SERCA Pump Optimizes Ca^{2+} Release by a Mechanism Independent of Store Filling in Smooth Muscle Cells

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ABSTRACT Thapsigargin-sensitive sarco/endoplasmic reticulum Ca^{2+} pumps (SERCAs) are involved in maintaining and replenishing agonist-sensitive internal stores. Although it has been assumed that release channels act independently of SERCA pumps, there are data suggesting the opposite. Our aim was to study the relationship between SERCA pumps and the release channels in smooth muscle cells. To this end, we have rapidly blocked SERCA pumps with thapsigargin, to avoid depletion of the internal Ca^{2+} stores, and induced Ca^{2+} release with either caffeine, to open ryanodine receptors, or acetylcholine, to open inositol 1,4,5-trisphosphate receptors. Blocking SERCA pumps produced smaller and slower agonist-induced $[Ca^{2+}]_i$ responses. We determined the Ca^{2+} level of the internal stores both indirectly, measuring the frequency of spontaneous transient outward currents, and directly, using Mag-Fura-2, and demonstrated that the inhibition of SERCA pumps did not produce a reduction of the sarco/endoplasmic reticulum Ca^{2+} levels to explain the decrease in the agonist-induced Ca^{2+} responses. It appears that SERCA pumps are involved in sustaining agonist-induced Ca^{2+} release by a mechanism that involves the modulation of Ca^{2+} availability in the lumen of the internal stores.

INTRODUCTION

A wide variety of cellular events are controlled by a transient rise in cytoplasmic $[Ca^{2+}]$ ($[Ca^{2+}]_i$). In general, the opening of Ca^{2+} permeable ion channels produces an elevation of the $[Ca^{2+}]_i$, whereas active Ca^{2+} transporters are involved in returning $[Ca^{2+}]_i$ to resting levels. The two main sources of Ca^{2+} are the external milieu and the internal Ca^{2+} stores. The sarco/endoplasmic reticulum (SR) constitutes the principal Ca^{2+} store participating in the initial rapid increase in $[Ca^{2+}]_i$ by supplying Ca^{2+} via two different types of Ca^{2+} release channels, i.e., the inositol 1,4,5-trisphosphate receptors (IP_3Rs) and the ryanodine receptors (RyRs). The SR also participates in the subsequent decrease in $[Ca^{2+}]_i$ by removing Ca^{2+} from the cytoplasm and refilling the internal Ca^{2+} stores by the action of SR Ca^{2+} pumps (SERCAs). It appears that the amplitude of the $[Ca^{2+}]_i$ transient reflects the Ca^{2+} level present in the internal Ca^{2+} stores (Rueda et al., 2002a; Flynn et al., 2001). However, the differences in the Ca^{2+} buffer capacity between the SR and the cytoplasm (Guerrero et al., 1994a), the different cell volume for the SR (~1.5–7.5% in smooth muscle, Somlyo, 1985), and the presence of feedback mechanisms in the Ca^{2+} release channels (Iino, 1990; Chu et al., 1993) originate a nonlinear relationship between the SR luminal Ca^{2+} level and the amplitude of the Ca^{2+} transient (Trafford et al., 2002).

The roles played by Ca^{2+} release and Ca^{2+} reuptake in a Ca^{2+} transient have been studied in several types of cells. However, in the majority of those studies, it has been assumed that Ca^{2+} release operates independently of Ca^{2+} reuptake, as long as the latter has replenished internal Ca^{2+} stores. Therefore, there is limited information on how these two processes are integrated to shape the Ca^{2+} signal. The evidence suggests that SERCA pumps could indirectly modulate the Ca^{2+} release process by determining the loading state of the internal Ca^{2+} stores (Santana et al., 1997; Lukyanenko et al., 1999; ZhuGe et al., 1999). This conclusion has been based on studies showing that the activity of both RyRs (Sitsapesan and Williams, 1994) and IP_3Rs (Missiaen et al., 1992) are regulated by luminal SR Ca^{2+} concentration ($[Ca^{2+}]_{SR}$), which depends on the SERCA pump activity (Steenbergen and Fay, 1996). However, the effect of SERCA pumps on $[Ca^{2+}]_{SR}$ has been inferred more than directly measured, hence it is not clear whether the effect of SERCA pumps on the Ca^{2+} release is exclusively due to changes in the $[Ca^{2+}]_{SR}$.

Studies in smooth muscle, HeLa, and HEK293 cells, where $[Ca^{2+}]_{SR}$ was directly measured, have shown that blocking SERCA pumps increases the agonist-induced depletion of the internal stores (Steenbergen and Fay, 1996; Arnaudeau et al., 2001; Yu and Hinkle, 2000; Shmigol et al., 2001). These data imply that SERCA pumps are active during the Ca^{2+} release process and also argue for SERCA pumps being able to limit depletion of the internal Ca^{2+} stores, although the turnover rate of SERCA pumps is slower than that of the release channels. Actually, the simultaneous recording of $[Ca^{2+}]_i$ and $[Ca^{2+}]_{SR}$ in rat uterine smooth muscle cells revealed that blocking SERCA pumps produces a smaller agonist-induced Ca^{2+} response, albeit there is a larger agonist-induced depletion of the internal stores (Shmigol et al., 2001). Moreover, blocking SERCA pumps in pancreatic acinar cells decreases the rate of Ca^{2+} release and also causes the agonist-induced Ca^{2+} gradient to vanish; these are effects that cannot be explained by depletion of the internal Ca^{2+} stores (Lee et al., 1997). Collectively, these data suggest a connection between SERCA pumps and the

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Ca^{2+} release process. Actually, it has been shown that SERCA pumps undergo conformational changes immediately after inducing Ca^{2+} release, suggesting that the Ca^{2+} reuptake process may play some role in the Ca^{2+} release event (Mészáros and Ikemoto, 1985). Additionally, studies in SR vesicles from skeletal muscle have suggested that Ca^{2+} release and reuptake are not acting independently, but are in fact coordinated (Saiki and Ikemoto, 1999; Ikemoto and Yamamoto, 2000).

In this work, we have assessed the role of SERCA pumps on Ca^{2+} release in single myocytes from guinea pig urinary bladder, by rapidly and completely inhibiting SERCA pumps and a few seconds later stimulating Ca^{2+} release from the SR with caffeine, an agonist of RyRs (Rousseau and Meissner, 1989). This short period of time was chosen to avoid depletion of internal Ca^{2+} stores as a consequence of an uncompensated Ca^{2+} leak from the SR. We show that the rapid inhibition of SERCA pumps decreases both the amplitude and the rate of rise of caffeine-induced $[Ca^{2+}]_i$ increase. These effects do not seem to be due to a partial reduction of Ca^{2+} stores as was demonstrated both indirectly using the frequency of spontaneous transient outward currents (STOCs) and directly measuring the SR Ca^{2+} levels with Mag-Fura-2. These findings suggest that active SERCA pumps are required to obtain an optimal Ca^{2+} release in smooth muscle cells. Additionally, we have observed that SERCA pump inhibition affects in a similar manner the IP_3-induced Ca^{2+} release, suggesting that the mechanism used by SERCA pumps to modulate Ca^{2+} release does not depend on the nature of the Ca^{2+} release channel. In conclusion, our results suggest that SERCA pumps play a significant role, not only in maintaining and refilling the internal Ca^{2+} stores, but also in modulating the Ca^{2+} release process by a mechanism that does not seem to be associated with the luminal Ca^{2+} level before inducing Ca^{2+} release. A preliminary account of this work has been presented (Gómez et al., 2001).

MATERIALS AND METHODS

Cell isolation

Single smooth muscle cells were enzymatically dissociated from the urinary bladder of guinea pig as previously described (Muñoz et al., 1998; Rueda et al., 2002a). Briefly, adult male albino guinea pigs (400–490 g) were killed by cervical dislocation followed immediately by exsanguination. The urinary bladder was quickly removed and placed in dissociation solution. The mucosa and submucosa layers were removed mechanically and 200 mg of detrusor muscle was cut into fragments of ~14 mg. These fragments were incubated in 2.5 ml of dissociation solution with previously activated proteases (2.6 mg collagenase/ml and 1.9 mg papain/ml) in a shaking water bath at 25°C for 90 min. This was followed by two washes of 15 min each with dissociation solution containing 50 Kunitz units of DNase I (Sigma Chemicals, St Louis, MO). Proteases were activated by preincubating papain (Sigma Chemicals) in 2.25 ml of dissociation solution with 200 μM each of EDTA and DTT for 20 min and collagenase (Type 1A, Sigma Chemicals) in 0.25 ml of dissociation solution containing 2 mM $CaCl_2$ (final $[Ca^{2+}]$ was 200 μM). Both enzyme solutions were combined before adding the tissue fragments. Relaxed single smooth muscle cells were obtained by gentle trituration of the digested tissue with a plastic pipette.

$[Ca^{2+}]_i$ measurements in single smooth muscle cells

Cells were loaded with Fura-2 by incubating the cell suspension in dissociation solution with 1–2 μM Fura-2/AM (Molecular Probes, Eugene, OR) in the dark at room temperature for 1 h. Cells were washed and resuspended in normal saline solution and kept at 4°C for no less than 2 h and used within the same day of isolation. The Fura-2 loaded cell suspension (10–20 μl) was added to a recording chamber containing normal saline solution. This chamber was on the stage of a TMD inverted microscope (Nikon, Japan) coupled to an RF-F3010 microfluorometer for determination of Fura-2 fluorescence (Photon Technology International, South Brunswick, NJ). Fura-2 fluorescence excitation ratio (340/380 nm) was recorded at a frequency of 20 Hz from single cells, as previously reported (Muñoz et al., 1998). Smoothed excitation ratios (with a running average of 11 points) were converted to $[Ca^{2+}]_i$ using the Grynkiewicz equation (Grynkiewicz et al., 1985) after background and viscosity corrections. The background fluorescence at 340 and 380 nm were obtained from a cell-free area of the chamber. K_d for Fura-2 was estimated at 200 nM from an in vitro Ca^{2+} titration curve (Muñoz et al., 1998; Gutiérrez et al., 1999) and the viscosity correction was based on the procedure reported by Poenie (1990). Under our recording conditions, the viscosity factor was 25%. Caffeine, acetylcholine, or thapsigargin were locally applied with a borosilicate micropipette (4–5 MΩ resistance, TW100F-4, WPI, Sarasota, FL) placed close to the cell. These chemicals were ejected with pneumatic pressure (4 psi) exerted with a PV830 PicoPump (WPI, Sarasota, FL) for the time period indicated in the figures. Calibration of the PicoPump was carried out with a pipette solution containing Fura-2 free acid to verify the reliability of the PicoPump system. In all cases, the reported $[Ca^{2+}]_i$ responses represent the average peak increase over corresponding basal levels. The rate of rise of $[Ca^{2+}]_i$ responses (dCa/dt) were calculated with the first derivatives of these $[Ca^{2+}]_i$ records obtained with Savitzky-Golay's algorithm (Savitzky and Golay, 1964) using a window of five points. Puffing 0.1% of dimethylsulfoxide (DMSO) decreased neither the caffeine-induced $[Ca^{2+}]_i$ transient (470.8 ± 132.1 nM, $n = 8$ vs. 420.4 ± 87.9 nM, $n = 4$; no puff versus DMSO puff) nor the rate of $[Ca^{2+}]_i$ rise (545.8 ± 159.1 nM/s, $n = 8$ vs. 500.8 ± 119.5 nM/s, $n = 4$; no puff versus DMSO puff).

Determination of SR Ca^{2+} level in single smooth muscle cells

To assess the luminal SR Ca^{2+} level, we used a low-affinity calcium indicator, Mag-Fura-2 ($K_d = 49$ μM, Sugiyama and Goldman, 1995). Cells were incubated with 5 μM Mag-Fura-2/AM (Molecular Probes) in the dark at room temperature for 1 h, washed with indicator-free dissociation solution, resuspended in normal saline solution and kept at 4°C for no less than 2 h before being used. The Mag-Fura-2 loaded cell suspension (10–20 μl) was added to the recording chamber containing normal saline solution. In intact cells the probe was trapped both inside intracellular organelles and the cytoplasm. To remove the cytoplasmic fraction of the dye, we dialyzed cells with normal, dye-free pipette solution using the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981). Cells were held at 0 mV and dialyzed for 15 min before initiating Mag-Fura-2 fluorescence recordings. Because this time was not enough to remove all the indicator from the cytoplasm, $[Ca^{2+}]_i$ was clamped by dialyzing cells with pipette solution containing the combination of 1 mM BAPTA and 0.75 mM $CaCl_2$. This solution displayed a $[Ca^{2+}]_i$ of 128.2 ± 22.7 nM ($n = 5$) measured directly with Fura-2. Mag-Fura-2 fluorescence excitation ratio (340/380 nm) was determined at 20 Hz frequency using the same system that was used for Fura-2 fluorescence measurements. The excitation ratios were smoothed, after background subtraction, with the algorithm of Savitzky Golay

(Savitzky and Golay, 1964) using a window of 41 points followed by a running average of 30 points. In all cases, the recorded fluorescence ratio (F340/F380) was normalized to the basal level.

Patch-clamp recording

Membrane currents were recorded using the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981). Ion channel currents were simultaneously recorded with Fura-2 fluorescence ratios and a transistor-transistor logic pulse synchronized both recordings. Ion channel currents were low pass-filtered at 200 Hz and recorded with an Axopatch 1D amplifier (Axon Instruments, Foster City, CA) coupled to a Digidata 1200 (Axon Instruments) running Axotape (Axon Instruments) at a sampling rate of 1 kHz. Gigaseals were obtained with TW100F-4 borosilicate micropipettes (WPI, Sarasota, FL) of 4–5 MΩ made with a PP-83 vertical puller (Narishige, Japan). Holding potential was 0 mV. Transient outward currents were considered as STOCs when they exceeded a threshold of 20 pA, as detected with a peak detection routine of Origin software (Origin 4); the same software was used to determine the amplitude and frequency of STOCs in a time window of 30 s.

Solutions, chemicals, and data analysis

The dissociation solution contained (in mM): 55 NaCl, 6 KCl, 5 MgCl2, 10 glucose, 80 NaOH, 80 glutamic acid, and 10 HEPES, pH 7.4 (NaOH). The normal saline solution contained (in mM): 137 NaCl, 5 KCl, 4 $NaHCO_3$, 2 $CaCl_2$, 2 $MgCl_2$, 0.42 KH_2PO_4, 10 glucose, and 10 HEPES, pH 7.4 (NaOH). The pipette solution contained (in mM): 80 K-Glutamate, 5 NaCl, 40 KCl, 2 $MgCl_2$, 2 Na_2ATP, 0.1 GTP, 20 HEPES, pH 7.2 (KOH). This pipette solution contained either 10 μM Fura-2 acid or the combination of 1 mM BAPTA and 0.75 mM $CaCl_2$ for determinations of $[Ca^{2+}]_i$ and luminal Ca^{2+} level, respectively. Mag-Fura-2/AM, Fura-2/AM and Fura-2 acid were from Molecular Probes (Eugene, OR). Thapsigargin was purchased from RBI. The rest of the chemicals were from SIGMA (St Louis, Mo). Fura-2/AM, Mag-Fura-2/AM and thapsigargin were dissolved as to give 0.1% final concentration of DMSO. Caffeine was freshly dissolved in normal saline solution. Acetylcholine was dissolved in normal saline solution and kept at 4°C until used. Fura-2 acid was dissolved in water. All experiments were carried out at room temperature. Data shown are the means ± SEM, where n represents the number of cells. Differences were considered significant for $P < 0.05$ using either Student's t-test or ANOVA test (Student-Newman-Keuls).

RESULTS

Characterization of caffeine-induced rise in $[Ca^{2+}]_i$ in single smooth muscle cells from guinea pig urinary bladder

Caffeine releases Ca^{2+} from internal stores by activation of RyRs in smooth muscle cells isolated from guinea pig urinary bladder (Ganitkevich and Isenberg, 1992). Under our experimental conditions, the application of caffeine (20 mM in the puffer pipette) for a period of 5 s produced a transient increase in $[Ca^{2+}]_i$ (Fig. 1, *dashed line*) of 629.0 ± 76.0 nM ($n = 12$) from a basal $[Ca^{2+}]_i$ of 64.2 ± 4.9 nM ($n = 12$). A second application of caffeine, 30 s after the first pulse, produced only a small rise in $[Ca^{2+}]_i$ (103.7 ± 18.9 nM, $n = 12$). A third application of caffeine (Fig. 1), after a 5-min period at resting conditions, produced only a partial recovery of the Ca^{2+} response (393.0 ± 56.0 nM, $n = 10$). The

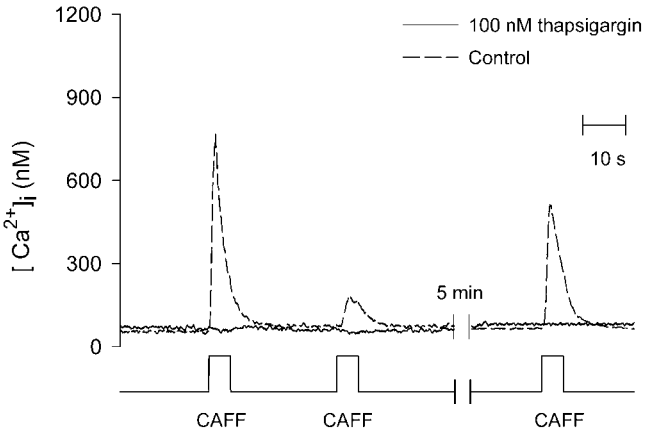

FIGURE 1 Effect of prolonged incubation in thapsigargin on $[Ca^{2+}]_i$ responses to the pulsatile application of caffeine. Single myocytes from the urinary bladder of guinea pig were incubated with (*solid line*) or without (*dashed line*) 100 nM thapsigargin (0.1% DMSO final concentration) for at least 30 min. Thapsigargin did not affect the basal $[Ca^{2+}]_i$, although abolished all the 20 mM caffeine (CAFF)-induced $[Ca^{2+}]_i$ responses (*solid line*). The previous application of caffeine to control cells produced a marked reduction of caffeine-induced $[Ca^{2+}]_i$ response (*dashed line*, second pulse). Some cells displayed a partial recovery to a third application of caffeine 5 min later. The timescale applies to both records, the bottom trace indicates the times when caffeine was applied to the cells.

transient nature of caffeine-induced rise in $[Ca^{2+}]_i$ suggests that the source of Ca^{2+} is an internal store. Accordingly, cells incubated with 100 nM thapsigargin for a prolonged period of time (up to 30 min) did not respond to the application of caffeine (Fig.1, *solid line*), presumably because the internal Ca^{2+} stores had been depleted. These data also imply that caffeine does not induce a Ca^{2+} influx as it is the case in myocytes from toad stomach (Guerrero et al., 1994b; Zou et al., 1999). Additionally, the incubation in thapsigargin did not modify the basal $[Ca^{2+}]_i$ suggesting the absence of a significant capacitative Ca^{2+} influx in this type of cells, which is in agreement with previous reports (Yoshikawa et al., 1996; Rueda et al., 2002b). The participation of RyRs in caffeine-induced $[Ca^{2+}]_i$ rise is further supported by ryanodine-induced use-dependent abolition of caffeine-triggered Ca^{2+} responses (Guerrero-Hernández et al., 2002; Rueda et al., 2002a).

The fact that the caffeine-induced Ca^{2+} response was only partially recovered suggests that internal Ca^{2+} stores did not refill completely under our recording conditions. It has been shown that refilling of internal Ca^{2+} stores depends on Ca^{2+} influx through L-type Ca^{2+} channels in guinea pig urinary bladder myocytes (Wu et al., 2002). Therefore, we studied the recovery of the internal stores in the presence of Bay K 8644 (Bay K), an agonist of L-type Ca^{2+} channels (Brown et al., 1984). In this case, the amplitude of the first caffeine-induced Ca^{2+} response was twofold higher than that obtained in the absence of Bay K (data not shown). Indeed, the recovery of the amplitude of the caffeine-induced Ca^{2+} response after 5 min was higher in the presence of Bay K

(83%) than that observed in control cells (61%). These data imply that augmenting Ca^{2+} influx facilitates refilling of internal stores. The partial recovery of the internal Ca^{2+} stores was further confirmed by the simultaneous recording of $[Ca^{2+}]_i$ and spontaneous transient outward currents. STOCs represent the activation of a cluster of Ca^{2+} and voltage-dependent K^+ channels in response to RyR-mediated Ca^{2+} sparks (Nelson et al., 1995; Bolton and Imaizumi, 1996). It has been shown that the frequency of STOCs in smooth muscle depends on the $[Ca^{2+}]_{SR}$ (ZhuGe et al., 1999). At a holding potential of 0 mV, the urinary bladder myocytes displayed STOCs without any change in the global $[Ca^{2+}]_i$ (Fig. 2 *A*). This is in line with the idea that STOCs are activated by spontaneous and localized Ca^{2+} release events. Nevertheless, the application of caffeine evoked both a transient $[Ca^{2+}]_i$ rise, of similar characteristics to those observed in intact cells (Fig. 1), and a Ca^{2+} sensitive outward current of a higher amplitude than of STOCs (Fig. 2 *A*). This Ca^{2+} release event partially reduced the amount of Ca^{2+} in the internal stores as reflected by the abolition of STOCs (Fig. 2 *A*; ZhuGe et al., 1999). After a period of 5 min without cell stimulation, STOCs have already returned, albeit with a lower frequency (2.2 ± 0.6 STOCs/s, $n = 3$, Fig. 2 *B*) than before the application of caffeine (3.6 ± 0.6 STOCs/s, $n = 10$, Fig. 2 *A*). In line with this, the amplitude of the second caffeine-induced Ca^{2+} response was also smaller (Fig. 2 *B*). Collectively, these data indicate that caffeine releases Ca^{2+} from internal stores by activating RyRs and that these internal stores recover only partially when Ca^{2+} influx is not stimulated in guinea pig urinary bladder myocytes.

Rapid inhibition of SERCA pumps diminishes caffeine-induced Ca^{2+} release

To assess the role played by SERCA pumps in the RyR-mediated Ca^{2+} release, we decided to inhibit SERCA pumps by exposing cells for only 5 s to a high concentration of thapsigargin (10 μM in the puffer pipette). The interaction of thapsigargin with SERCA pumps is rapid, potent, highly specific, and practically irreversible (Lytton et al., 1991; Thastrup et al., 1990; Kirby et al., 1992). This fast inhibition of SERCA pumps should provide a time window where Ca^{2+} release can be activated before Ca^{2+} depletion of internal stores. Fig. 3 shows a $[Ca^{2+}]_i$ response to the application of caffeine in a naive cell (*dashed line*) or a cell exposed to thapsigargin where indicated (*solid line*). The peak amplitude of the control $[Ca^{2+}]_i$ response (*dashed line*) was 653.8 ± 32.6 nM ($n = 72$) from a basal $[Ca^{2+}]_i$ level of 79.7 ± 1.8 nM ($n = 72$). As we have demonstrated previously, internal Ca^{2+} stores recovered only partially under these recording conditions. Accordingly, a second application of caffeine, 5 min after the first pulse, increased $[Ca^{2+}]_i$ by only 398.1 ± 31.9 nM ($n = 65$).

The exposure to thapsigargin, 10 s before the application of caffeine, produced two unexpected changes in the caffeine-induced Ca^{2+} response. 1), The amplitude of the

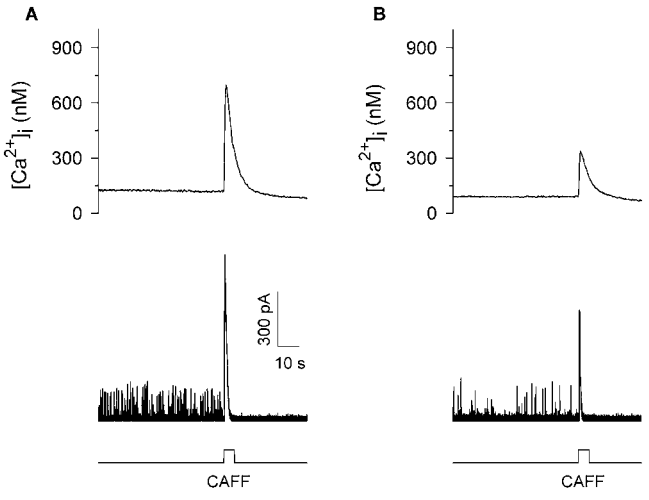

FIGURE 2 Simultaneous recording of $[Ca^{2+}]_i$ and outward currents in response to the application of caffeine. (*A*) Single smooth muscle cells in the whole-cell configuration of patch-clamp technique were exposed to 20 mM caffeine (CAFF) for the time indicated and the $[Ca^{2+}]_i$ and outward currents were recorded and compared in the same cells after a resting period of 5 min (*B*). Neither the frequency of the spontaneous transient outward currents nor the amplitude of the caffeine-induced $[Ca^{2+}]_i$ transients recovered completely, suggesting that internal Ca^{2+} stores replenished only partially under our recording conditions. Timescale applies to all records.

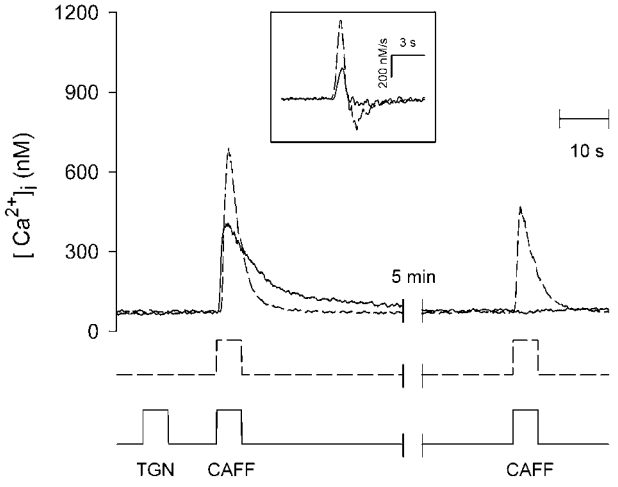

FIGURE 3 Effect of rapidly blocking SERCA pumps with thapsigargin on caffeine-induced $[Ca^{2+}]_i$ transients. It is compared with the 20 mM caffeine (CAFF)-induced $[Ca^{2+}]_i$ response between cells exposed (*solid line*) or not (*dashed line*) to 10 μM thapsigargin (TGN, 0.1% DMSO final concentration) at the time indicated. Note that the exposure to thapsigargin for only 5 s produced a smaller Ca^{2+} transient, a slower rate of recovery of $[Ca^{2+}]_i$, and a lack of response to a second application of caffeine. Timescale applies to all records. Inset shows the rate of rise (dCa/dt) for the first caffeine-induced Ca^{2+} response for cells either exposed (*solid line*) or not (*dashed line*) to thapsigargin. These data indicate a clear reduction in the rate of rise of the caffeine-induced Ca^{2+} response.

Ca^{2+} transient was significantly reduced to 307.9 ± 45.9 nM (Fig. 3, *solid line*, $n = 12$), which represented a 53% reduction in the Ca^{2+} rise. 2), There was also a significant decrease of the maximal rate of rise of caffeine-induced Ca^{2+} response (see *inset* in Fig. 3). The rate of $[Ca^{2+}]_i$ rise was reduced by 64% on the application of thapsigargin (311.7 ± 54.6 nM/s, $n = 12$) with respect to the control rate (860.9 ± 61.5 nM/s, $n = 77$). A second application of caffeine, 5 min after the first pulse, produced no Ca^{2+} response (2.0 ± 3.3 nM, $n = 12$), confirming that SERCA pumps have been inhibited with this approach. Because inhibition of SERCA pumps could deplete internal stores, one possible explanation for the decrease in the caffeine-induced Ca^{2+} response is that internal stores of urinary bladder myocytes have a large Ca^{2+} leak. This leak is necessary to reduce the Ca^{2+} content of the internal stores in a short period of time between SERCA pump inhibition and the application of caffeine. However, the following data suggest that internal Ca^{2+} stores present rather a small Ca^{2+} leak.

The effect of inhibition time of SERCA pumps on the caffeine-induced Ca^{2+} release

Assuming that internal Ca^{2+} stores initiate depletion via a Ca^{2+} leak as soon as SERCA pumps are inhibited, then an exponential decay in the amplitude of caffeine-induced Ca^{2+} transient should be expected (Nazer and van Breemen, 1998). To verify this, we studied the effect of inhibition time of SERCA pumps on caffeine-induced Ca^{2+} release, by applying caffeine at different times to the same cells that had been previously exposed to thapsigargin for the same time period of only 5 s (Fig. 4). Under these recording conditions, basal $[Ca^{2+}]_i$, the peak amplitude of the Ca^{2+} response and the maximal rate of $[Ca^{2+}]_i$ rise were determined. The basal $[Ca^{2+}]_i$ was not modified by the different inhibition times of SERCA pumps (Fig. 4 *A*). Nevertheless, thapsigargin reduced both the amplitude ($\Delta[Ca^{2+}]_i$) and the rate of rise (dCa/dt) of caffeine-induced Ca^{2+} response (Fig. 4, *B* and *C*, respectively). This was true for all the times tested, except when caffeine was applied immediately after the pulse of thapsigargin (0 s). Unexpectedly, the maximal inhibition by thapsigargin on the amplitude (53%) and the rate of rise (64%) of the caffeine-induced Ca^{2+} transient was completely established by 10 s, and stayed at this level even when caffeine was applied 300 s after the exposure to thapsigargin. Importantly, all cells responded to caffeine, except for inhibition times of 60 and 300 s, where two out of 19 and two out of 12 cells showed no response at all, respectively. These data demonstrated a rather rapid onset without an exponential time course of the inhibitory effect on caffeine-induced Ca^{2+} response by blocking SERCA pumps. Thus, it appears that the explanation of the effect of thapsigargin on caffeine-induced Ca^{2+} release requires a more complex mechanism than simply the depletion of internal Ca^{2+} stores.

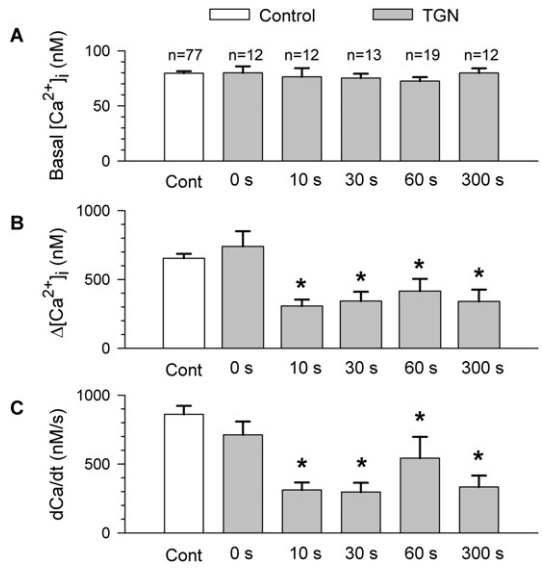

FIGURE 4 Time course of the effect of thapsigargin on basal $[Ca^{2+}]_i$ and caffeine-induced $[Ca^{2+}]_i$ response. A 5-s pulse of 10 μM thapsigargin (TGN) was applied followed by a pulse of 20 mM caffeine at the times indicated and compared with the values obtained in cells not exposed to thapsigargin (Control). The times indicated represent the period between the end of the exposure to thapsigargin and the beginning of the application of caffeine. The ''0 s'' column implies that caffeine was applied immediately after the end of thapsigargin. (*A*) Effect of thapsigargin on the basal $[Ca^{2+}]_i$ before the application of caffeine. (*B*) Displays the peak increase in $[Ca^{2+}]_i$ in response to the application of caffeine. (*C*) The maximal rate of rise of the caffeine-induced $[Ca^{2+}]_i$ response. The number of cells (*n*) studied for each condition is indicated on top of each column. $*P < 0.05$ with respect to the control value (Newman Keuls, ANOVA unifactorial).

SERCA pump inhibition does not affect the frequency of STOCs

As we have indicated before, the frequency of STOCs is extremely sensitive to the SR Ca^{2+} levels (ZhuGe et al., 1999; Cheranov and Jaggar, 2002; McCarron et al., 2002). We have used this property to indirectly assess the effect of a rapid application of thapsigargin on the luminal Ca^{2+} level of SR in smooth muscle cells from urinary bladder of guinea pig. We recorded STOC activity at a membrane potential of 0 mV and applied thapsigargin (10 μM in the puffer pipette) for a period of 5 s (Fig. 5 *A*). The application of thapsigargin did not induce the disappearance of STOCs suggesting that the SR Ca^{2+} level was not greatly lowered. In fact, the overall frequency of STOCs determined for time periods of 30 s was comparable before (3.6 ± 0.6 STOCs/s, $n = 10$) and after (3.2 ± 0.7 STOCs/s, $n = 7$) the application of thapsigargin (Fig. 5 *A*). This lack of modification of STOC frequency contrasts with the significant reduction observed when stores were not completely refilled after a pulse of 20 mM caffeine (2.2 ± 0.6 STOCs/s, Fig. 2 *B*). We further explored the effect of thapsigargin on the frequency of STOCs by binning the STOCs in three groups of different amplitudes (Fig. 5 *B*). It is evident that the effect of thapsigargin was to decrease the amplitude of STOCs,

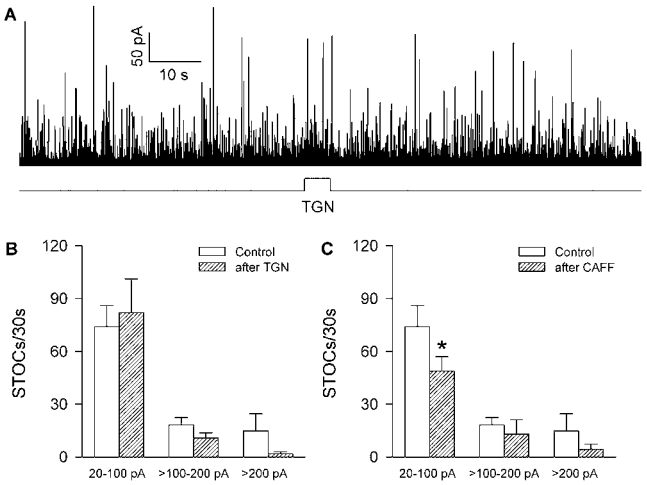

FIGURE 5 The frequency of spontaneous transient outward currents was not significantly altered by acute exposure to thapsigargin. STOCs represent the coordinated activation of a cluster of large conductance Ca^{2+}-dependent K^+ channels in response to superficial Ca^{2+} sparks. (*A*) shows a recording of STOC activity at 0 mV before and after the application of 10 μM thapsigargin (TGN, *bottom trace*). (*B*) The amplitude of STOCs was binned in three different groups during a period of 30 s before (*open columns*, $n = 10$) and after (*shaded columns*, $n = 7$) the application of thapsigargin. (*C*) Shows the frequency for STOCs of different amplitude before (*open columns*, $n = 10$) and 5 min after the application of 20 mM caffeine (CAFF, *shaded columns*, $n = 3$). *$P < 0.05$ (Student's *t*- test). Note how partial depletion of internal stores reduces the activity of the most frequent STOCs, whereas thapsigargin did not affect, and if anything increased, the frequency of the most common STOCs.

because the frequency of STOCs higher than 200 pA was lowered and the frequency of STOCs smaller than 100 pA was slightly increased (Fig. 5 *B*). This situation explains why the overall frequency was not significantly modified. Nevertheless, in conditions where there is only a partial recovery of internal Ca^{2+} stores, the frequency of STOCs was decreased at all amplitudes (Fig. 5 *C*), which led to a significant reduction of the overall frequency. Assuming that the frequency of STOCs reflects luminal Ca^{2+} levels then it can be argued that the application of thapsigargin did not reduce importantly the luminal Ca^{2+} level in the SR of urinary bladder myocytes.

Rapid inhibition of SERCA pumps does not produce a decrease in luminal Ca^{2+} levels before stimulation with caffeine

To directly assess the effect of blocking SERCA pumps on the Ca^{2+} level from internal stores, we measured the fluorescence ratio of a low-affinity Ca^{2+} indicator, Mag-Fura-2. To remove the cytoplasmic fraction of Mag-Fura-2, cells were dialyzed using the whole-cell configuration of patch-clamp technique. The holding potential was set at 0 mV throughout all the experiments. Additionally, the pipette solution included 1 mM BAPTA and 0.75 mM $CaCl_2$ to clamp $[Ca^{2+}]_i$ to an average value of 128.2 ± 22.7 nM ($n = 5$), as determined by Fura-2 recording (see Methods).

Fig. 6 shows the time course of the reduction in the Mag-Fura-2 fluorescence ratio induced by the application of caffeine in both the absence and presence of thapsigargin (*A* and *B*, respectively). In the absence of thapsigargin, the application of 20 mM caffeine for 5 s resulted in a rapid decrease of the luminal Ca^{2+} level, which remained low as long as caffeine was applied to the cell (Fig. 6 *A*). Few seconds after halting caffeine application, luminal Ca^{2+} level started to recover. Interestingly, we did not observe a complete depletion of the internal Ca^{2+} store in response to the application of caffeine. On average, caffeine diminished the Mag-Fura-2 fluorescence ratio by 8.96 ± 0.98% ($n = 9$). The time for recovery of luminal Ca^{2+} level varied from cell to cell. In general, Mag-Fura-2 fluorescence ratio returned to the prestimulation level before 60 s, although there was a fraction of cells that did not recover completely (three out of eight cells) even after 5 min. A second caffeine pulse, 5 min after the first pulse (Fig. 6 *A*), lowered luminal Ca^{2+} level similarly to the first application (10.3 ± 2.0%, $n = 7$).

To assess the effect of rapidly blocking SERCA pumps on the luminal Ca^{2+} level, we applied a pulse of 10 μM thapsigargin (with another puffer pipette) 60 s before the application of caffeine (Fig. 6 *B*). Importantly, SERCA pump inhibition did not change luminal Ca^{2+} level, because the

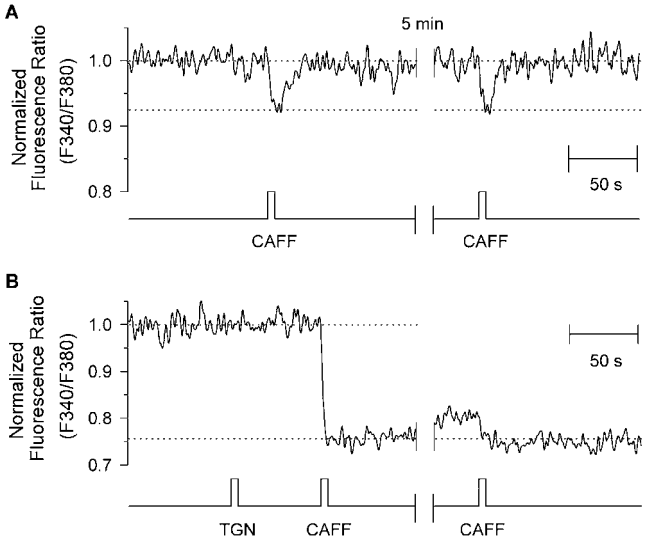

FIGURE 6 Determination of SR Ca^{2+} levels with Mag-Fura-2 in single myocytes. (*A*) The normalized fluorescence ratio was reversibly decreased by the application of 20 mM caffeine (CAFF, $n = 9$). B) However, this ratio was irreversibly reduced when the application of caffeine was preceded by the exposure to 10 μM thapsigargin (TGN, $n = 4$). Note that blocking SERCA pumps with thapsigargin produced both no change in the luminal Ca^{2+} levels for the initial 1 min and a larger reduction of the Mag-Fura-2 fluorescence ratio in response to caffeine. These data also show that the application of thapsigargin totally blocked recovery of the internal Ca^{2+} stores even after a period of 5 min without stimulation.

Mag-Fura-2 fluorescence ratio (0.995 ± 0.003) remained unaltered after the application of thapsigargin (0.995 ± 0.009, $n = 4$). This implies that the SR Ca^{2+} leak is rather minuscule under our recording conditions. After blocking SERCA pumps, the application of caffeine decreased luminal Ca^{2+} level by 17.0 ± 3.0% ($n = 4$), which was significantly larger than the control response (Fig. 6 *A*). This suggests that there was a substantial refilling of internal stores during Ca^{2+} release to the extent that active SERCA pumps limited significantly the depletion of SR. In the presence of thapsigargin, luminal Ca^{2+} levels recovered either minimally or not at all even after a 5-min period at resting conditions. The lack of recovery of the internal Ca^{2+} stores was further tested by a second application of caffeine (Fig. 6 *B*). Caffeine induced either a small drop or failed lowering luminal Ca^{2+} levels. These data suggest that thapsigargin-sensitive Ca^{2+} pumps are needed to restore the luminal Ca^{2+} level in the internal Ca^{2+} stores.

Collectively, these data demonstrated that blockade of SERCA pumps does not produce a rapid depletion of internal stores, at least not enough to explain the reduction of the caffeine-induced Ca^{2+} response. Additionally, it appears that active SERCA pumps play a significant role in limiting depletion of internal stores during Ca^{2+} release induced by caffeine.

Rapid inhibition of SERCA pumps also reduces the IP_3-induced Ca^{2+} release

Internal Ca^{2+} stores can also be released by activation of IP_3Rs in smooth muscle (Walker et al., 1987). Therefore, we assessed the participation of SERCA pumps in agonist-induced Ca^{2+} release to determine whether the role of SERCA pumps was specific for RyRs. To this end, cells were stimulated with 10 μM acetylcholine for 5 s to activate IP_3Rs. Fig. 7 shows Ca^{2+} responses obtained in cells that were either exposed (*solid line*) or unexposed (*dashed line*) to thapsigargin 10 s before the challenge with acetylcholine. The exposure to thapsigargin decreased the amplitude of acetylcholine-induced Ca^{2+} response by 48%, similarly to the inhibition observed with caffeine (53%). The acetylcholine-induced Ca^{2+} response was reduced from 922.6 ± 95.7 nM (basal $[Ca^{2+}]_i$ of 84.6 ± 6.9 nM, $n = 12$) to 476.8 ± 84.8 nM (basal $[Ca^{2+}]_i$ of 68.0 ± 4.8, $n = 13$) by the previous application of thapsigargin. More importantly, thapsigargin also diminished the maximal rate of rise of acetylcholine-induced Ca^{2+} response (570.4 ± 132.1 nM/s, $n = 13$) by 61% with respect to the value obtained for control cells (1470.5 ± 295.8 nM/s, $n = 12$). This effect was similar to that observed for cells stimulated with caffeine (64%). One important difference between caffeine-induced Ca^{2+} response and acetylcholine-induced Ca^{2+} release is that the latter was significantly delayed from 3.1 ± 0.4 s ($n = 12$) to 5.5 ± 0.8 s ($n = 13$) by the previous exposure to 10 μM thapsigargin. In summary, these results imply that active SERCA pumps are necessary for an efficient Ca^{2+} release process, regardless of the type of Ca^{2+} release channel activated in smooth muscle cells.

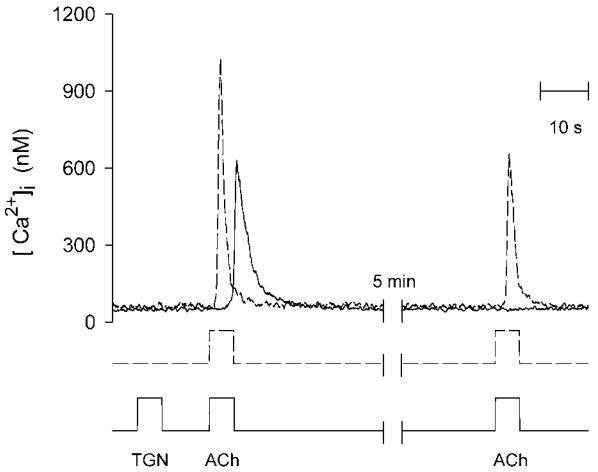

FIGURE 7 Blocking SERCA pumps with thapsigargin reduces both the amplitude and the rate of rise of acetylcholine-induced Ca^{2+} release. Naive cells were stimulated with 10 μM acetylcholine (ACh) where indicated (*dashed line*, $n = 12$) or acetylcholine was applied to cells previously exposed to 10 μM thapsigargin (TGN, *solid line*, $n = 13$). Note that there was a partial recovery of the acetylcholine-induced $[Ca^{2+}]_i$ response after a 5-min period in resting conditions, whereas the application of thapsigargin inhibited any further $[Ca^{2+}]_i$ response to the application of acetylcholine.

DISCUSSION

Rapid and irreversible inhibition with thapsigargin of SERCA pumps

We have studied the effect of rapidly blocking SERCA pumps on Ca^{2+} release from internal stores in smooth muscle cells. The aim was to study Ca^{2+} release without the counteracting effect of SERCA pumps but at the same time having unaltered the Ca^{2+} content of internal stores. This was achieved by using a short pulse of a high concentration of thapsigargin. This condition produced a complete block of SERCA pumps as determined by the lack of recovery of both caffeine-induced $[Ca^{2+}]_i$ response and the SR Ca^{2+} level. Thapsigargin was the correct choice to inhibit SERCA pumps because it is rapid, potent, highly specific, and essentially irreversible (Lytton et al., 1991; Thastrup et al., 1990; Kirby et al., 1992). These characteristics helped us obtain basically an irreversible inhibition of SERCA pumps even when myocytes were exposed to thapsigargin for only a short period of time. Additionally, it is known that thapsigargin neither affects the activity of RyRs recorded in planar lipid bilayers (Lukyanenko et al., 1999) nor when the activity of RyRs is indirectly detected with Ca^{2+} sparks (Gomez et al., 1996). Moreover, the use of caffeine to release Ca^{2+} from the internal stores facilitated the interpretation of our results because this alkaloid activates directly RyRs

without the requirement of a complicated signal transduction mechanism.

Low Ca^{2+} leak from the SR of urinary bladder myocytes

A continuous Ca^{2+} influx through the plasma membrane is one of the key components of the superficial buffer barrier model (for review see van Breemen et al., 1995). In the absence of this influx, the SR would not be overloaded, so there would be no need of a Ca^{2+} leak in the SR to release the excess Ca^{2+}. Under our recording conditions, it appears that Ca^{2+} influx through the plasma membrane is rather low based mainly on the observation that the internal stores did not recover completely after caffeine stimulation. This conclusion is supported by three different approaches. One, the absence of a complete recovery of the frequency of STOCs (an indicator of luminal Ca^{2+} levels), which was observed 5 min after the application of caffeine. Two, the direct recording of the SR Ca^{2+} levels with Mag-Fura-2 showed that these levels did not recover completely in all cells after the exposure to caffeine. Three, the stimulation of Ca^{2+} influx via L-type Ca^{2+} channels with Bay K increased the amplitude of the second caffeine-induced Ca^{2+} response. These data imply that increasing Ca^{2+} influx facilitates refilling of internal stores in these cells. Collectively, these data argue for a limited Ca^{2+} influx through the plasma membrane in nonstimulated myocytes from the urinary bladder of guinea pig.

Additionally, blocking SERCA pumps with thapsigargin revealed an extremely low Ca^{2+} leak from the SR. This was evident, as the inhibition of SERCA pumps reduced neither the overall frequency of STOCs nor the basal level of the Mag-Fura-2 fluorescence ratio. It has been reported that the frequency of STOCs is markedly sensitive to the SR Ca^{2+} levels in smooth muscle cells (ZhuGe et al., 1999; Cheranov and Jaggar, 2002; McCarron et al., 2002). Indeed, the Ca^{2+} content from the SR needs to fall by only a relatively small amount to suppress STOCs. McCarron et al. (2002) reported that a decrease of only 16% of SR Ca^{2+} level resulted in a 70% inhibition of STOCs, whereas ZhuGe et al. (1999) observed that STOCs display a steep recovery of its frequency when the Ca^{2+} content of the internal stores has reached a level close to 80%. Moreover, Rueda et al. (2002b) have shown in the same type of cells used here, that STOCS disappear by histamine-induced Ca^{2+} release although the caffeine-induced $[Ca^{2+}]_i$ response indicates that internal stores still contain Ca^{2+}. Collectively, these data suggest that STOC frequency is a sensitive indicator, albeit indirect, of the SR Ca^{2+} levels. We also used Mag-Fura-2 to directly assess the effect of thapsigargin on SR Ca^{2+} levels in urinary bladder myocytes, an approach that has been used already in different types of smooth muscle cells (Sugiyama and Goldman, 1995; Steenbergen and Fay, 1996; ZhuGe et al., 1999). In our hands, this approach required to buffer $[Ca^{2+}]_i$ to avoid interference by the cytoplasmic Mag-Fura-2. Nevertheless, the reversible decrease in Mag-Fura-2 fluorescence ratio induced by caffeine and the lack of recovery due to the application of thapsigargin imply that our signal reflected mainly a thapsigargin-sensitive internal Ca^{2+} store releasable by caffeine. These two different approaches to measure the SR Ca^{2+} level produced the same picture, i.e., a rather small Ca^{2+} leak in the SR of urinary bladder myocytes. Other studies looking directly to the SR Ca^{2+} levels, have also demonstrated an extremely low Ca^{2+} leak in the SR of smooth muscle cells from both the toad stomach (Steenbergen and Fay, 1996; ZhuGe et al., 1999) and human myometrium (Young and Mathur, 1999). Although a small Ca^{2+} leak from the SR does not seem to be the case in all types of smooth muscle cells. Actually, the same type of studies has shown that blocking SERCA pumps produces a significant depletion of the internal Ca^{2+} stores in A7r5 cells (Tribe et al., 1994), rat gastric (White and McGeown, 2002), and uterine (Shmigol et al., 2001) myocytes. The reason for this difference in the SR Ca^{2+} leak among the different types of smooth muscle cells is not evident.

Active SERCA pumps limits depletion of the SR Ca^{2+} level during Ca^{2+} release

Our determinations of SR Ca^{2+} levels demonstrated that blocking SERCA pumps with thapsigargin induced a higher depletion of internal stores in response to caffeine. Similar role for SERCA pumps in reducing depletion of internal stores during Ca^{2+} release has been seen in other types of cells stimulated with different agonists (Steenbergen and Fay, 1996; Arnaudeau et al., 2001; Yu and Hinkle, 2000; Shmigol et al., 2001). Although it is not clear how SERCA pumps impede store depletion, it is evident that the intervention of the SERCA pumps during Ca^{2+} release is critical to produce optimal cytoplasmic Ca^{2+} elevations. This is apparent when it is compared to the effect of thapsigargin on the SR Ca^{2+} levels with the corresponding change in $[Ca^{2+}]_i$. Interestingly, similar observations have been reported for the first time in rat uterine smooth muscle cells when $[Ca^{2+}]_i$ and SR Ca^{2+} levels were simultaneously recorded (Shmigol et al., 2001). One possible explanation is that the activity of SERCA pumps is necessary during Ca^{2+} release to keep high levels of free Ca^{2+} in the SR to produce a sustained Ca^{2+} efflux. One of the limitations with this idea is that the Ca^{2+} flux through SERCA pumps is much slower than the flux rate through Ca^{2+} release channels. Considering both that the unitary Ca^{2+} current of RyRs under quasiphysiological conditions amounts to 0.35 pA (Mejia-Alvarez et al., 1999), which represents a Ca^{2+} flux of $\sim 1 \times 10^6$ Ca^{2+} ions/s, and because the turnover rate of SERCA 2b pump, which is the predominant isoform in smooth muscle (Lytton et al., 1989; Wu and Lytton, 1993), is only $\sim$3.5 Ca^{2+} ions/s (Lytton et al., 1992), then it is necessary to have $\sim$312,000 SERCA pumps/RyR to compensate the Ca^{2+}

efflux from the SR. Assuming that in smooth muscle cells from the urinary bladder, the density of SERCA pumps is similar to what has been reported for smooth muscle cells from aorta (87 pmol SERCAs/100 mg tissue, Wu and Lytton, 1993) and because we have determined with [^{3}H]-ryanodine binding that myocytes from the urinary bladder of guinea pig contain 168 fmol RyR/100 mg tissue, then there are ~518 SERCAs/RyR. This number of SERCA pumps is not sufficient to compensate the Ca^{2+} efflux via RyRs. This contention will be true even if 20 mM caffeine would be activating only 1% of the RyRs present in urinary bladder myocytes, a situation that seems unlikely, because 20 mM is a saturating concentration of caffeine in the activation of RyRs.

Rapid SERCA pump inhibition decreases Ca^{2+} responses to agonists

Typically SERCA pumps are involved in maintaining and replenishing internal Ca^{2+} stores (Hussain and Inesi, 1999). A role that was clearly demonstrated in our cells by using Mag-Fura-2 to determine the luminal Ca^{2+} levels in response to the application of either thapsigargin, to block SERCA pumps, or caffeine, to release Ca^{2+} from internal stores via the RyRs. Nevertheless, blocking SERCA pumps produced smaller and slower agonist-induced Ca^{2+} responses, to a similar extent whether the release channels activated were either RyRs or IP_3Rs. Our data suggest that these effects of thapsigargin on $[Ca^{2+}]_i$ response cannot be explained by a reduction in the SR Ca^{2+} levels. Some of the evidence are the absence of a reduction in either the STOCs frequency or the Mag-Fura-2 signal. Additionally, the effect of thapsigargin on $[Ca^{2+}]_i$ responses was maximally established in only 10 s, a time too short to allow depletion of internal Ca^{2+} stores considering the small Ca^{2+} leak from the SR present in these cells. Moreover, basal $[Ca^{2+}]_i$ was not modified by the application of thapsigargin in our cells. Conceivably, a large Ca^{2+} leak, which is necessary to deplete the SR, would produce a transient increase in $[Ca^{2+}]_i$, as it is observed in other types of cells when SERCA pumps are blocked with thapsigargin (Ribeiro et al., 1997).

In many different types of cells blocking SERCA pumps either does not affect the peak Ca^{2+} transient (Gomez et al., 1996), increase the frequency of Ca^{2+} oscillations (Petersen et al., 1993) or increase the Ca^{2+} wave velocity (Lukyanenko et al., 1999). Nevertheless, there are also other types of cells where blocking SERCA pumps, without depleting internal Ca^{2+} stores, produces either smaller (Shmigol et al., 2001) or slower (Lee et al., 1997; Aguilar-Maldonado et al., 2003) Ca^{2+} responses. The effect of rapid application of thapsigargin in our cells produced the second type of effect on agonist-induced Ca^{2+} responses. Lee et al, (1997) proposed that active SERCA pumps are needed to load adjacent Ca^{2+} pools to allow Ca^{2+} wave propagation. This implies a larger depletion of the internal stores with active SERCA pumps due to the recruitment of additional Ca^{2+} pools. However, our Mag-Fura-2 data showed the opposite, i.e., that internal stores depleted more when SERCA pumps were blocked. Another possibility is that the activity of SERCA pumps could be involved in inhibiting the Ca^{2+}-dependent inactivation of the release channels. Because the Ca^{2+} sensitivity of the inactivation site of RyRs (Chu et al., 1993) is much lower than of IP_3Rs (Iino, 1990), it is expected that blocking SERCA pumps affects more the inactivation of IP_3Rs than that of RyRs. Nevertheless, the effect of thapsigargin was similar between the two types of release channels. Additionally, a lower depletion of internal Ca^{2+} stores with a faster inactivation of the release channels is expected, but our Mag-Fura-2 data showed a larger depletion after blocking SERCA pumps. Therefore, the need for active SERCA pumps for an efficient Ca^{2+} release appears to involve another mechanism. We speculate that active SERCA pumps are somehow modulating Ca^{2+} availability in the internal stores.

Interestingly, SERCA 2b appears to affect Ca^{2+} release differently with respect to the other types of SERCA pumps. There are studies showing that SERCA 2b can modulate Ca^{2+} release despite being slower than the other types of SERCA pumps. The expression of SERCA 2b in *Xenopus* laevis oocytes produces Ca^{2+} waves of a higher amplitude compared to those induced by the overexpression of SERCA 1 or SERCA 2a (Lechleiter et al., 1998). Additionally, the overexpression of SERCA 2b in heart led to a significant increase in the work-performing heart mechanism, although the maximal activity of Ca^{2+} uptake was not modified (Greene et al., 2000). Moreover, SERCA 2b is the only SERCA pump that contains a long carboxy terminal that appears to interact with luminal Ca^{2+} binding proteins (John et al., 1998).

All these data suggest that active SERCA pumps in smooth muscle cells are required to obtain optimal Ca^{2+} release from internal stores. This work also indicates that SERCA pumps are another element involved in the dynamic regulation of the Ca^{2+} release process. Further work will be needed to establish the mechanism used by active SERCA pumps to help Ca^{2+} release in smooth muscle cells.

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