Role of Sarcoplasmic Reticulum and Mitochondria in Ca^{2+} Removal in Airway Myocytes

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ABSTRACT The aim of this study was to use both a theoretical and experimental approach to determine the influence of the sarco-endoplasmic Ca^{2+}-ATPase (SERCA) activity and mitochondria Ca^{2+} uptake on Ca^{2+} homeostasis in airway myocytes. Experimental studies were performed on myocytes freshly isolated from rat trachea. $[Ca^{2+}]_i$ was measured by microspectrofluorimetry using indo-1. Stimulation by caffeine for 30 s induced a concentration-graded response characterized by a transient peak followed by a progressive decay to a plateau phase. The decay phase was accelerated for 1-s stimulation, indicating ryanodine receptor closure. In Na^{2+}-Ca^{2+}-free medium containing 0.5 mM La^{3+}, the $[Ca^{2+}]_i$ response pattern was not modified, indicating no involvement of transplasmalemmal Ca^{2+} fluxes. The mathematical model describing the mechanism of Ca^{2+} handling upon RyR stimulation predicts that after Ca^{2+} release from the sarcoplasmic reticulum, the Ca^{2+} is first sequestrated by cytosolic proteins and mitochondria, and pumped back into the sarcoplasmic reticulum after a time delay. Experimentally, we showed that the $[Ca^{2+}]_i$ decay after Ca^{2+} increase was not altered by the SERCA inhibitor cyclopiazonic acid, but was slightly but significantly modified by the mitochondria uncoupler carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone. The experimental and theoretical results indicate that, although Ca^{2+} pumping back by SERCA is active, it is not primarily involved in $[Ca^{2+}]_i$ decrease that is due, in part, to mitochondrial Ca^{2+} uptake.

INTRODUCTION

Cell calcium homeostasis and calcium signaling are the result of the dynamic interactions between the ON mechanisms that lead to cytosolic Ca^{2+} concentration increase via extracellular calcium influx and/or calcium release from intracellular Ca^{2+} stores and the OFF mechanisms that tend to decrease $[Ca^{2+}]_i$ (Berridge et al., 2000). Although it is known that Ca^{2+} pumping back by sarco-endoplasmic Ca^{2+}-ATPase (SERCA) and pumping out by plasma membrane Ca^{2+}-ATPase (PMCA) and Na^+-Ca^{2+} exchanger are not the unique processes involved in the OFF mechanisms and that mitochondrial Ca^{2+} uptake and Ca^{2+} binding to cytosolic proteins may also participate in the maintenance of a low $[Ca^{2+}]_i$, SERCA and PMCA are generally considered as the major and the first structures implicated in $[Ca^{2+}]_i$ decrease after $[Ca^{2+}]_i$ increase due to cell stimulation (Berridge et al., 2000; Sanders, 2001). However, although theoretical studies have pointed out that Ca^{2+} binding to cytosolic proteins and mitochondrial uptake may greatly influence the Ca^{2+} response pattern (Marhl et al., 2000), few studies have been performed to investigate the relative participation of SERCA activity and mitochondrial Ca^{2+} uptake in Ca^{2+} signaling of airway smooth muscle cells.

Caffeine is a cell-permeant agonist of ryanodine receptors (RyR) that is largely used to investigate calcium signaling. In particular, pharmacological stimulation of RyRs by caffeine can be used to trigger calcium release from the sarcoplasmic reticulum and investigate the mechanisms activated by the initial $[Ca^{2+}]_i$ increase (Guibert et al., 1996; Roux et al., 1998; Drummond and Tuft, 1999; Pacher et al., 2000; Vallot et al., 2001; Kamishima and Quayle, 2002). On the other hand, RyRs are expressed in a large variety of cells, including airway myocytes, and may be implicated in their physiological response (Kannan et al., 1997; Prakash et al., 1998). The understanding of the effect of pharmacological stimulation of ryanodine receptors by caffeine as well as their physiological involvement in cell stimulation would be greatly improved by use of a theoretical model of calcium handling upon RyR stimulation.

The aim of the present study was first to characterize the calcium response to caffeine stimulation in rat freshly isolated cells and to build a theoretical model of calcium handling upon caffeine stimulation based on these experimental results. The model included calcium release from the sarcoplasmic reticulum (SR) through RyR stimulation and pumping back by SERCAs. In vascular smooth muscle cells, several recent studies have shown that mitochondria Ca^{2+} uptake occurs after SR Ca^{2+} release and $[Ca^{2+}]_i$ increase (Drummond and Fay, 1996; Drummond and Tuft, 1999; Pacher et al., 2000; Vallot et al., 2001; Kamishima and Quayle, 2002; Szado et al., 2003). It is also known that Ca^{2+} may bind to two classes of protein binding sites in the cytosol. The first class represents the buffering proteins such as parvalbumin, calbindin, and also C-domains of calmodulin, which bind calcium relatively slowly but with a high affinity (Falke et al., 1994; Smith et al., 1996). The second class, which is referred to as signaling proteins, comprises binding sites like N-domains of calmodulin that have very

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high rate constants of binding and dissociation with respect to calcium, but low affinity. Hence the model also included, as additional OFF mechanisms, mitochondrial Ca^{2+} uptake and Ca^{2+} binding to buffering proteins.

This model was further used, in combination with experimental investigations, to evaluate the involvement of these various Ca^{2+} intracellular compartments in Ca^{2+} dynamics upon Ca^{2+} release from the SR. In particular, we determined the influence of SERCA activity and mitochondrial Ca^{2+} uptake in comparison with the other buffering mechanisms on Ca^{2+} homeostasis and on $[Ca^{2+}]_i$ variations after caffeine-induced $[Ca^{2+}]_i$ increase.

MATERIALS AND METHODS

Tissue preparation

Rat tracheae were obtained from male Wistar rats 10–15 weeks old, weighing 300–400 g. For each experiment, a rat was stunned and killed by cervical dissociation. Heart and lungs were removed en bloc, and the trachea was rapidly dissected out. The muscular strip located on the dorsal face of the trachea was further dissected under binocular control, as previously described (Roux et al., 2002). The epithelium-free muscular strip was cut into several pieces and the tissue was then incubated overnight (14 h) in low-Ca^{2+} (200 μM) physiological saline solution (PSS; composition given below) containing 0.5 mg ml^{-1} collagenase, 0.35 mg ml^{-1} pronase, 0.03 mg ml^{-1} elastase, and 3 mg ml^{-1} bovine serum albumin at 4°C. After this time, the muscle pieces were triturated in a fresh enzyme-free solution with a fire polished Pasteur pipette to release cells. Cells were stored for 1–3 h to attach on glass coverslips at 4°C in PSS containing 0.8 mM Ca^{2+} and used on the same day. In control experiments, immunocytochemistry was performed using monoclonal mouse anti-smooth muscle α-actin antibodies and fluorescein-5′-isothiocyanate-conjugated anti-mouse immunoglobulin G antibodies to verify that the isolated cells obtained by dissociation were smooth muscle cells (data not shown).

Fluorescence measurement and estimation of $[Ca^{2+}]_i$

Changes in $[Ca^{2+}]_i$ were monitored fluorimetrically using the Ca^{2+}-sensitive probe indo-1 as previously described (Roux et al., 2002). Briefly, freshly isolated cells were loaded with indo-1 by incubation in PSS containing 1 μM indo-1 acetoxymethylester for 25 min at room temperature and then washed in PSS for 25 min. Coverslips were then mounted in a perfusion chamber and continuously superfused at room temperature. A single cell was illuminated at 360 ± 10 nm. Emitted light from that cell was counted simultaneously at 405 nm and 480 nm by two photomultipliers (P100, Nikon, Tokyo, Japan). $[Ca^{2+}]_i$ was estimated from the 405/480 ratio using a calibration for indo-1 determined within cells.

Caffeine was applied to the tested cell by a pressure ejection from a glass pipette located close to the cell. No changes in $[Ca^{2+}]_i$ were observed during test ejections of PSS (data not shown). Generally, each record of $[Ca^{2+}]_i$ response to caffeine was obtained from a different cell. Each type of experiment was repeated for the number of cells indicated in the text.

Solutions, chemicals, and drugs

Normal PSS contained (in mM): 130 NaCl, 5.6 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 11 glucose, 10 Hepes, pH 7.4 with NaOH. In Ca^{2+}-free solution, Ca^{2+} was removed and 0.4 mM EGTA was added. In "Ca^{2+}-confining" solution, Ca^{2+} and Na^+ were omitted and 130 mM *N*-methyl-D-glucamine, 0.4 mM EGTA, and 0.5 mM $LaCl_3$ were added to the solution. Such a solution is Ca^{2+} confining, i.e., inhibits any Ca^{2+} fluxes throughout the plasma membrane (Tribe et al., 1994), because removal of external Ca^{2+} inhibits any Ca^{2+} influx, removal of external Na^+ inhibits the Na^+/Ca^{2+} exchanger, and lanthanum is a nonspecific inhibitor of Ca^{2+}-dependent transport activities, in particular the PMCA (Herscher and Rega, 1996) and the Na^+/Ca^{2+} exchanger (Iwamoto and Shigekawa, 1998).

Collagenase (type CLS1) was from Worthington Biochemical Corp. (Freehold, NJ). Bovine serum albumin, cyclopiazonic acid (CPA), thapsigargin, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), and caffeine (CAF) were purchased from Sigma (Saint Quentin Fallavier, France). Indo-1 AM was from Calbiochem (France Biochem, Meudon, France). Indo-1 AM, CPA, and FCCP were dissolved in dimethyl sulphoxide, for which the maximal concentration used in our experiments was <0.1% and had no effect on the resting value of the $[Ca^{2+}]_i$ nor on the variation of the $[Ca^{2+}]_i$ induced by caffeine (data not shown).

Data analysis and statistics

Cytosolic calcium concentrations ($[Ca^{2+}]_i$) in control conditions are expressed as mean ± SE. For each protocol, experiments were repeated on two or more rats, control cells were obtained in each rat, and $[Ca^{2+}]_i$ values recorded for each protocol are expressed as a percentage of the corresponding values obtained in control cells from the rats used for that protocol. $[Ca^{2+}]_i$ values that characterize the Ca^{2+} response, i. e., baseline, peak, and plateau values, were compared between control and each specific experimental condition using unpaired student *t*-tests (peak and plateau values were calculated above baseline). Time-dependent decrease in $[Ca^{2+}]_i$ induced by SERCA inhibition was fitted by a sigmoidal equation using the Levenberg-Marquardt algorithm with ORIGIN software (Microcal Software Inc., Northhampton, MA). Parameters derived form curve fitting are given with SE. Comparison of the decay phase following the maximal $[Ca^{2+}]_i$ value in control versus specific conditions (i.e., Ca^{2+} confining solution, CPA, and FCCP) were performed as follows: for each cell tested, $[Ca^{2+}]_i$ measurements were repeated every 0.2 s for 10 s, time 0 corresponding to the maximal $[Ca^{2+}]_i$. Comparison between the points obtained in control versus specific condition was done by analysis of variance (ANOVA) for repeated measurements, using SPSS software (SPSS, Chicago, IL).

Computational

Simulations were performed using the Runge-Kutta routine for numerical solving of differential equations. The software used was MADONNA (University of Berkeley, Berkeley, CA).

RESULTS

Characterization of caffeine-induced $[Ca^{2+}]_i$ response

$[Ca^{2+}]_i$ response to caffeine stimulation

In this series of experiments, cells were stimulated with caffeine for 30 s in a concentration range from 0.01 to 5 mM. Stimulation with caffeine induced a $[Ca^{2+}]_i$ response characterized by a first transient peak followed by a $[Ca^{2+}]_i$ decay to a plateau slightly above the resting $[Ca^{2+}]_i$ value. At the end of the stimulation the $[Ca^{2+}]_i$ rapidly returned to baseline. Both the amplitude of the peak and the plateau depended on caffeine concentration. Typical traces of $[Ca^{2+}]_i$ response to 0.1 and 5 mM caffeine are shown in Fig. 1 *A*. Fig. 1 *B* shows the mean peak (*left panel*) and

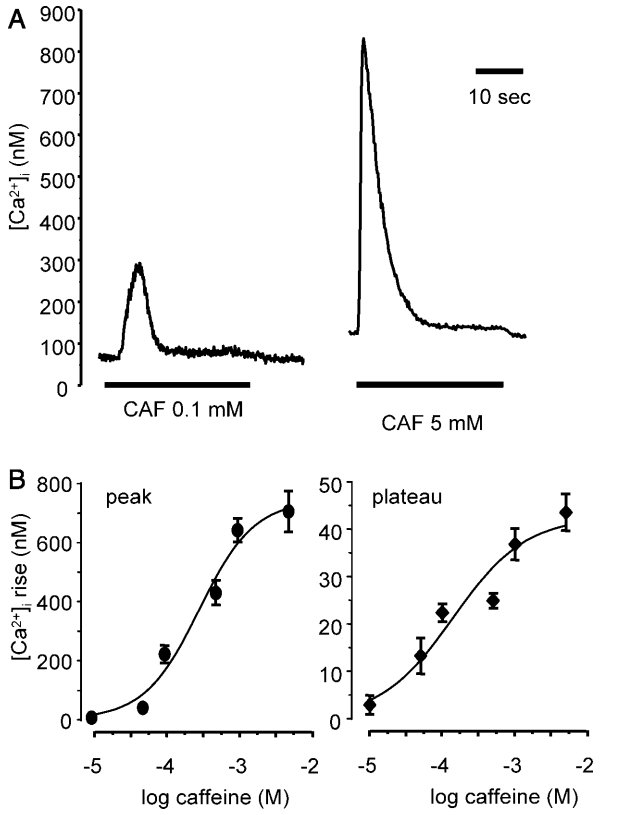

FIGURE 1 Effect of various caffeine concentrations on $[Ca^{2+}]_i$ responses. (*A*) Original traces of calcium response to 0.1 mM (*left trace*) and 5 mM (*right trace*) CAF (30-s stimulation). (*B*) Amplitude of the calcium peak (●, *left panel*) and plateau (♦, *right panel*) for CAF concentrations from 0.01 mM to 5 mM. Each point is a mean value from 10 to 35 cells. Vertical bars are SE.

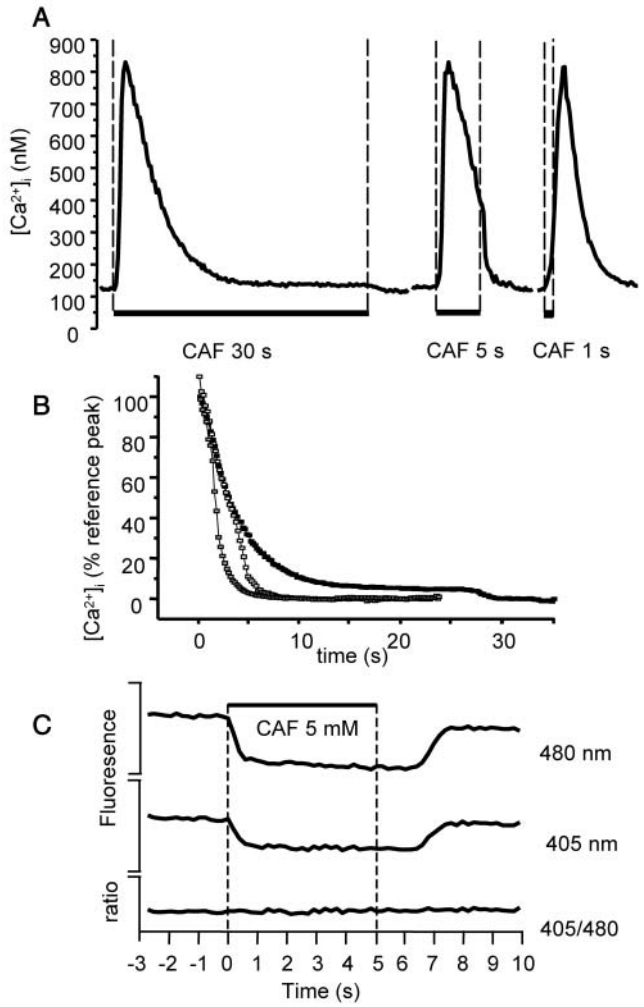

FIGURE 2 Effect of caffeine stimulation for various durations on $[Ca^{2+}]_i$. (*A*) Original traces of cells stimulated by 5 mM CAF for 30 s, 5 s, and 1 s. (*B*) Mean $[Ca^{2+}]_i$ decay upon stimulation with 5 mM CAF for 30 s (■), 5 s (□), and 1 s (*shaded squares*). Time 0 corresponds to max $[Ca^{2+}]_i$ increase (peak). Values are expressed as percent of the peak obtained when cells were stimulated for 30 s (reference peak), 0 corresponding to baseline. Each curve is the mean value from 10 to 12 cells. (*C*) Effect of CAF ejection on fluorescence intensities and ratio. CAF was ejected for 5 s on a cell pre-incubated for 11 min with 10 μM CPA. During CAF ejection, fluorescence quenching at both wavelengths is observed, without alteration in the fluorescence ratio. Fluorescence intensities and ratio are in arbitrary units.

plateau (*right panel*) values for the various caffeine concentrations are given in (n = 10–35 cells for each concentration). Log IC_{50}, i.e., the logarithm of the half-maximal response concentration determined from curve fitting, was -3.51 ± 0.07 logM and -3.85 ± 0.19 logM for the peak and the plateau, respectively.

The peak and plateau values in response to 30-s stimulation by 5 mM caffeine were 697 ± 59 nM and 43 ± 4.0 nM, respectively (n = 19). When the stimulation was stopped after 1 s, the amplitude of the Ca^{2+} peak was not significantly modified (681 ± 66 nM; n = 24), but the $[Ca^{2+}]_i$ decreased more rapidly and returned to baseline. When cell stimulation was stopped after 5 s, i.e., during the decay phase, the $[Ca^{2+}]_i$ decrease rate augmented at the end of caffeine exposure and became similar to that observed for 1-s stimulation. Typical traces are shown in Fig. 2 *A*.

Fig. 2 *B* shows specifically the $[Ca^{2+}]_i$ decay phase following the peak, when cells were stimulated with caffeine for 1 s, 5 s, and 30 s. Values are mean values calculated from 10 to 12 cells for each condition, and are expressed as the percent of the mean peak obtained for 30-s caffeine stimulation. Time 0 corresponds to peak, i. e., the maximal $[Ca^{2+}]_i$ increase. The fact that the $[Ca^{2+}]_i$ decay was quicker for 1-s ejection than for 5-s or 30-s ejection indicates that caffeine was rapidly washed out at the end of the ejection, inducing RyR closure. Caffeine has the property to decrease the indo-1 fluorescence intensity at both wavelengths, without altering the fluorescence ratio (O'Neill et al., 1990). The analysis of fluorescent intensity confirmed the quick penetration and washout of caffeine, as shown in Fig. 2 *C*.

Role of transmembrane Ca^{2+} fluxes in the response to caffeine stimulation

The possible implication of calcium fluxes through the plasma membrane was assessed by a series of experiments in Ca^{2+}-free medium and in Ca^{2+}-confining solution. In normal PSS, baseline value was 139.7 ± 4.2 nM ($n = 42$). Removal of external Ca^{2+} or incubation in Ca^{2+}-confining solution did not significantly modify the resting $[Ca^{2+}]_i$ value (88.0 ± 3.2% control, $n = 23$ and 99.9 ± 3.9% control, $n = 23$, respectively). When cells in Ca^{2+}-free or Ca^{2+}-confining medium were stimulated with 5 mM caffeine for 30 s, neither the peak nor the plateau was significantly different from control. Fig. 3 *A* shows the mean results, expressed as percent to control conditions, for the peak (*left panel*) and the plateau (*right panel*).

Due to the superficial SR that may accumulate Ca^{2+} that enters through the plasmalemma, the so-called buffer barrier, Ca^{2+} influx may reload the SR without increasing the $[Ca^{2+}]_i$ (van Breemen et al., 1995; Marin et al., 1999; Sanders, 2001). To test whether such a mechanism was triggered by caffeine stimulation, cells were stimulated by two successive 1-s ejections of 5 mM caffeine at a 15-s interval. The first stimulation induced a Ca^{2+} peak followed by a quick decay, the RyRs being closed. The second stimulation 15 s after the first one induced a second Ca^{2+} peak, the amplitude of which was used as an estimate of Ca^{2+} loading of the SR after the first stimulation. In control experiments, the mean $[Ca^{2+}]_i$ response to the first and the second stimulation was 695 ± 82 nM and 206 ± 26 nM, respectively ($n = 35$). The amplitude of the first peak was not significantly modified in the absence of extracellular calcium; neither was the amplitude of the second one ($n = 13$). Original traces are shown in Fig. 3 *B*, and mean values in Fig. 3 *C* (*left panel*). A similar stimulation protocol was used in Ca^{2+}-confining solution. As in Ca^{2+}-free medium, neither the amplitude of the first response nor that of the second one was altered compared to normal medium ($n = 23$), Fig. 3, *B* (*left trace*) and *C* (*left panel*). To see whether Ca^{2+} fluxes through the plasma membrane may be significantly involved during cytosolic Ca^{2+} removal upon cell stimulation, we analyzed the $[Ca^{2+}]_i$ phase after 1-s caffeine ejection in control versus Ca^{2+}-confining solution. Mean values obtained in control condition ($n = 22$, *filled circles*) and in Ca^{2+}-confining solution ($n = 23$, *open circles*) are shown in Fig. 3 *C* (*right panel*). ANOVA analysis of the decay phase showed no significant difference in normal and Ca^{2+}-confining solution.

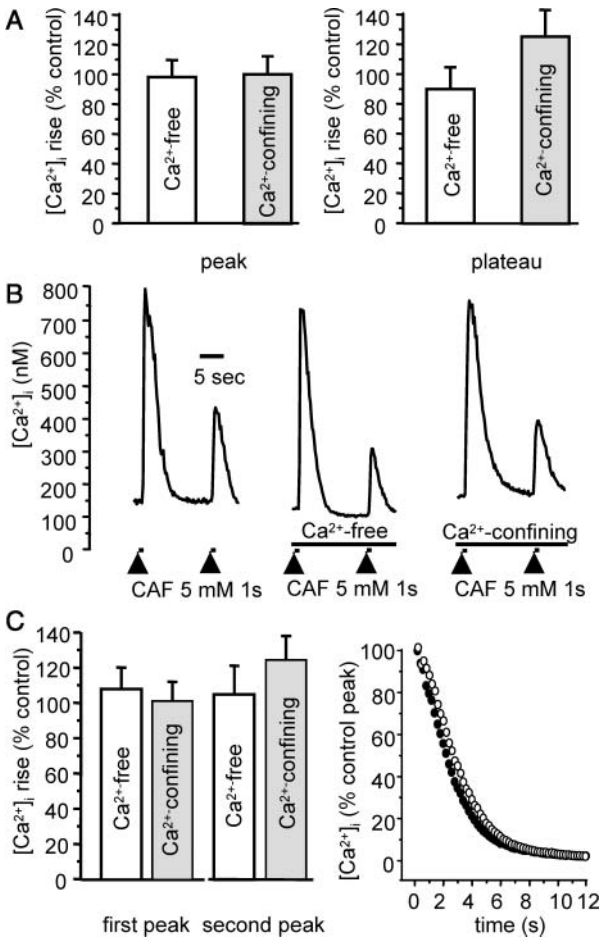

FIGURE 3 Effect of Ca^{2+}-free and Ca^{2+}-confining solutions on response to 5 mM caffeine. (*A*) Peak and plateau values in response to 30-s CAF stimulation, in Ca^{2+}-free medium (*open columns*, $n = 21$) and Ca^{2+}-confining medium (*shaded columns*, $n = 11$). (*B*) Original trace of Ca^{2+} response to two successive stimulations by CAF for 1 s, at a 15-s interval in the presence of external Ca^{2+} (*left*), in Ca^{2+}-free medium (*middle*), and in Ca^{2+}-confining solution (*right*). (*C*) Left panel, mean values of the Ca^{2+} response to two successive stimulations by CAF for 1 s, at a 15-s interval in Ca^{2+}-free medium (*open columns*, $n = 13$) and Ca^{2+}-confining solution (*shaded columns*, $n = 23$). (*C*) Right panel, mean $[Ca^{2+}]_i$ decay after the first CAF-induced peak in control (●, $n = 22$) and Ca^{2+}-confining solution (○, $n = 23$), as percentage of the control peak, 0 corresponding to baseline. Vertical bars are SE.

Mathematical modeling

Presentation of the model

In accordance with our experiments, the model takes into account the calcium exchange between the cytosol and the intracellular calcium stores, neglecting any transplasmalemmal Ca^{2+} fluxes. We focus on four different intracellular calcium stores: the SR, mitochondria, and two classes of calcium-binding proteins in the cytosol, i.e., signaling and buffering proteins. The signaling proteins characterize protein-binding sites with high affinity and low capacity, whereas the buffering proteins characterize protein-binding sites with low affinity and high capacity. Regarding the SR, three different calcium fluxes are included in the model: the ATP-dependent calcium uptake from the cytosol into the SR (J_{SERCA}), the Ca^{2+} efflux from the SR through ryanodine sensitive Ca^{2+} channels following the calcium-induced calcium release (CICR) mechanism (J_{RyR}), and an additional Ca^{2+} leak flux from the SR into the cytosol (J_{leak}). For the

exchange of Ca^{2+} between the mitochondria and the cytosol we take into account active Ca^{2+} uptake by mitochondrial uniporters (J_{in}) and calcium release through Na^+/Ca^{2+} and H^+/Ca^{2+} exchangers (J_{out}). In the cytosol Ca^{2+} binding to signaling and buffering proteins is considered.

The concentration of free-Ca^{2+} binding sites on signaling proteins, SPr, can be calculated by applying a rapid-equilibrium approximation to the fast binding reactions (see, e.g., Wagner and Keizer, 1994; Heinrich and Schuster, 1996; Marhl et al., 1998a; Höfer et al., 2001). Together with the conservation relation for the total concentration of Ca^{2+} binding sites on signaling proteins, SPr_{tot}, the concentration of free-Ca^{2+} binding sites on signaling proteins, SPr, is given by the following equation:

$$SPr = \frac{K_{SPr} SPr_{tot}}{K_{SPr} + Ca_i}, \tag{1}$$

where K_{SPr} is the dissociation constant of binding sites on signaling proteins.

The rapid-equilibrium approximation for the fast Ca^{2+}-binding to signaling proteins is justified in view of the very high values of the rate constants for signaling proteins (Wagner and Keizer, 1994; Smith et al., 1996). The rate constants for binding and dissociation of 90–500 $\mu M^{-1}s^{-1}$ and 300–500 s^{-1}, respectively (Falke et al., 1994; Smith et al., 1996), imply a time constant of <0.01 s. Even under consideration of diffusional resistance and competition with Mg^{2+}, this time constant is much smaller than the duration of any calcium pulse.

Using the conservation relation for the total concentration of Ca^{2+} binding sites on signaling proteins, SPr_{tot}, the concentration of bound Ca^{2+} binding sites on signaling proteins can be calculated as:

$$CaSPr = SPr_{tot} - SPr. \tag{2}$$

Taking into account the conservation relations for the total cellular calcium, Ca_{tot}, and the total concentration of Ca^{2+} binding sites on buffering proteins, BPr_{tot}, we obtain equations for bound and free-Ca^{2+} binding sites on buffering proteins, $CaBPr$ and BPr, respectively:

$$CaBPr = Ca_{tot} - Ca_i - \frac{\rho_{SR}}{\beta_{SR}} Ca_{SR} - \frac{\rho_m}{\beta_m} Ca_m - CaSPr, \tag{3}$$

$$BPr = BPr_{tot} - CaBPr. \tag{4}$$

Here ρ_{SR} and ρ_m represent the volume ratio between the SR and the cytosol and between the mitochondria and the cytosol, respectively. Assuming very fast unsaturated buffering of Ca^{2+} in the SR and mitochondrial compartments, we use factors β_{SR} and β_m, which are the free/total calcium concentration ratios, in the SR and in the mitochondria, respectively (Marhl et al., 1998b; Haberichter et al., 2001). Applying the rapid-equilibrium approximation for relating the free-calcium concentration in each organelle, Ca_j (j stands for SR and m), with the total concentration in the corresponding organelle, $Ca_{tot,j}$, leads to $Ca_j = (K_j/(K_j+B_j))\, Ca_{tot,j}$, where K_j is the dissociation constant of the binding sites on buffering proteins B_j in the organelle. Taking into account that $B_j \gg Ca_j$, we can assume that B_j nearly equals the total buffer concentration, $B_{tot,j}$. Therefore, we consider $\beta_j = (K_j/(K_j+B_{tot,j})) = \text{const}$. Because the values for β_{SR} and β_m have not been experimentally determined for airway myocytes, we take values that are in the range of experimentally obtained values for other cell types. For β_{SR} values usually span from 0.0025 (Li et al., 1995) to 0.01 (Smith et al., 1996), whereas β_m can be found in the range from 0.00001 to 0.01 (Babcock et al., 1997; Fall and Keizer, 2001; Chalmers and Nicholls, 2003).

The time dependence of the free cytosolic calcium concentration, Ca_i, is determined by Ca^{2+} fluxes across the SR membrane, by Ca^{2+} exchange between the cytosol and mitochondria, and by Ca^{2+} binding to signaling and buffering proteins in the cytosol:

$$\frac{dCa_i}{dt} = \frac{1}{1 + K_{SPr} SPr_{tot}/(K_{SPr} + Ca_i)^2} \times [J_{RyR} - J_{SERCA} + J_{leak} + J_{out} - J_{in} + k_{off} CaBPr - k_{on} Ca_i BPr]. \tag{5}$$

Here k_{off} and k_{on} denote the off and on rate constants, respectively, of the Ca^{2+} binding to the buffering proteins. The first factor in Eq. 5, characterizing Ca^{2+} binding to signaling proteins, is obtained by the method of eliminating fast reactions (see Heinrich and Schuster, 1996; Marhl et al., 1998a).

The equation for the free-calcium concentration in the SR, Ca_{SR}, is linked with the fluxes across the SR membrane as follows:

$$\frac{dCa_{SR}}{dt} = \frac{\beta_{SR}}{\rho_{SR}} [J_{SERCA} - J_{RyR} - J_{leak}]. \tag{6}$$

The equation for the free-Ca^{2+} concentration in mitochondria, Ca_m, reads:

$$\frac{dCa_m}{dt} = \frac{\beta_m}{\rho_m} [J_{in} - J_{out}]. \tag{7}$$

Here, β_j relates fluxes, i.e., $\beta_j = \mathrm{d}Ca_j/\mathrm{d}Ca_{tot,j}$, like in previous papers by Wagner and Keizer (1994) and Smith et al. (1996). Under the condition of very fast unsaturated buffering of Ca^{2+} in the organelles used above, this is in

agreement with using the constant β_j for relating the concentrations in Eq. 3. Differentiating $Ca_j = \beta_j Ca_{tot,j}$ with respect to time gives $dCa_j/dt = \beta_j\, dCa_{tot,j}/dt$ and dividing by $dCa_{tot,j}/dt$ gives the above formula $\beta_j = dCa_j/dCa_{tot,j}$.

There are three genetically distinct isoforms of RyR in Mammals, RyR1, RyR2, and RyR3. In smooth muscle, several isoforms may be expressed (Marin et al., 1999; Ogawa et al., 2000). In airway smooth muscle, RT-PCR in porcine trachea has revealed that both RyR2 and RyR3 mRNA were present (Kannan et al., 1997). However, in human bronchial smooth muscle, we have previously showed using RT-PCR and RNase protection assay that RyR3 was the unique isoform expressed (Hyvelin et al., 2000). On this basis, our model was built on RyR3 activity. The Ca^{2+} flux through ryanodine channels, J_{RyR}, is caffeine-activated. The open probability depends on caffeine concentration, *Caff*, and follows the Hill kinetics with $n = 2$. As driving force for the Ca^{2+} channel flux the concentration gradient across the SR membrane is taken into account and the equation for J_{RyR} reads:

$$J_{RyR} = k_{RyR} \frac{Caff^2}{K_{Caff}^2 + Caff^2} CICRMg(Ca_{SR} - Ca_i), \qquad (8)$$

where k_{RyR} is the rate constant of the ryanodine channels and K_{Caff} represents the half-saturation constant of the ryanodine channels for caffeine. The kinetics for caffeine activation was obtained by fitting our experimental data (see Fig. 1 *B*). This is also fully in accordance with previous experimental studies in airway smooth muscle cells (Roux et al., 1998). On the basis of experimental investigations (Ogawa et al., 2000) on the Ca^{2+}-induced Ca^{2+} release (CICR) mechanism of ryanodine channels, *CICRMg*, the effect of Mg^{2+} on the activity of ryanodine receptors is taken into account as follows:

$$CICRMg = \frac{Ca_i}{(Ca_i + K_{A,Ca}(1 + Mg_i/K_{A,Mg}))(1 + Ca_i/K_{I,Ca} + Mg_i/K_{I,Mg})}, \qquad (9)$$

where $K_{A,Ca}$ and $K_{A,Mg}$ represent the half-saturation constants of activate sites for Ca^{2+} and Mg^{2+}, respectively, and $K_{I,Ca}$ and $K_{I,Mg}$ represent the half-saturation constants of inactivate sites for Ca^{2+} and Mg^{2+}, respectively.

For the SERCA flux into the SR lumen, J_{SERCA}, the Hill kinetics with $n = 2$ is taken:

$$J_{SERCA} = k_{SERCA} \frac{Ca_i^2}{K_{SERCA}^2 + Ca_i^2}, \qquad (10)$$

where k_{SERCA} is the rate constant of the SERCAs and K_{SERCA} stands for the half-saturation constant of SERCAs.

The leak flux J_{leak} depends on the concentration gradient across the SR membrane and a simple relation is taken:

$$J_{leak} = k_{leak}(Ca_{SR} - Ca_i), \qquad (11)$$

where k_{leak} is the rate constant for Ca^{2+} leak flux through the SR membrane and has been estimated on the basis of our experimental data.

There is experimental evidence of a very fast and effective calcium sequestration by mitochondria through a specific uniporter (Hehl et al., 1996; Applegate et al., 1997; Babcock et al., 1997). In some cases the Ca^{2+} uptake by mitochondria can be extremely fast due to a mechanism called the rapid mode (RaM) (Gunter et al., 2000; Rizzuto et al., 2000). Based on the experimental results that Ca^{2+} sequestration takes place at free cytosolic calcium levels of $>\sim$0.5–1.0 μM (Jouaville et al., 1995; Bernardi and Petronilli, 1996; Hehl et al., 1996; Herrington et al., 1996; Hoth et al., 1997; Ricken et al., 1998) a step-like kinetics is considered for the mitochondrial Ca^{2+} uptake by uniporters, J_{in} (Marhl et al., 1998a; Grubelnik et al., 2001; Haberichter et al., 2001).

$$J_{in} = k_{in} \frac{Ca_i^8}{K_m^8 + Ca_i^8}, \qquad (12)$$

where k_{in} represents the maximal permeability of the uniporters in the mitochondrial membrane, and K_m represents the half-saturation for Ca^{2+} (Marhl et al., 2000). As in our previous publication (Marhl et al., 1998a), in factor k_{in} the constant value of the mitochondrial transmembrane potential, $\Delta\psi^{(m)}$, is implicitly included. The potential difference $\Delta\psi^{(m)}$ is usually strongly changed only by fast release of calcium through the permeability transient pore (PTP). Note that under normal physiological conditions just a slow release of calcium from the mitochondria takes place (Bernardi and Petronilli, 1996; Eriksson et al., 1999; Svichar et al., 1999).

For the mitochondrial Ca^{2+} release through Na^+/Ca^{2+} and H^+/Ca^{2+} exchangers we consider a simple linear dependency on Ca_m (Marhl et al., 1998b; Grubelnik et al., 2001):

$$J_{out} = k_{out} Ca_m, \qquad (13)$$

where k_{out} is the maximal rate for calcium efflux from mitochondria.

Model parameters used in our calculations are given in figure captions. The parameter values for Ca^{2+} fluxes across the SR membrane were estimated in accordance with our

own experimental results. Data regarding the RyR activity were taken from the experiments done by Ogawa et al. (2000). The parameter values regarding Ca^{2+} binding to signaling and buffering proteins were taken from the experiments in the literature and were extensively discussed in our previous paper (Marhl et al., 1998a). The parameter values regarding the mitochondrial Ca^{2+} handling were also taken from the experimental results published in the literature and were exhaustively discussed in our previous papers (Marhl et al., 1998b, 2000; Haberichter et al., 2001).

Modeling of caffeine-induced $[Ca^{2+}]_i$ response

Cell stimulation with different concentrations of caffeine was simulated by varying the model parameter *Caff*. The calculated traces for *Caff* = 0.1 mM and 5 mM for 30 s are shown in Fig. 4 *A*, and correspond to the experimental results presented in Fig. 1 *A*. Fig. 4 *B* represents the calculated concentration-dependent RyR activity. The sigmoidal relationship between caffeine concentration and RyR activity is similar to that between caffeine concentration and $[Ca^{2+}]_i$ response showed in Fig. 1 *B*. Model calculations of $[Ca^{2+}]_i$ responses to 5-mM caffeine exposures for 1 s, 5 s, and 30 s are shown in Fig. 4 *C* (*dotted line*, *dashed line*, and *solid line*, respectively). They correspond to the experimental traces shown in Fig. 2 *A*, and reflect fast closure of RyR at the end of caffeine exposure.

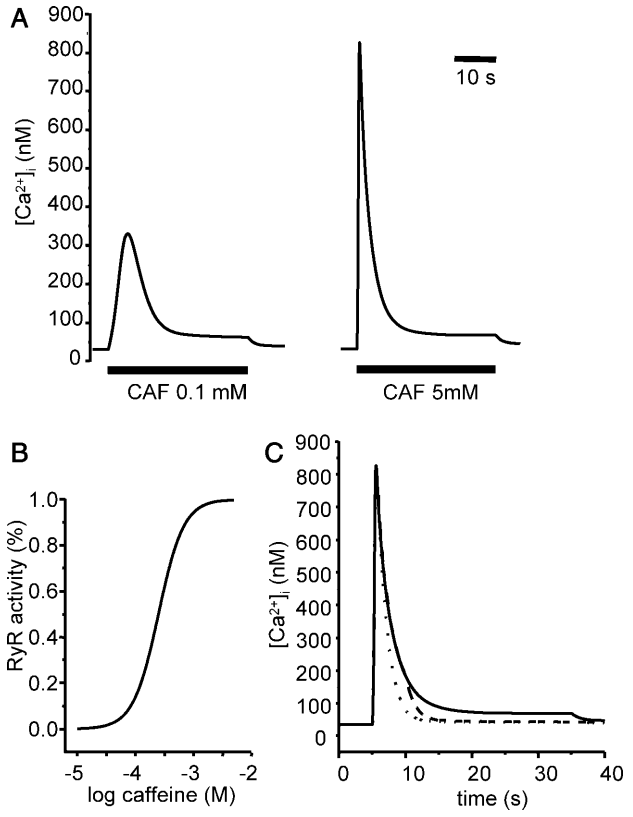

FIGURE 4 Simulation of Ca^{2+} response to caffeine. (*A*) Simulated response to 0.1 (*left*) and 5 (*right*) mM CAF for 30 s. (*B*) Concentration-activity curve of RyR. (*C*) Simulated responses to 30-s (*solid line*), 5-s (*dashed line*), and 1-s (*dotted line*) caffeine (5 mM). Parameter values are $\rho_{SR} = 0.01$; $\rho_m = 0.01$; $\beta_{SR} = 0.0025$; $\beta_m = 0.0025$; $Ca_{tot} = 50\ \mu M$; $SPr_{tot} = 90\ \mu M$; $BPr_{tot} = 120\ \mu M$; $Mg_i = 500\ \mu M$; $k_{RyR} = 2000\ s^{-1}$; $k_{SERCA} = 1\ \mu M\ s^{-1}$; $k_{leak} = 0.02\ s^{-1}$; $k_{in} = 20\ \mu M\ s^{-1}$; $k_{out} = 0.1\ s^{-1}$; $k_{on} = 0.1\ \mu M^{-1}\ s^{-1}$; $k_{off} = 0.01\ s^{-1}$; $K_{Caff} = 250\ \mu M$; $K_{A,Ca} = 2.5\ \mu M$; $K_{A,Mg} = 75\ \mu M$; $K_{I,Ca} = 400\ \mu M$; $K_{I,Mg} = 300\ \mu M$; $K_{SERCA} = 0.1\ \mu M$; $K_m = 1\ \mu M$; and $K_{SPr} = 5\ \mu M$.

Modeling of Ca^{2+} dynamics upon calcium release from the SR

The model was used to predict a scenario of how Ca^{2+} is distributed among the calcium stores that may be involved in the OFF mechanisms after Ca^{2+} release from the SR. Ca^{2+} release was triggered by simulating 1-s caffeine stimulation (5 mM), and Fig. 5 presents the predicted scenario. Time 0 corresponds to the beginning of caffeine stimulation. During the $[Ca^{2+}]_i$ decay phase, the RyRs are closed, allowing effective Ca^{2+} pumping back by the SERCAs without further Ca^{2+} release through open RyRs. However, the increase in luminal Ca^{2+} concentration is slower than $[Ca^{2+}]_i$ decrease. The first process that takes place is Ca^{2+} binding to fast protein binding sites quickly followed by Ca^{2+} sequestration into mitochondria. After a short time delay, Ca^{2+} is shifted to the slow protein binding sites, and finally, after a considerably longer time delay, it is pumped back into the SR. The predictions of the model for the first 5 s after 1-s stimulation are shown in Fig. 5, *A* and *B*. Fig. 5 *C* represents the predicted long time variations in SR Ca^{2+} concentration. As in Fig. 5, *A* and *B*, stimulation runs up for 1 s. Simulations were performed for 0.1 and 5 mM caffeine. It can be seen that complete Ca^{2+} reuptake into the SR after maximal Ca^{2+} release is a slow process, that takes ~10 min. Caffeine (0.1 mM) triggers a smaller Ca^{2+} release from the SR, and faster recovery.

Involvement of SERCA in cytosolic Ca^{2+} clearance

Model prediction of SERCA blockade

According to the predictions of the model presented above, SERCA activity is not necessarily the key mechanism in the $[Ca^{2+}]_i$ decrease after Ca^{2+} release from the SR. To test this hypothesis, we compared model predictions of Ca^{2+} dynamics with active versus blocked SERCAs. We simulated two successive stimulations by 5 mM caffeine of 1 s at a 15-s interval, with and without active SERCAs. Fig. 6 shows the predicted calcium response and the decay phase after the first Ca^{2+} peak. There is no significant difference in $[Ca^{2+}]_i$ decay upon Ca^{2+} release from the SR in case of active versus blocked SERCAs, which indicates that in our model Ca^{2+} pumping back into the SR is not primarily involved in Ca^{2+} clearance from the cytosol.

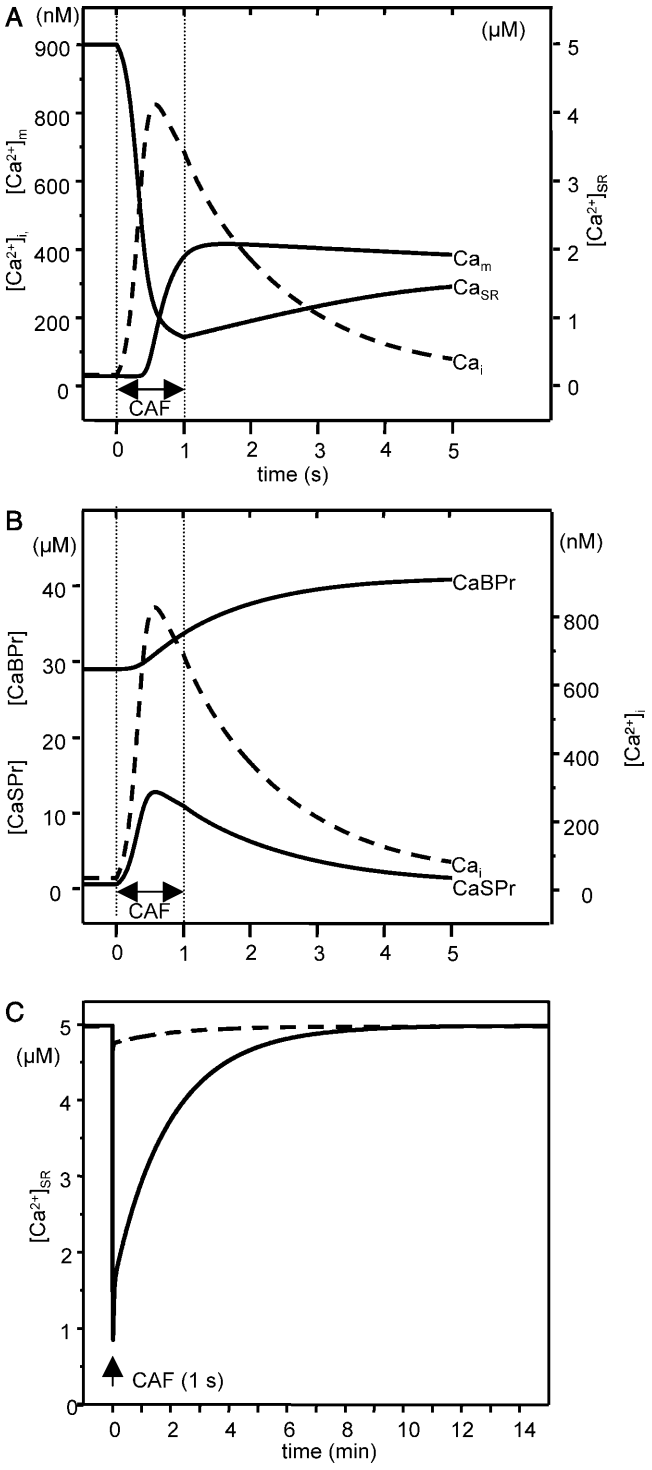

FIGURE 5 Model prediction of Ca^{2+} distribution after Ca^{2+} release from the SR. (*A* and *B*) Model predictions on Ca^{2+} handling upon 5-mM CAF stimulation. Stimulation begins at time 0 s and runs up to 1 s (*double arrow*). Parameter values are the same as in Fig. 4. Concentration in the cytosol (Ca_i) is indicated by the dashed line. (*A*) Concentration in the SR (Ca_{SR}) and mitochondria (Ca_m). (*B*) Ca^{2+} bound to fast-kinetics (CaSPr) and to slow-kinetics (CaBPr) cytosolic proteins. (*C*) Predicted long-time variations in SR Ca^{2+} concentration for 0.1 (*dashed line*) and 5 mM CAF (*solid line*). Stimulation begins at time 0 s and runs up to 1 s (*arrow*). Parameter values are the same as in Fig. 4.

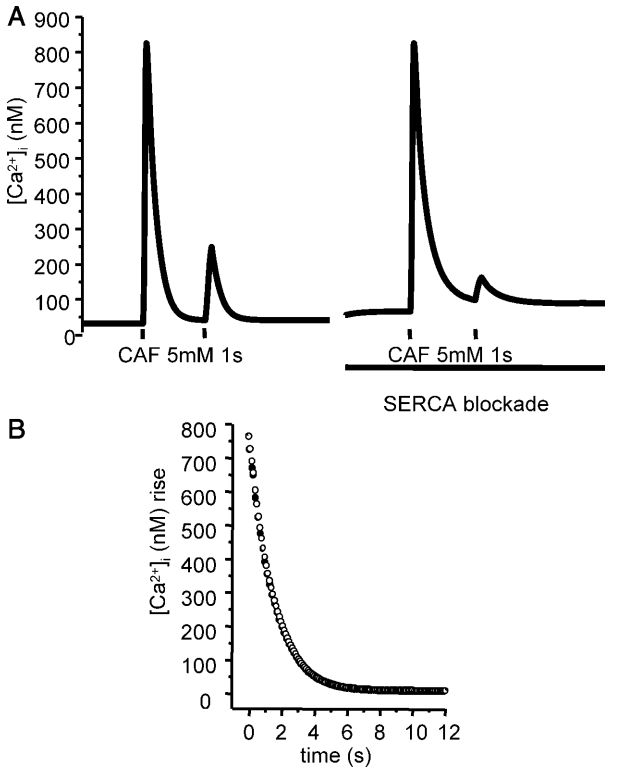

FIGURE 6 Model prediction of the effect of SERCA blockade on the Ca^{2+} response to two successive stimulations by 5 mM caffeine (1 s). (*A*) Predicted $[Ca^{+}]_i$ response to two successive 1-s stimulations by 5 mM CAF at a 15-s interval, with active (*left trace*) and inactivated (*right trace*) SERCA. (*B*) Predicted values of the decay phase after the first $[Ca^{+}]_i$ rise, with active (●) and inactivated (○) SERCA. Parameter values are the same as in Fig. 4.

Experimental effects of SERCA blockade

To test this prediction, experiments were performed using the same protocol as for simulated experiments, i.e., each cell was stimulated by two successive stimulations of 5 mM caffeine of 1 s at a 15-s interval, in the absence and in the presence of CPA, a reversible SERCA inhibitor. For each cell tested, exposure to 10 μM CPA was initiated 30 s before the first caffeine stimulation. Exposure to 10 μM CPA alone ($n = 14$) for 1 min induced no change or, in some cells, a slight increase in $[Ca^{2+}]_i$ 5–15 s after the beginning of exposure. This CPA-induced $[Ca^{2+}]_i$ increase, for which the mean value was 28.4 ± 7.4 nM, was transient and the $[Ca^{2+}]_i$ at the end of CPA exposure was not significantly altered compared to baseline before CPA exposure (99.3 ± 0.9% control). When cells were stimulated by caffeine 30 s after the beginning of exposure to CPA, the first peak was not significantly modified versus control (96.4 ± 6.9% control; $n = 21$), in accordance with the predicted results, but the Ca^{2+} response to the second stimulation was greatly decreased (49.2 ± 8.1% control; $p < 0.05$), indicating that the SERCA was blocked by CPA and that, in the absence of SERCA inhibition, the Ca^{2+} response to the second caffeine stimulation was mainly due to effective Ca^{2+} pumping back

into the SR. Typical traces and mean values are given in Fig. 7.

Under these conditions, we analyzed the $[Ca^{2+}]_i$ decay phase after the first caffeine stimulation for 12 s, i.e., a time sufficient for the $[Ca^{2+}]_i$ to return to a steady-state value. Mean values calculated from 21 cells in each condition are shown in Fig. 8. Statistical comparison showed no significant difference in the absence versus in the presence of 10 μM CPA.

To verify that, because of low passive Ca^{2+} leak, short-time SERCA blockade does not significantly deplete the SR, we assessed the time-dependent effect of the SERCA inhibitors CPA and thapsigargin (TG) on Ca^{2+} homeostasis. Experiments were performed in Ca^{2+}-free medium to avoid any calcium influx that may be involved in long-term SERCA blockade. Each cell tested was stimulated by two successive ejections of 5 mM caffeine for 5 s at a 15-s interval. The Ca^{2+} response to the first stimulation was used as an estimate of SR Ca^{2+} load. The loss of response to the second stimulation ensured that Ca^{2+} pumping back by SERCAs was actually inhibited. $[Ca^{2+}]_i$ responses in Ca^{2+}-free medium without SERCA inhibition were fitted by linear regression and the slope did not significantly differ from zero (Fig. 8 *B*, *crosses* fitted by *solid straight line*). Exposure to 10 μM CPA up to 25 min in the absence of external Ca^{2+} did not significantly alter the resting $[Ca^{2+}]_i$ (100.3 $\pm$ 4.2% control, $n = 20$). Decrease in the calcium response to caffeine was observed several minutes after the beginning of exposure to CPA, and complete abolishment of the response needed >10 min. Typical traces are shown in Fig. 8 *A*. Similar results were obtained with the irreversible inhibitor TG (1 μM) ($n = 14$). The time-dependent decrease of the amplitude of the first Ca^{2+} peak was sigmoidal with both SERCA inhibitors, and the duration of exposure to CPA (10 μM) and TG (1 μM) needed to induce 50% inhibition was 10.8 $\pm$ 1.0 min and 8.0 $\pm$ 0.5 min, respectively. Fig. 8 *B* shows the experimental values obtained with CPA (*solid*

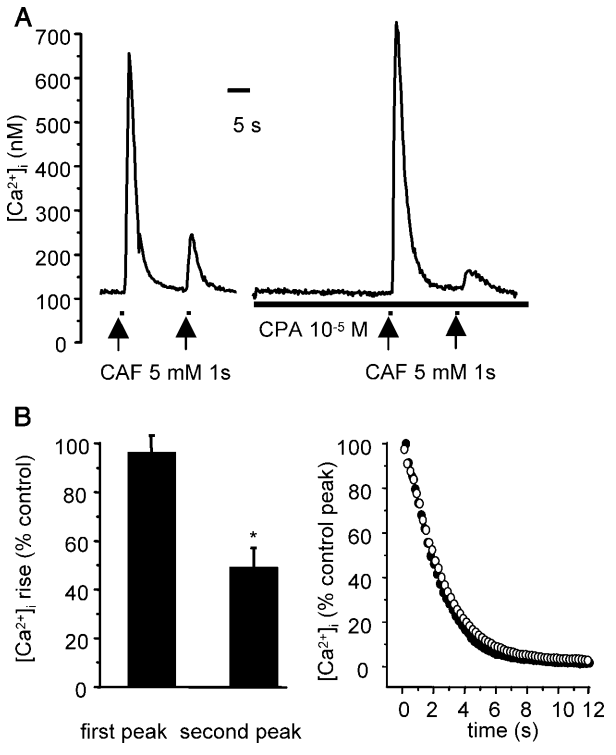

FIGURE 7 Effect of CPA (10 μM) on $[Ca^{2+}]_i$ response to successive 1-s stimulations with 5 mM caffeine. (*A*) Original traces of cells stimulated in the absence (*left trace*) and in the presence of CPA (*right trace*). (*B*) Effect of CPA on the calcium response to two successive CAF stimulations. Values were obtained from 21 cells, and are expressed as a percentage of control. Vertical bars are SE. *$p < 0.05$. (*C*) Mean $[Ca^{2+}]_i$ decay after the first CAF stimulation in the absence (●) and in the presence (○) of 10 μM CPA. Each curve in the mean value of 21 cells in each experimental conditions, expressed as percentage of the control peak, 0 corresponding to baseline.

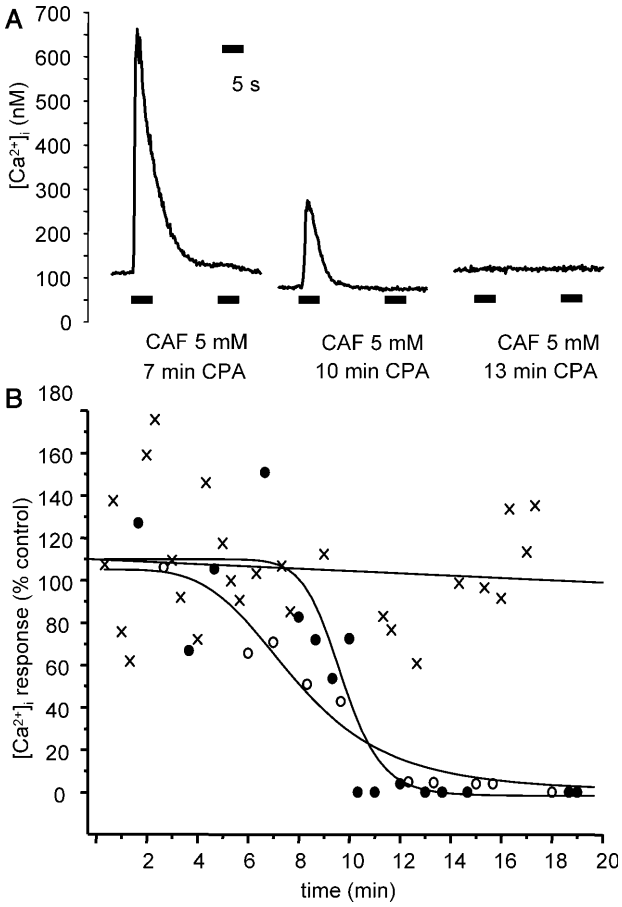

FIGURE 8 Effect of SERCA blockade on SR Ca^{2+} load. (*A*) Typical traces of $[Ca^{2+}]_i$ response to two successive 5-s CAF stimulations in cells preincubated with 10 μM CPA for 7 min (*left trace*), 10 min (*middle trace*), and 13 min (*right trace*). (*B*) Individual Ca^{2+} response to 5 mM CAF of cells incubated in Ca^{2+}-free medium (*crosses*) and incubated in Ca^{2+}-free medium with 10 μM CPA (●) or with 1 μM TG (○). Abscissa: duration of incubation. Ordinate: first peak amplitude in percent of control, i.e., response of cells in normal PSS. $[Ca^{2+}]_i$ in Ca^{2+}-free medium are fitted by linear regression; $[Ca^{2+}]_i$ responses in the presence of CPA and TG are fitted by a sigmoidal equation.

circles) and TG (*open circles*). These results indicate that spontaneous Ca^{2+} leak from the SR at rest was small.

Model prediction of mitochondrial Ca^{2+} uptake blockade

The theoretical and experimental results presented above show that Ca^{2+} pumping back by SERCA is not significantly responsible for the $[Ca^{2+}]_i$ decrease after Ca^{2+} release from the SR and, according to the model, Ca^{2+} uptake into mitochondria may be more important. To test this hypothesis, we compared model predictions of Ca^{2+} dynamics with active versus blocked mitochondrial Ca^{2+} uptake. We simulated the $[Ca^{2+}]_i$ response to 1-s stimulation by 5 mM caffeine, with and without active Ca^{2+} uniporter, and determined the respective $[Ca^{2+}]_i$ decays, as shown Fig. 9 *A*. According to the model, mitochondrial inhibition results in a slight change in the slope of the Ca^{2+} decay, indicating that mitochondrial Ca^{2+} uptake is actually involved in cytosolic Ca^{2+} clearance after Ca^{2+} release from the SR, but has a slight influence on the shape of the $[Ca^{2+}]_i$ decay.

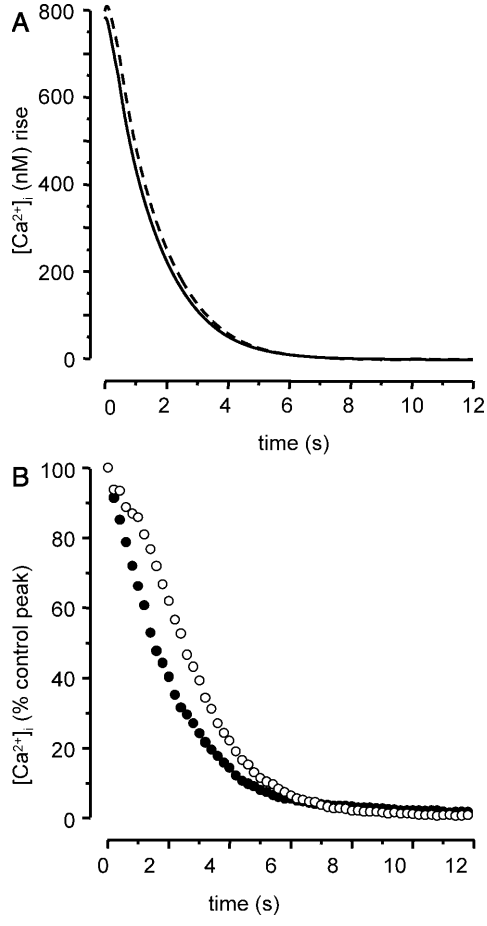

FIGURE 9 Predicted and experimental effect of mitochondrial Ca^{2+} uptake inhibition on the Ca^{2+} response to 5 mM caffeine (1 s). (*A*) Predicted values of the decay phase after CAF stimulation, with active (*solid line*) and inactivated (*dashed line*) mitochondrial Ca^{2+} uptake. Parameter values are the same as in Fig. 4. (*B*) Mean $[Ca^{2+}]_i$ decay after CAF-induced peak in the absence ($n = 21$, ●) and in the presence ($n = 25$, ○) of 5 μM FCCP, as a percentage of the control peak, 0 corresponding to baseline.

Experimental effects of mitochondrial Ca^{2+} uptake blockade

To test this prediction, experiments were performed in the presence of 5 μM FCCP, an uncoupler of mitochondrial function usually used at 1–5 μM (Gurney et al., 2000; Albrecht et al., 2002; Kang et al., 2003; Szado et al., 2003). FCCP (5 μM) assures a quick mitochondrial inhibition (Kang et al., 2003). For each cell tested, exposure to 5 μM FCCP was initiated 30 s before 1-s caffeine stimulation. Exposure to 5 μM FCCP alone for 30 s induced no change in $[Ca^{2+}]_i$ (data not shown). When cells were stimulated by caffeine 30 s after the beginning of exposure to FCCP, the first peak was not significantly modified versus control (102.4 ± 6.2% control; $n = 25$). We hence analyzed the $[Ca^{2+}]_i$ decay phase after the caffeine-induced peak. Mean values calculated in control condition ($n = 21$) and in the presencve of FCCP ($n = 25$) are shown in Fig. 9 *C*. In the presence of FCCP, the $[Ca^{2+}]_i$ decay was slower than that in control condition, and ANOVA analysis showed that the difference was statistically significant. However, the subsequent resting $[Ca^{2+}]_i$ value was not modified compared to control.

DISCUSSION

Our experimental study has shown that calcium signaling upon short caffeine stimulation results in Ca^{2+} dynamics within the cell without significant involvement of Ca^{2+} fluxes through the plasma membrane. On the basis on these experimental data, we build a relevant theoretical model of RyR stimulation and subsequent Ca^{2+} handling. Using this model in combination with the experimental approach, we used short-time stimulation by caffeine to investigate the OFF mechanisms, i.e., the mechanisms implicated in the return of $[Ca^{2+}]_i$ to baseline after $[Ca^{2+}]_i$ increase. Our experimental results, in accordance with our theoretical model, indicate that although Ca^{2+} pumping back by SERCA is active after Ca^{2+} release from SR upon RyR stimulation, it is not primarily involved in $[Ca^{2+}]_i$ decrease. Ca^{2+} uptake by mitochondria slightly but significantly shapes the $[Ca^{2+}]_i$ decay that may also depend on additional intracellular buffering processes.

Our cell model of RyR stimulation is a ''closed cell'' one, i.e., in which Ca^{2+} fluxes through the plasma membrane are not considered and the total Ca^{2+} concentration within the cell is kept constant. Although transmembrane Ca^{2+} fluxes are likely to be present in these cells, our results showed that such an approximation is relevant in our experimental conditions. Indeed, Ca^{2+} influx was not triggered by caffeine-induced Ca^{2+} release, because the $[Ca^{2+}]_i$ response was not altered in Ca^{2+}-free medium. Store-operated channels, activated by the depletion of intracellular Ca^{2+}

stores, have been described in a variety of smooth muscle cells. Some authors have speculated that the main physiological role of store-operated channels may be the refilling of the SR (Marin et al., 1999; Ng and Gurney, 2001). The fact that the second Ca^{2+} response to two successive caffeine stimulations, which is actually due to SR refilling because it is abolished in the presence of the SERCA blocker CPA, was not modified in Ca^{2+}-free medium indicates that such a mechanism is not involved in our experimental conditions. Usually, Ca^{2+} extrusion by PMCA and/or Na^+/Ca^{2+} exchanger is considered as an important OFF mechanism (Berridge et al., 2000; Sanders, 2001). In Na^+- and Ca^{2+}-free solution containing 0.5 mM lanthanum, that inhibits any Ca^{2+} influx or efflux (Tribe et al., 1994), the decay phase of the Ca^{2+} response was not modified, indicating that Ca^{2+} extrusion is not implicated in the quick $[Ca^{2+}]_i$ return to baseline. This is in accordance with previous studies that showed that the $[Ca^{2+}]_i$ response to contractile agonists like acetylcholine is not altered by removal of extracellular Ca^{2+} (Roux et al., 1996, 1997; Prakash et al., 1998; Bergner and Sanderson, 2002). In the absence of compensatory Ca^{2+} influx, fast Ca^{2+} extrusion would rapidly induce a complete loss of intracellular Ca^{2+} pool and hence loss of Ca^{2+} response.

The predicted values for the resting Ca^{2+} levels, that are ~40 nM, are lower than the experimental values, that were ~130 nM, in accordance with our previous results (Roux et al., 1997, 1998). However, due to relative imprecision of the calculation of absolute $[Ca^{2+}]_i$ values and interindividual variations so that values measured in some cells from our experiments were similar to that predicted but the model, the predicted values, though lower than the mean estimated $[Ca^{2+}]_i$, can be considered as in a physiological range.

We analyzed the OFF mechanisms after $[Ca^{2+}]_i$ increase using RyR stimulation. Single-channel activity measurement in lipid bilayer indicates that RyR2 and RyR3 differ in their sensitivity to Ca^{2+} inactivation and Mg^{2+} inhibition, so that for RyR3 Ca^{2+}-induced Ca^{2+} release may be reduced in the presence of physiological Mg^{2+} concentration (Ogawa et al., 2000). In accordance with the simulated Ca^{2+} response, stimulation by various caffeine concentrations showed a sigmoidal relationship between the amplitude of the Ca^{2+} response and the logarithmic concentration of caffeine. The fact that the response is not an all-or-none phenomenon suggests that Ca^{2+}-induced Ca^{2+} release is limited, which is consistent with a predominant RyR3 expression.

To investigate the mechanisms responsible for Ca^{2+} removal in smooth muscle cells, some authors have used voltage pulse to induce Ca^{2+} influx through voltage-dependent L-type Ca^{2+} channels (Kamishima and McCarron, 1998; Shmigol et al., 1999). In such a protocol, initial $[Ca^{2+}]_i$ increase is due to additional Ca^{2+} influx into the cell without SR Ca^{2+} depletion. However, in airway smooth muscle, although L-type Ca^{2+} channels may be activated during stimulation (Tomasic et al., 1992), contractile agonists such as acetylcholine act primarily via Ca^{2+} release from the SR (Roux et al., 1996, 1997; Prakash et al., 1998; Bergner and Sanderson, 2002). Hence, in this type of cells, it seems physiologically relevant to induce Ca^{2+} release from the SR rather than extracellular Ca^{2+} influx to investigate the subsequent OFF mechanisms. Analysis of fluorescence quenching and comparison of the decay phase after 1-, 5-, and 30-s caffeine stimulation indicate that caffeine washout and RyR closure occurred ~2–3 s after the beginning of caffeine ejection. Because the maximal $[Ca^{2+}]_i$ increase occurred approximately in the same time (see Fig. 2), we assumed that during the decay phase, the duration of which was ~10 s after maximal $[Ca^{2+}]_i$ increase, the RyRs were closed. Hence the mechanisms involved in cytosolic clearance in our experiments do not seem to be specific to RyR stimulation and are likely to be also implicated upon airway smooth muscle stimulation by physiological agonists that induce Ca^{2+} release from the SR.

Usually, Ca^{2+} uptake by SERCAs is considered as the main OFF mechanism (Marin et al., 1999; Berridge et al., 2000; Sanders, 2001). The analysis of the decay phase after short caffeine stimulation in vitro and in silico demonstrated that, although Ca^{2+} pumping back is actually active and does reload the SR, SERCA activity does not significantly modulate the shape of the $[Ca^{2+}]_i$ decay. Several authors have used CPA to inhibit the SERCA activity, with significant effect on Ca^{2+} decrease, including in airway myocytes (Sims et al., 1996, 1997; Yoshikawa et al., 1996; Shmigol et al., 1999). Global comparison of these different studies is difficult because the experimental protocols used to trigger $[Ca^{2+}]_i$ increase as well as to analyze $[Ca^{2+}]_i$ decay differed between studies. However, it appears in these studies that even if the $[Ca^{2+}]_i$ decay rate was altered in the presence of CPA, the general pattern was not deeply modified. Hence, $[Ca^{2+}]_i$ return to baseline should be attributed to mechanisms other than SERCA activity.

The model prediction about Ca^{2+} sequestration in mitochondria is in agreement with experimental results in some other systems, showing that mitochondria indeed sequester a large amount of Ca^{2+} released from the sarco/endoplasmic reticulum (for review see Schuster et al., 2002). For example, in chromaffin cells, around 80% of the Ca^{2+} released from the ER is cleared first into mitochondria (Babcock and Hille, 1998). In vascular smooth muscle cells, several recent studies have shown that mitochondria Ca^{2+} uptake occurs after SR Ca^{2+} release and $[Ca^{2+}]_i$ increase (Drummond and Fay, 1996; Drummond and Tuft, 1999; Pacher et al., 2000; Vallot et al., 2001; Kamishima and Quayle, 2002; Szado et al., 2003) but, to the best of our knowledge, this issue has not been previously addressed in airway smooth muscle cells. It should be noted that although our model predicts that mitochondrial Ca^{2+} uptake is effective after $[Ca^{2+}]_i$ increase, simulated inhibition of mitochondria uniporter does not greatly alter the shape of the $[Ca^{2+}]_i$ decay after 1-s caffeine stimulation, and this was

confirmed by our experimental results. In particular, the $[Ca^{2+}]_i$ decay phase itself was slowed in the presence of FCCP, but the subsequent resting value did not differ from control.

In conclusion, we have built a relevant theoretical model of Ca^{2+} handling upon RyR stimulation in airway myocytes. We have experimentally confirmed the model predictions that Ca^{2+} pumping back into the SR by SERCA, though effective, is not primarily involved in $[Ca^{2+}]_i$ decay upon Ca^{2+} release from the SR, and that Ca^{2+} uptake by mitochondria slightly but significantly shapes the $[Ca^{2+}]_i$ decay. The model provides a possible scenario for Ca^{2+} handling in which cytosolic Ca^{2+}-binding proteins play a key role in cytosolic Ca^{2+} clearance. Further studies are needed to check the model predictions about the role of these buffering processes in $[Ca^{2+}]_i$ modulation.

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