reducing the rate of electrical stimulation (Satoh et al., 1997). Sparks were counted manually in those experiments. Altering $[Ca^{2+}]_o$ changed $[Ca^{2+}]$ in both cytosolic and SR compartments, making it difficult to directly relate the changes in spark frequency to changes in $[Ca^{2+}]_{SR}$. In addition, those results could have been influenced by altered detectability of sparks with different amplitudes against the background noise (Song et al., 1997). In the present study, we manipulated the SR $Ca^{2+}$ content by selectively stimulating or inhibiting the efficiency of the $Ca^{2+}$ pump (by an anti-phospholamban antibody or thapsigargin) at constant (buffered) cytosolic $[Ca^{2+}]$. We used a computer algorithm for objective detection and measurement of sparks and corrected the counts for missed events. Our results provide compelling evidence that the probability of release site activation is controlled by the SR $Ca^{2+}$ content.

The observations of this study are also consistent with the results of lipid bilayer experiments, which showed that elevation of luminal $Ca^{2+}$ leads to an increase in open probability of the RyR (Sitsapesan and Williams, 1994; Lukyanenko et al., 1996; Györke and Györke, 1998; Ching et al., 2000). A growing body of evidence suggests that the regulation of RyRs by luminal $Ca^{2+}$ involves luminal $Ca^{2+}$-sensing sites (Györke and Györke, 1998; Ching et al., 2000); however, $Ca^{2+}$ passing through the channel pore might also affect RyR activity by interacting with the cytosolic $Ca^{2+}$ regulatory sites (Xu and Meissner, 1998). The precise location of the luminal $Ca^{2+}$ sensor is not known; nevertheless, it must be situated on either the RyR or a closely associated regulatory protein such as calsequestrin, triadin, or junctin.

## The role of leak in setting SR $Ca^{2+}$ content

At basal conditions, we observed a sparking frequency of $f = 2–6$ events/s/100 $\mu$m. These values are within the range of frequencies of spontaneous $Ca^{2+}$ sparks reported in intact rat ventricular myocytes in the presence of 1–2 mM extracellular [$Ca^{2+}$] (1.5–5 events/s/100 $\mu$m; Song et al., 1997; Satoh et al., 1997; Wier et al., 1997; Lukyanenko and Györke, 1999). Assuming a line scan acquisition volume ($V$) of 100 $\mu m^3$ (Lukyanenko et al., 2000), a single spark current ($i_{spark}$) of 10–20 pA (Izu et al., 2001), and a spark current duration ($t_{spark}$) of 5 ms (Lukyanenko et al., 1998), we can estimate the diastolic leak flux as $q_{leak} = 5–30$ $\mu$M/s ($q_{leak} = [i_{spark} \times t_{spark} \times f]/[z \times F \times V]$, where $z$ = valence of calcium ion and $F$ = Faraday). These flux rates are consistent with the SR leak flux derived by Balke et al. (1994) from [$Ca^{2+}$] transients in rat ventricular myocytes (18 $\mu$M/s). At the same time they are significantly higher than the unidirectional SR $Ca^{2+}$ leak estimated from measuring the time-dependent loss of SR $Ca^{2+}$ in rabbit and rat ventricular myocytes exposed to $Na^+$-free solutions and thapsigargin ($\sim$0.5 $\mu$M/s; Bassani and Bers, 1995). We ascribe this apparent discrepancy to the luminal $Ca^{2+}$ dependency of the leak. Whereas our measurements were performed at constant SR $Ca^{2+}$ load, where the leak is relatively high, the measurements of Bassani and Bers were performed as the SR was being gradually depleted and the $Ca^{2+}$-dependent leak reduced.

Our leak values, estimated from steady-state $Ca^{2+}$ sparks, should be sufficiently large to effectively counterbalance $Ca^{2+}$ uptake by the SR $Ca^{2+}$ pump at resting cytosolic [$Ca^{2+}$] (Ginsburg et al., 1998). Furthermore, consistent with previous findings (Györke et al., 1997; Díaz et al., 1997; Lukyanenko et al., 2000), the SR $Ca^{2+}$ content could be increased dramatically by employing tetracaine or $Mg^{2+}$ to reduce the number of available release sites. These results suggest that, during diastole, the SR $Ca^{2+}$ load is set not only by the ability of the $Ca^{2+}$ pump to move $Ca^{2+}$ but also by the dissipative loss of $Ca^{2+}$ through RyRs. In other words, the leak predominates over the thermodynamic limit for the $Ca^{2+}$-ATPase. This conclusion is consistent with the results of a recent theoretical analysis of the role of the leak in determining the SR $Ca^{2+}$ content (Snyder et al., 2000).

## RyR modulation has only transient effects on $Ca^{2+}$ sparks

We demonstrated that maintained potentiation of RyRs by RyR agonists such as caffeine results in only brief increases in frequencies of both spontaneous and depolarization-induced $Ca^{2+}$ sparks. This is the first time the transient nature of RyR-agonist-induced effects has been shown at the local release level. Consistent with our previous observations in intact resting myocytes (Györke et al., 1997), the RyR inhibitors tetracaine and $Mg^{2+}$ transiently suppressed the frequency of $Ca^{2+}$ sparks. In the present study, all these effects could be attributed to alterations of SR $Ca^{2+}$ load compensating the primary changes in availability of the RyR channels. In general, our results suggest that, because of load-sensitive $Ca^{2+}$ efflux and the limited nature of the SR $Ca^{2+}$ stores, any maintained and selective modulation of RyRs would have only temporary effects on SR $Ca^{2+}$ release.

It is interesting to consider why certain substances such as cADPR, nitric oxide, and ruthenium red, which are thought to specifically interact with RyRs, can have maintained modulatory effects on $Ca^{2+}$ sparks (Lukyanenko and Györke, 1999; Cui et al., 1999; Meszaros and Lukyanenko, 1998; Ziolo et al., 1999; Lukyanenko et al., 2000). One possible explanation is that some of these agents may affect $Ca^{2+}$ transport mechanisms other than RyRs. For example, preliminary data obtained in our laboratory suggest that cADPR effects on $Ca^{2+}$ sparks are mediated by potentiation of the SR $Ca^{2+}$ pump with subsequent luminal $Ca^{2+}$-dependent increase in sparking activity (Lukyanenko et al., 2001). In some instances, the impact of the primary effects on RyRs may exceed the capacity of the compensatory mechanisms to restore sparking activity. For example, high blocking concentrations of ruthenium red induce a steady inhibition of $Ca^{2+}$ sparks. The number of RyRs that remains available for activation is simply not sufficient to recover the initial sparking rate (Lukyanenko et al., 2000). Finally, with certain agents, the ability of the release mechanism to respond transiently to pharmacological disturbances may be compromised if the agent affects the sensitivity of the RyR to luminal $Ca^{2+}$. Indeed, disabling the luminal $Ca^{2+}$ sensor should interrupt the feedback interactions between $[Ca]_{SR}$ and RyR activity that are responsible for recovering the basal sparking rate. This might explain why withdrawal of the RyR potentiator ATP from the cytosol leads to sustained inhibition of $Ca^{2+}$ sparks (Smith and O'Neill, 2001). In this regard, we have reported that in reconstitution studies the presence of ATP is required for luminal $Ca^{2+}$ to affect RyRs (Lukyanenko et al., 1996).

### Implications for understanding how alterations of $Ca^{2+}$ transport affect E-C coupling

Our study extends the previous work of Eisner and his colleagues in periodically stimulated ventricular myocytes (Eisner et al., 1998, 2000). They demonstrated that maintained modulation of RyRs produces only transient effects on global systolic $Ca^{2+}$ and attributed these results to a form of auto-regulation in which the balance of systolic $Ca^{2+}$ fluxes generated by the various competing $Ca^{2+}$ transport mechanisms determines the SR $Ca^{2+}$ content and release. In essence, they ascribed the transient nature of the effects to changes in the amount of releasable $Ca^{2+}$ retained in the SR upon disturbing the ratio of $Ca^{2+}$ fluxes in and out of the cell during systole. Our results reveal two new factors: 1) a luminal $Ca^{2+}$ sensor that tunes the functional activity of the RyR (i.e., its responsiveness to the cytosolic $Ca^{2+}$ trigger) in accordance with changes in the SR $Ca^{2+}$ load and provides an ultimate set point for $[Ca^{2+}]$ in the SR lumen (via the $[Ca^{2+}]$-dependent leak) and 2) high rates of diastolic SR $Ca^{2+}$ recycling that allow for more rapid compensatory changes in the SR $Ca^{2+}$ content than provided by changes in systolic fluxes only. These factors endow the SR with a capacity to control calcium cycling in a more robust manner than previously thought.

The importance of these new $Ca^{2+}$ control mechanisms also becomes evident when comparing the consequences of changes in RyRs with those of alterations in their coupling to dihydropyridine receptor (DHPR) channels. Impaired DHPR-RyR coupling has been suggested to underlie reduced $Ca^{2+}$ release in certain models of heart failure (Gomez et al., 1997). Analyses based on consideration of only systolic $Ca^{2+}$ fluxes predict that reduced recruitment of release sites associated with impaired DHPR-RyR coupling should produce temporary effects on systolic $Ca^{2+}$, similar to the effects observed with the RyR blocker tetracaine (Eisner et al., 1998). The results here demonstrate that high diastolic SR $Ca^{2+}$ cycling can explain how the consequences of reduced systolic $Ca^{2+}$ release (due to impaired DHPR-RyR coupling) could be different from those of influences reducing the activity of RyRs during both systole and diastole (i.e., tetracaine).

Overall, it appears the intrinsic auto-regulation at the level of RyRs shown in this study and the auto-regulation based on balance of systolic fluxes (Eisner et al., 1998) should work synergistically to account for the dynamics of $Ca^{2+}$ in both cytosolic and luminal compartments of beating cardiac cells.

### Implications for understanding the stability of CICR

In the CICR process, where $Ca^{2+}$ is both the trigger signal and output signal, the danger of spontaneous oscillations is always present. Theoretical studies suggest that to maintain a stable CICR, the cells may have to self-regulate their release gain by adjusting the activity of the RyRs to the SR $Ca^{2+}$ load (Stern, 1992). We have shown that the functional state of RyRs is under continuous control of luminal $[Ca^{2+}]$. At the same time, the activity of RyRs determines the SR $Ca^{2+}$ content, thereby forming a closed control loop (Fig. 8). Any change in RyR activation is compensated by a change in SR $Ca^{2+}$ load. On the other hand, all alterations in SR $Ca^{2+}$ content are countered by a leak pathway. Therefore, this dynamic control mechanism would be advantageous for stabilizing CICR in the face of perturbations in $Ca^{2+}$ uptake and release. This dynamic control mechanism comes with a price. Indeed, high leak fluxes clearly are costly and inefficient from an energy consumption standpoint. Further, this mechanism can operate effectively only within a certain range of alterations of load. When the increases in SR Ca content fall outside the normal, "correctable" range (i.e. Ca overload), enhanced channel activity mediated by elevated luminal Ca would tend to exacerbate the problem of instability resulting in more regenerative Ca waves (Lukyanenko et al., 1999).

## CONCLUSIONS

We have found that openings of RyRs manifested as spontaneous $Ca^{2+}$ sparks mediate a substantial diastolic release that is controlled by the levels of $[Ca^{2+}]$ inside the SR. This luminal $Ca^{2+}$-sensitive release appears to be an essential part of a dynamic regulatory system that allows cells to auto-regulate the size and functional state of their SR $Ca^{2+}$ pool. These results have a general relevance to understanding the regulation of intracellular $Ca^{2+}$ release and contractility in cardiac muscle.

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