ORIGINAL RESEARCH

Ryanodine Receptor Sensitization Results in Abnormal Calcium Signaling in Airway Smooth Muscle Cells

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Abstract

Intracellular Ca^{2+} dynamics of airway smooth muscle cells (ASMCs) are believed to play a major role in airway hyperresponsiveness and remodeling in asthma. Prior studies have underscored a prominent role for inositol 1,4,5-triphosphate (IP_3) receptors in normal agonist-induced Ca^{2+} oscillations, whereas ryanodine receptors (RyRs) appear to remain closed during such Ca^{2+} oscillations, which mediate ASMC contraction. Nevertheless, RyRs have been hypothesized to play a role in hyperresponsive Ca^{2+} signaling. This could be explained by RyRs being "sensitized" to open more frequently by certain compounds. We investigate the implications of RyR sensitization on Ca^{2+} dynamics in ASMC using a combination of mathematical modeling and experiments with mouse precision-cut lung slices. Caffeine is used to increase the sensitivity of RyRs to cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and sarcoplasmic reticulum Ca^{2+} ($[Ca^{2+}]_{SR}$). In ASMCs, high caffeine concentrations (>10 mM) induce a sustained elevation of $[Ca^{2+}]_i$. Our mathematical model accounts for this by the activation of store-operated Ca^{2+} entry that results from a large increase in the RyR sensitivity to $[Ca^{2+}]_{SR}$ and the associated Ca^{2+} release, which leads to a reduction of $[Ca^{2+}]_{SR}$. Importantly, our model also predicts that: (*1*) moderate RyR sensitization induces slow Ca^{2+} oscillations, a result experimentally confirmed with low concentrations of caffeine; and (*2*) high RyR sensitization suppresses fast, agonist-induced Ca^{2+} oscillations by inducing substantial store-operated Ca^{2+} entry and elevated $[Ca^{2+}]_i$. These results suggest that RyR sensitization could play a role in ASMC proliferation (by inducing slow Ca^{2+} oscillations) and in airway hyperresponsiveness (by inducing greater mean $[Ca^{2+}]_i$ for similar levels of contractile agonist).

Keywords: precision-cut lung slice; mathematical modeling; asthma; hypersensitivity; Ca^{2+} oscillations

Clinical Relevance

Our work shows that increased ryanodine receptor (RyR) sensitivity can underlie unexpected complications in airway smooth muscle cell Ca^{2+} signaling and therefore in the downstream physiology. This suggests RyR as a target for therapeutic drugs.

Intracellular Ca^{2+} dynamics control a wide variety of cellular processes. In particular, airway smooth muscle cell (ASMC) contraction is mediated by oscillations in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), which correspond to the cyclic release and reuptake of Ca^{2+} from the sarcoplasmic reticulum (SR). The release of Ca^{2+} from the SR occurs via two types of channels, the inositol 1,4,5-triphosphate (IP_3) receptor (IP_3R) and the ryanodine receptor (RyR), whereas Ca^{2+} reuptake into the SR is performed by sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPases (SERCAs). Ca^{2+} may also enter the cell via receptor-operated, store-operated, and voltage-operated Ca^{2+} channels, and be extruded from the cell across the plasma membrane by Ca^{2+} ATPases (PMCAs). In ASMCs, G protein–coupled receptor stimulation (by agonists) and membrane depolarization (by

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KCl) induce two types of Ca^{2+} oscillations (1, 2). KCl-induced Ca^{2+} oscillations have a low frequency (~3/min) as compared with faster agonist-induced Ca^{2+} oscillations (~10/min in human and ~30/min in mouse at room temperature; these are even faster at 37°C). In mouse ASMCs, KCl-induced Ca^{2+} oscillations induce twitching of the ASMCs and substantially less airway contraction as compared with agonist-induced contraction, but the relative magnitudes of KCl versus agonist-induced contraction is species dependent (2, 3).

We have previously (4) proposed a mechanism for how IP_3R and RyR interact to give these two oscillatory behaviors. The pathways are summarized in Figure 1. In brief:

- Agonist stimulation, and subsequent production of IP_3, leads to release of Ca^{2+} through the IP_3R, and a cycle of Ca^{2+} release and reuptake to and from the SR, via the mechanism of Ca^{2+}-induced Ca^{2+} release. This causes sufficient depletion of the SR to inactivate the RyR, which thus play no significant role in on-going agonist-induced Ca^{2+} oscillations (*see* Figure E1A in the online supplement).
- KCl stimulation causes overfilling of the SR, which activates the RyR and leads to Ca^{2+} release from the SR, again in a process of Ca^{2+}-induced Ca^{2+} release (Figure E1B).
- The IP_3R and RyR interact via their joint effects on $[Ca^{2+}]_i$ and sarcoplasmic reticulum Ca^{2+} ($[Ca^{2+}]_{SR}$). The fact that RyRs become desensitized when the SR is depleted plays a crucial role in determining the relative contributions of IP_3R and RyR to the control of Ca^{2+} oscillations.

As such, increasing the Ca^{2+} sensitivity of RyR (which we refer to in this work as RyR "sensitization") could have important implications for the participation of RyRs in Ca^{2+} dynamics in hyperresponsive conditions. In addition to agonist-induced calcium signaling being intrinsically altered in asthmatic airway (e.g., Ref. 5), proinflammatory factors present in asthma enhance $[Ca^{2+}]_i$ responses of (nonasthmatic) ASMCs to agonist stimulation and/or to store depletion (6–12). There is evidence that the RyR could play a role in these augmented Ca^{2+} responses (13, 14). This role for RyR sensitivity can be explored experimentally

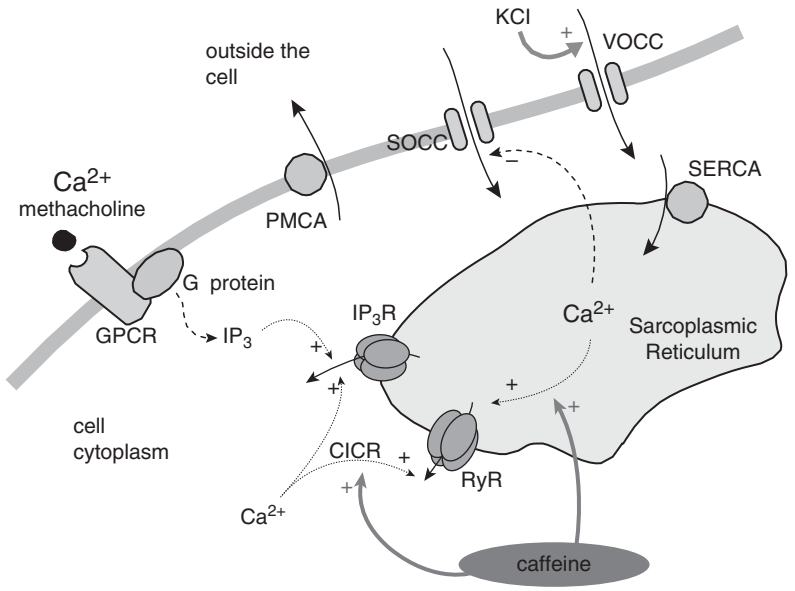

Figure 1. Schematic diagram of the major pathways involved in the model of Ca^{2+} dynamics in airway smooth muscle cells (ASMCs). *Solid black lines* denote Ca^{2+} fluxes. Stimulation of cell surface G protein–coupled receptors (GPCR) leads to production of inositol 1,4,5-triphosphate (IP_3) and activation of the IP_3 receptor (IP_3R). Subsequent release of Ca^{2+} from the sarcoplasmic reticulum (SR) leads to Ca^{2+}-induced Ca^{2+} release (CICR) through either IP_3R or ryanodine receptors (RyRs). Depletion of the SR induces the opening of store-operated Ca^{2+} channels (SOCCs) in the plasma membrane but also inactivates RyR. Ca^{2+} is removed from the cytoplasm by plasma membrane ATPase (PMCA) or by sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA). Addition of extracellular KCl leads to membrane depolarization and opening of voltage-operated Ca^{2+} channels (VOCCs). The consequent increase in Ca^{2+} influx causes (in the absence of agonist) overfilling of the SR and activation of RyR. IP_3R and RyR interact via their effects on the concentration of Ca^{2+} ($[Ca^{2+}]$) in the cytosol and the SR. Here, we focus on the predicted effects of caffeine (and other compounds), which sensitizes RyR to cytosolic and luminal Ca^{2+}.

with compounds, such as caffeine, that increase the sensitivity of RyR to $[Ca^{2+}]_{SR}$ and $[Ca^{2+}]_i$ (15, 16). Therefore, in this work, we integrate both IP_3R and RyR dynamics into an extension of our previous mathematical model of Ca^{2+} dynamics in ASMC (17), and experimentally test the predictions of this model. The consequences of RyR sensitization on ASMC Ca^{2+} dynamics is evaluated in the absence and presence of agonist stimulation.

Materials and Methods

Experimental Methods

Precision-cut lung slices (PCLSs) were prepared from female BALB/c mice (7–12 wk old) as described in Section E1.1 of the online supplement. All experiments were conducted at body temperature (37°C) in a custom-made, temperature-controlled microscope chamber as described in (3).

Measurement of Ca^{2+} oscillations: PCLSs were incubated in Hanks' buffered salt solution supplemented with 20 mM HEPES (sHBSS) containing 20 μM Oregon Green 488 BAPTA-1-AM (Invitrogen, Carlsbad, CA), a Ca^{2+}-sensitive dye, 0.1% Pluronic F-127 (Invitrogen), and 200 μM sulfobromophthalein in the dark at 30°C for 1 hour. Subsequently, the PCLSs were incubated in 200 μM sulfobromophthalein for 30 minutes. Slices were mounted on a cover glass and held down with 200-μm nylon mesh. A smaller cover glass was placed on top of the mesh and sealed at the sides with silicone grease to facilitate solution exchange. Mounted lung slices were continuously perfused with sHBSS or sHBSS containing the required compounds. ASMC Ca^{2+} signals were examined with a custom-built two-photon scanning laser microscope with a 40× oil immersion objective (NA 1.35) and images recorded at 15 or 30 images s^{-1}. Changes in fluorescence intensity (which represent changes in $[Ca^{2+}]_i$) were analyzed in an ASMC of interest by averaging the gray value of up to a 10 × 10-pixel region using

custom written software. Relative fluorescence intensity was expressed as a ratio of the fluorescence intensity (F_t/F_0; i.e., fluorescence intensity at a particular time [F_t] normalized with respect to the initial fluorescence intensity [F_0]).

Statistical Analysis

Results on Ca^{2+} oscillation frequency are shown as mean (±SEM). Student's *t* test was used to compute the 95% confidence interval of the mean (mean $\pm t^{n-1}_{0.975}$ SEM, where $t^{k}_{0.975}$ denotes the quantile at 97.5% of the student distribution with *k* degrees of freedom), and to evaluate the significance of the difference between means at different caffeine concentrations.

Mathematical Model

The model is an extension of previous work (17). In brief, the dynamics of $c = [Ca^{2+}]_i$ and $c_s = [Ca^{2+}]_{SR}$ are governed by:

$$\frac{dc}{dt} = J_{in} - J_{PMCA} + J_{rel} - J_{SERCA},$$
$$\frac{dc_s}{dt} = \gamma(J_{SERCA} - J_{rel}), \quad (1)$$

where J_{in} models Ca^{2+} influx (principally through store-operated Ca^{2+} channels), J_{PMCA} and J_{SERCA} are the Ca^{2+} fluxes through the PMCA and SERCA pumps, respectively, and J_{rel} models the flux out of the SR through IP_3R and RyR. Full details of each of these fluxes are given in the online supplement (Section E1.2).

For our purposes, the most important term is J_{rel}, which contains a model of the RyR flux. In this flux, the open probability of the RyR is assumed to be given by:

$$P_{RyR}(c, c_s) = \frac{c^3}{K_{cR}^3 + c^3} \frac{c_s^4}{K_{sR}^4 + c_s^4}. \quad (2)$$

Thus, the RyR sensitivity to cytosolic and luminal Ca^{2+} is governed by the two parameters, K_{cR} and K_{sR}, respectively.

Results

Effect of RyR Sensitization on Resting Ca^{2+}

Model predictions. Sensitization of the RyR is obtained in the model by decreasing the cytosolic Ca^{2+} activation threshold (K_{cR}) or the luminal Ca^{2+} activation threshold (K_{sR}) from their resting values (Equation 2 and Table E1). We define the indices, cytosolic sensitivity (r_c) and luminal sensitivity (r_s), to reflect the degree of cytosolic and luminal sensitization, respectively, as follows: $r_c = (K_{cR0} - K_{cR})/K_e$ and $r_s = (K_{sR0} - K_{sR})/K_s$, where K_{cR0} and K_{sR0} are the resting values of K_{cR} and K_{sR} in Table E1. Thus, increasing values of r_c and r_s represent increasing cytosolic and luminal sensitization, respectively, of RyR to Ca^{2+}.

Figures 2A and 2B show the effect of increasing r_c and r_s, respectively, on $[Ca^{2+}]_i$ dynamics. Both moderate cytosolic and luminal sensitization of RyR induce low-frequency RyR-mediated Ca^{2+} oscillations (*panels i* and *ii*), with a frequency that increases with increased sensitization. We note that the induction of Ca^{2+} oscillations by an increase in r_s can be understood as the mirror mechanism by which KCl induces Ca^{2+} oscillations: the increase in $[Ca^{2+}]_{SR}$ induced by membrane depolarization, which activates RyR (Figure E1B), is effectively replaced by a decrease in the $[Ca^{2+}]_{SR}$ half-activation threshold, K_{sR} (i.e., overcoming the threshold is equivalent to lowering it sufficiently).

At higher RyR sensitization (Figures 2A and 2B [*iii*]), the Ca^{2+} oscillations disappear and are replaced by a steady $[Ca^{2+}]_i$, with one important difference: the steady $[Ca^{2+}]_i$ induced by high cytosolic RyR sensitization remains close to the resting $[Ca^{2+}]_i$, whereas the steady $[Ca^{2+}]_i$ induced by high luminal sensitization is substantially elevated. This is worth noting for comparison with experimental observation (Ref. 1 and Figure 2C [*iii*]; *see* subsequent text). The model indicates that this difference in steady state is due to the different levels of store-operated Ca^{2+} entry (SOCE) activation. Indeed, as we have previously shown (17), a sustained increase in Ca^{2+} influx (i.e., a Ca^{2+} flux from the extracellular medium into the cytosol) is required for a sustained increase in $[Ca^{2+}]_i$. An increase in RyR r_s, but not r_c, induces a sustained Ca^{2+} influx, because it allows RyR to remain open at lower $[Ca^{2+}]_{SR}$, and thus SOCE is substantially activated. Figure 2E illustrates this mechanism: with increased RyR luminal sensitivity, the RyRs open more frequently (*magenta curve*), because the $[Ca^{2+}]_{SR}$ (Figure 2D) reaches the lower threshold for RyR activation sooner. Simultaneously, because the $[Ca^{2+}]_{SR}$ remains lower, a larger fraction of the store-operated Ca^{2+} channel remains open (Figure 2E, *blue curve*). The results of Figures 2A and 2B are summarized and extended in the online supplement (Figure E3).

Experimental validation. Caffeine has been shown by other groups to increase RyR sensitivity to both cytosolic and luminal Ca^{2+} in artificial lipid bilayers (15, 16). In addition, in ASMCs, high caffeine concentrations (20 mM) induce a sustained, elevated $[Ca^{2+}]_i$ (1), which is a signature for luminal RyR sensitization according to our model (Figure 2B [*iii*]). Consequently, to validate the predictions of our model that RyR sensitization influences the Ca^{2+} dynamics of ASMCs, we used varying concentrations of caffeine to sensitize the RyR of ASMCs in mouse lung slices (Figure 2C).

Stimulation of ASMCs with 1–4 mM caffeine (at 1-mM intervals) induced low-frequency $[Ca^{2+}]_i$ oscillations (Figures 2C [*i* and *ii*]). The experimental dose–response curve for caffeine (for a total of four different caffeine concentrations) is given in Figure E4. It can be seen that the oscillation frequency increases as a function of caffeine concentration. Specifically, the mean oscillation frequency increased from 1.4 (±0.49)/min for 1 mM caffeine, to 3.5 (±1.1)/min for 3 mM caffeine, the increase between the two means being statistically significant at the 1% level. This is consistent with an increase in RyR sensitization, as shown by Figures 2A and 2B. We note, however, substantial variation in the Ca^{2+} response, shape, and frequency between cells (Figure E5). A similarly large cell variability is observed for KCl-induced Ca^{2+} oscillations (Figure E6).

By contrast, stimulation of ASMCs with higher concentrations of caffeine (>6 mM caffeine; 10 mM shown in Figure 2C [*iii*]) induced a steady, elevated $[Ca^{2+}]_i$. These results are explained by the model predictions for luminal sensitization of RyR (Figure 2B [*iii*]), and are similar to prior results obtained with 20 mM caffeine (1). In summary, these experimental results and model predictions indicate that RyR sensitization can lead to significant changes in the Ca^{2+} dynamics of ASMCs in resting conditions.

Effect of RyR Sensitization on Agonist-Induced Ca^{2+} Oscillations

Because ASMCs use Ca^{2+} oscillations to induce contraction, and this contraction may be related to airway hypersensitivity, we used our model to explore how RyR sensitivity may alter agonist-induced Ca^{2+} oscillations. There is evidence that $[Ca^{2+}]_{SR}$

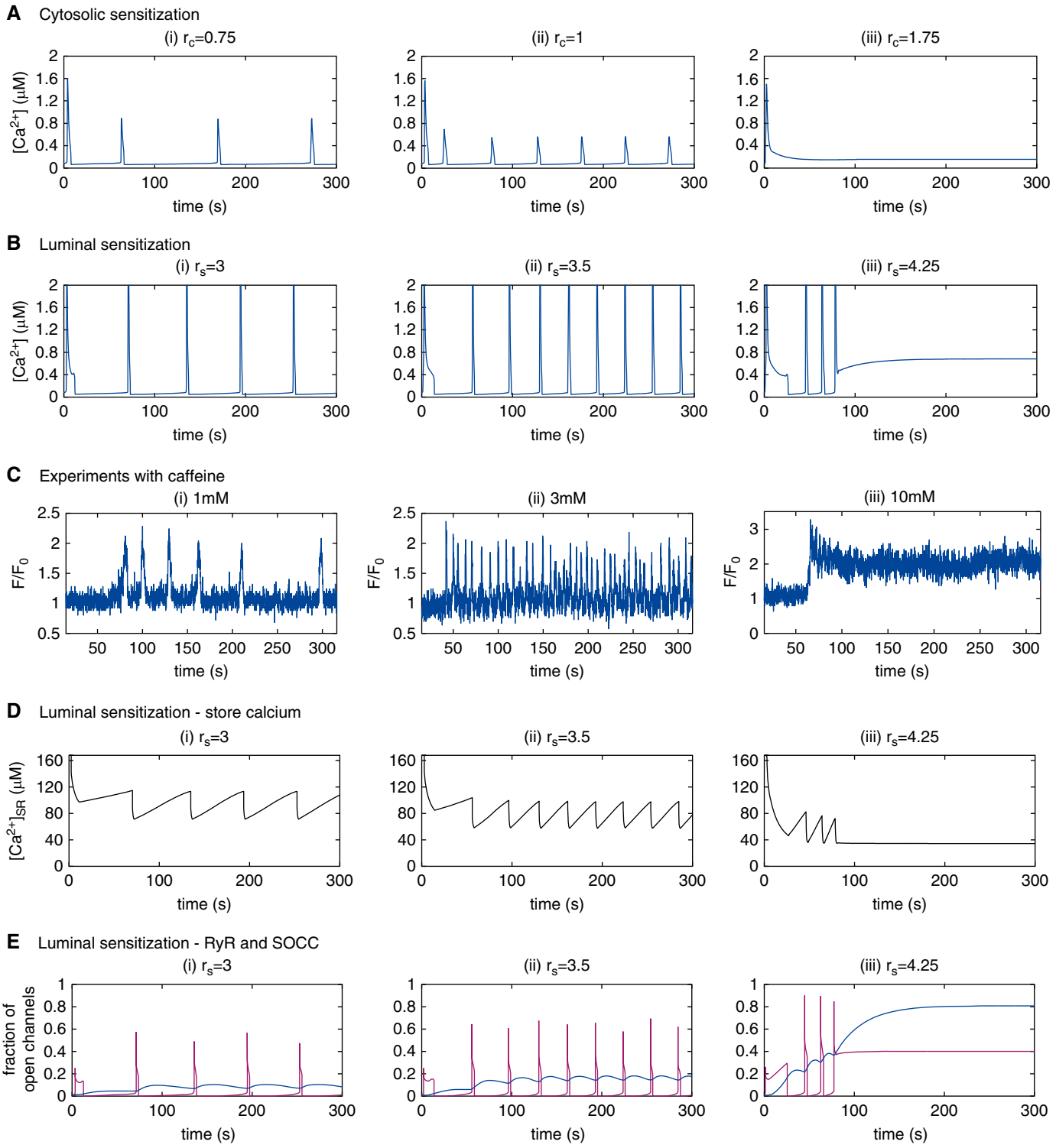

Figure 2. (*A*) Simulations of the effect of increasing levels of cytosolic RyR sensitization (r_c) on cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) dynamics, starting from (r_c, r_s) = (0, 1) (*red dot* in Figure E3A). (*B*) Simulations of the effect of increasing levels of luminal RyR sensitization (r_s) on $[Ca^{2+}]_i$ dynamics, starting from (r_c, r_s) = (0, 0) (no sensitization; *black dot* in Figure E3A). (*C*) Relative fluorescence signals indicating the $[Ca^{2+}]_i$ responses induced by caffeine in ASMCs of mouse lung slices (*see* Materials and Methods) for (*i*) 1 mM caffeine, (*ii*) 3 mM caffeine, and (*iii*) 10 mM caffeine. (*D*) The predicted sarcoplasmic reticulum Ca^{2+} ($[Ca^{2+}]_{SR}$) associated with the $[Ca^{2+}]_i$ dynamics modeled in *B*. (*E*) The predicted fraction of open RyRs (*magenta*) and open SOCCs (*blue*) associated with the $[Ca^{2+}]_i$ dynamics in *B*.

is reduced during agonist-induced Ca^{2+} oscillations (Ref. 4 and Figure E1A). We therefore expect that cytosolic sensitization of RyR, if not accompanied by substantial luminal sensitization, will have negligible effect on agonist-induced Ca^{2+} oscillations. This is because, in the absence of luminal sensitization, the opening probability of RyR decreases quickly with decreasing $[Ca^{2+}]_{SR}$. As a consequence, we begin the discussion of our study of the effect of RyR sensitization on agonist-induced Ca^{2+} oscillations by assuming luminal RyR sensitization only, and then discuss how these results are modified if there is concomitant cytosolic RyR sensitization.

We simulate pre-existing luminal RyR sensitization by setting r_s to specific values (Figures 3A and 3B) and mimic agonist application by increasing the IP_3 concentration (p) from zero to either 0.5 (Figure 3A) or 1.5 (Figure 3B) at a t of 200 seconds. These simulations show that low or moderate luminal RyR sensitization has little effect on Ca^{2+} oscillations induced by low IP_3 concentrations ($p = 0.5$); a small effect on Ca^{2+} oscillation frequency is noticeable with an r_s of 3 (compare *middle* and *bottom panels* in Figure 3A; *see also red curve* in Figure 3C).

By contrast, moderate luminal RyR sensitization ($r_s = 3$) has a greater effect on Ca^{2+} oscillations induced by higher IP_3 concentration ($p = 1.5$, Figure 3B). The Ca^{2+} oscillations are abolished and replaced with sustained elevated $[Ca^{2+}]_i$ (*blue trace*). With higher levels of luminal RyR sensitization ($r_s = 4.25$), the substantially elevated $[Ca^{2+}]_i$ that follows the few initial slow Ca^{2+} oscillations is not significantly affected by an increase in $[IP_3]$ (*green trace*). Unfortunately, our experimental approach to test these predictions of RyR sensitization effect on agonist-induced Ca^{2+} oscillations with caffeine were confounded by the inhibitory action of caffeine on the IP_3R (18, 19) (*see also* DISCUSSION). We found the frequency of agonist-induced Ca^{2+} oscillations to be smaller (instead of larger) in the presence of

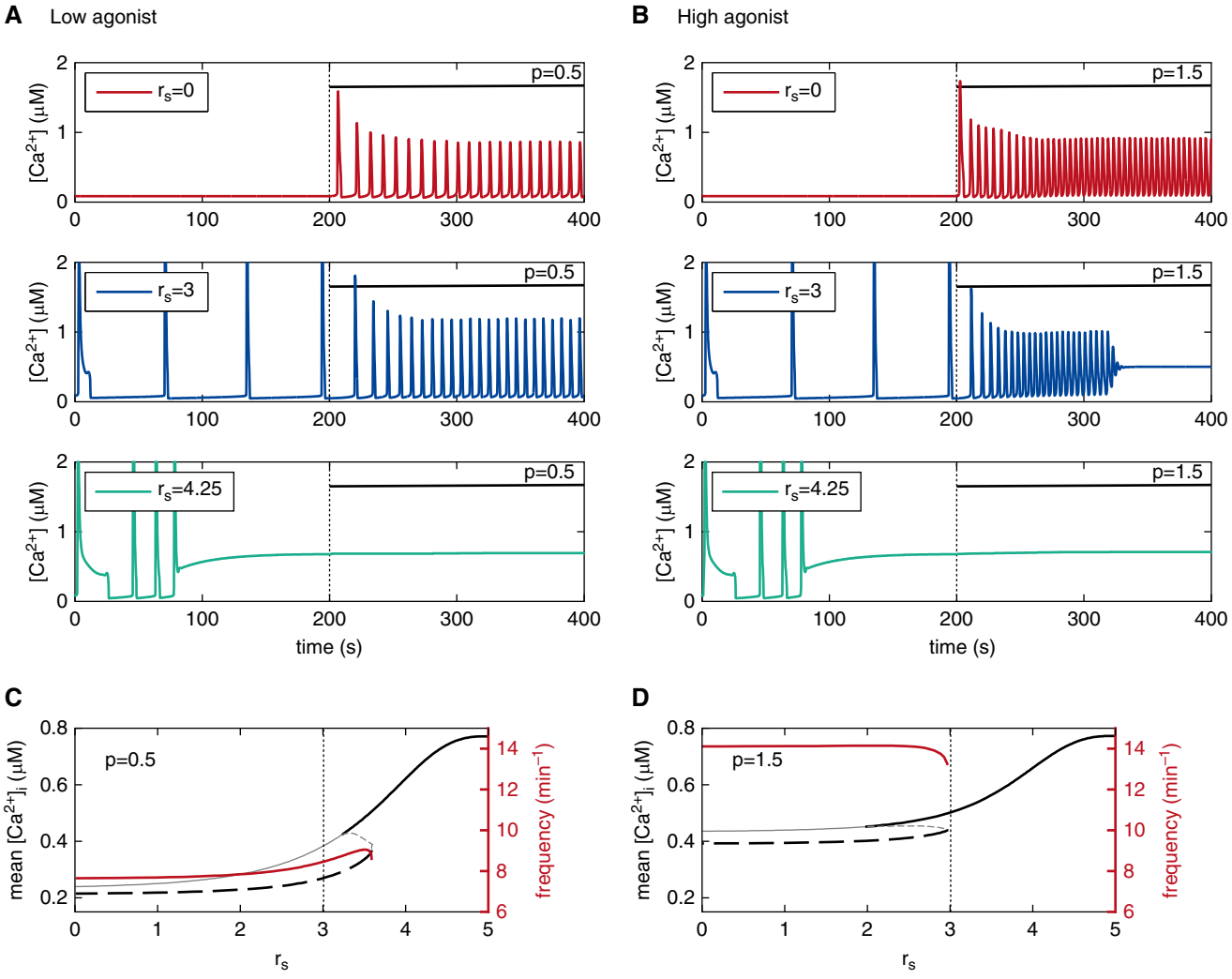

Figure 3. (*A* and *B*) Effect of agonist application on ASMC Ca^{2+} dynamics with different levels of pre-existing luminal RyR sensitization (r_s; increasing from *top* to *bottom*—*red* to *blue* to *green*), in the absence of cytosolic RyR sensitization ($r_c = 0$). (*A*) Low $[IP_3]$ ($p = 0.5$), and (*B*) high $[IP_3]$ ($p = 1.5$), applied at t of 200 seconds ($p = IP_3$ concentration). (*C* and *D*) Long-term mean $[Ca^{2+}]_i$ (*black*) and Ca^{2+} oscillation frequency (*red*) as a function of luminal RyR sensitization for the two agonist levels in *A* and *B*.

caffeine than in its absence (Figures E7–E9), even at high agonist concentrations (800 nM methacholine [MCh]). This outcome can be reproduced with the model by assuming that caffeine inhibits the IP_3R (e.g., decreases IP_3R conductance) in addition to increasing luminal RyR sensitization (simulations not shown).

A summary of the interaction between luminal RyR sensitization and agonist stimulation is shown in Figures 3C and 3D. The frequency of Ca^{2+} oscillations (*red curve*) at low or high IP_3 concentrations is relatively constant for increasing RyR sensitivity up to an r_s of 2.5. The same applies for mean $[Ca^{2+}]_i$ (*dashed black curve*). However, at higher RyR sensitivities ($r_s > 3$), the mean $[Ca^{2+}]_i$ increases gradually with increasing r_s, and Ca^{2+} oscillations are suppressed.

Figure 4 shows how Figure 3A (*middle panel*) and Figure 3C are modified if we assume cytosolic RyR sensitization ($r_c = 0.5$ or $r_c = 1$) in addition to luminal RyR sensitization ($r_s = 3$ in Figure 4A). Moderate cytosolic sensitization ($r_c = 0.5$) increases the frequency of agonist-induced Ca^{2+} oscillations (Figure 4A, *cyan trace*), whereas large cytosolic sensitization ($r_c = 1$) suppresses the Ca^{2+} oscillations (Figure 4A, *magenta trace*). Figure 4B shows that cytosolic sensitization reduces the range of luminal sensitization (r_s) in which agonist-induced Ca^{2+} oscillations persist, and renders the Ca^{2+} oscillation frequency sensitive to luminal sensitization (compare with Figure 3C).

Figure 5 underscores the important implications of our model findings by showing how the mean $[Ca^{2+}]_i$ as a function of $[IP_3]$ is modified by increasing RyR sensitization. For any given agonist concentration (*dotted vertical lines* represent $p = 0.5$ and 1.5, used in Figure 3), the mean $[Ca^{2+}]_i$ is always higher in the presence of RyR sensitization; that is, the greater the RyR sensitization, the greater the mean $[Ca^{2+}]_i$. These response curves also show that the range of IP_3 concentrations that can induce Ca^{2+} oscillations (*dashed lines*) shrinks and disappears as luminal RyR sensitization is increased. In particular, the overlap between Ca^{2+} oscillations for an r_s of 0 (*dashed blue curve*) and steady Ca^{2+} for an r_s of 3 (*solid red curve*) over a large range of $[IP_3]$ ($p = 0.8$–2.3 for $r_c = 0$) implies that moderate changes in RyR sensitivity can have a large impact on the dynamics of ASMC Ca^{2+} signaling. In summary, increasing luminal RyR sensitization progressively impairs agonist-induced Ca^{2+} oscillations and increases mean $[Ca^{2+}]_i$. A simultaneous increase in cytosolic RyR sensitization accelerates the suppression of the Ca^{2+} oscillations, but does not induce an additional increase in mean $[Ca^{2+}]_i$ at high IP_3 concentrations, where $[Ca^{2+}]_i$ is already steady (compare *blue* and *magenta curves*).

Discussion

In this work, we have investigated the effect of RyR sensitization on Ca^{2+} dynamics in ASMCs using a dual approach of mathematical modeling and experimental observation of ASMCs in mouse lung slices. Our objective was to use our model to explore the hypothesis that an increase in RyR sensitivity, which may result from ongoing airway inflammation, will alter the basic Ca^{2+} dynamics of ASMCs and their responses to contractile agonists. Any changes in the Ca^{2+} dynamics of ASMCs would be expected to have consequences for ASMC proliferation and airway hyperresponsiveness that are typical of asthma.

A key advance in our model of the Ca^{2+} dynamics of ASMCs that allows us to explore the effect of RyR sensitization is the

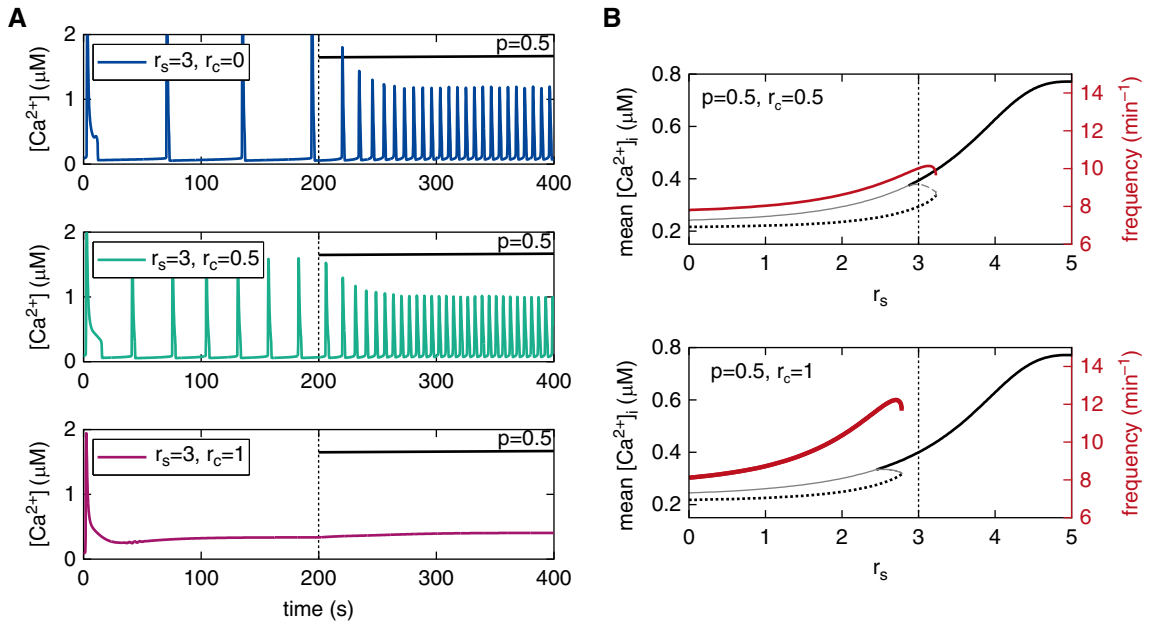

Figure 4. Effect of simultaneous cytosolic and luminal RyR sensitization on agonist-induced Ca^{2+} oscillations for low $[IP_3]$ ($p = 0.5$). (*A*) Increasing levels of cytosolic sensitization (from *top* to *bottom*) with RyR luminal sensitization ($r_s = 3$; *top panel* is identical to *middle panel* in Figure 3A). (*B*) Long-term mean $[Ca^{2+}]_i$ (*black curves*) and oscillation frequency (*red curve*) as a function of luminal RyR sensitization for the two non-zero sensitization levels used in *A* (compare with Figure 3C).

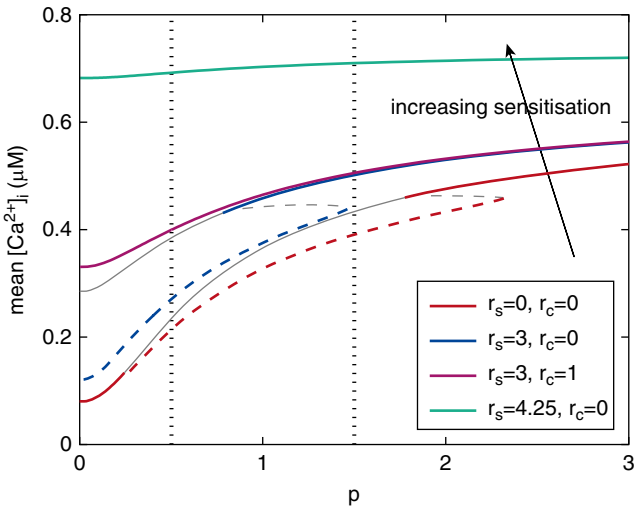

Figure 5. Effect of different levels of RyR sensitization (increasing as indicated by the *arrow*) on the entire dose–response curve relating mean $[Ca^{2+}]_i$ to $[IP_3]$ during agonist stimulation. *Dashed curves* correspond to Ca^{2+} oscillations and *solid curves* to steady Ca^{2+}. *Dotted vertical lines* indicate the $[IP_3]$ values used in Figures 3A and 3B. The *bottom* (*red*) *curve* is the dose–response curve in the absence of sensitization; hence, it corresponds to the response curve in Figure E2A. *Gray curves* represent unstable solutions.

incorporation of SOCE and the interaction of the luminal Ca^{2+} concentration of the SR with RyR. The regulation of SOCE is determined by $[Ca^{2+}]_{SR}$, which, in turn, is highly dependent on the open probability of the RyR, which is itself dependent on $[Ca^{2+}]_{SR}$. Agonists that produce IP_3 cause Ca^{2+} release from the SR, a gradual decrease of $[Ca^{2+}]_{SR}$ and gradual buildup of SOCE. KCl, on the other hand, causes increased Ca^{2+} influx, overloading of the SR, and release of Ca^{2+} through activated RyR. In this latter case, the SR is overloaded, and so SOCE does not occur; even the Ca^{2+} spikes do not deplete the SR enough to cause significant SOCE. Caffeine, however, enables RyR activation in the absence of SR overloading. Hence, the Ca^{2+} spikes induced by caffeine may deplete the SR sufficiently to result in increased SOCE. Thus, although caffeine and KCl both induce RyR opening, they have quite different effects on SOCE.

It is also important to note that, in our model, SOCE acts like a low-pass filter, responding not to each individual Ca^{2+} spike, but to mean levels of SR depletion or overfilling. Thus, individual Ca^{2+} spikes do not activate spikes of Ca^{2+} entry through store-operated Ca^{2+}.

The idea that the RyR may be involved in on-going inflammatory processes and may influence airway responsiveness has been previously suggested. For example, the cytokines, TNF-α and IL-13, were reported to increase the $[Ca^{2+}]_i$ responses of ASMC to contractile agonists, a mechanism that correlates with activation of CD38, the production of cyclic ADP-ribose, and the modification of the RyR (8, 12). Experimental results obtained with β-escin–permeabilized ASMCs suggest that cyclic ADP-ribose has a direct effect on RyR (13).

RyR Sensitization Stimulates Slow Ca^{2+} Oscillations

The first prediction of the model that is experimentally confirmed is that small increases in cytosolic and/or luminal RyR sensitivity result in low-frequency Ca^{2+} oscillations. Although the consequences of these Ca^{2+} oscillations for ASMC physiology are not currently known, we propose two possibilities. The first and most obvious would be an increase in ASMC tone. However, in mouse airways, low-frequency Ca^{2+} oscillations do not induce substantial contraction; each ASMC is observed to twitch asynchronously. This is a result of the combination of a low sensitivity of mouse ASMCs to Ca^{2+} and the short duration of myosin light-chain kinase activation. By contrast, other species, including human, show greater contraction to low-frequency Ca^{2+} oscillations. The second, but less obvious, consequence of low-frequency oscillations could be a change in gene expression. In previous studies, Ca^{2+} oscillations were generated with the repetitive photolytic release of IP_3 (20) or the alternate application of calcium and calcium-free solutions (21) to demonstrate that gene expression was initiated by low-frequency Ca^{2+} oscillations. Ca^{2+} oscillations generated by IP_3 at 1/min stimulated more gene expression than at 0.5/min or 2/min or by a sustained plateau (20). The effectiveness of Ca^{2+} oscillations with a period of over 100 seconds declined when using alternating Ca^{2+} solutions (21). In a similar manner, Ca^{2+} oscillations may also affect enzyme activity as part of a frequency-modulated control system (22). Thus, the stimulation of Ca^{2+} oscillations may have significant effects on the basic phenotype of the ASMCs. The idea that slow Ca^{2+} oscillations are associated with asthma is supported by the finding of spontaneous Ca^{2+} oscillations in human ASMCs from biopsies (23).

RyR Sensitization Increases Mean $[Ca^{2+}]_i$

A second major prediction of the model is that moderate or high luminal RyR sensitization results in higher mean (or elevated) $[Ca^{2+}]_i$ when the ASMCs are exposed to any concentration of contractile agonist. This prediction is consistent, irrespective of whether luminal RyR sensitization is moderate, and does not abolish the agonist-induced Ca^{2+} oscillations, or whether the RyR sensitization is high and converts the Ca^{2+} oscillations into a sustained $[Ca^{2+}]_i$ elevation. Cytosolic RyR sensitization can enhance the effect of luminal RyR sensitization on mean $[Ca^{2+}]_i$ when luminal sensitization is moderate and not capable by itself of abolishing agonist-induced Ca^{2+} oscillations. The immediate implication of a higher mean $[Ca^{2+}]_i$ is that, in the presence of agonist, RyR sensitization could generate greater ASMC contraction. This, in turn, suggests a possible mechanism for airway hyperresponsiveness.

The concept that increased (sustained) mean $[Ca^{2+}]_i$ results in increased contraction is demonstrated in Ca^{2+}-permeabilized human lung slices (2), where, for a given agonist concentration, airway contraction increases with constant $[Ca^{2+}]_i$ imposed. Thus, with RyR sensitization, the mean $[Ca^{2+}]_i$ would be higher than normal, resulting in additional contraction.

Another potential consequence of the replacement of agonist-induced Ca^{2+} oscillations with a sustained elevation in $[Ca^{2+}]_i$ is stimulation of Ca^{2+}-dependent

mechanisms that have slow response kinetics. It is already well known that the frequency and shape of Ca^{2+} oscillations are important controllers of contraction (24). However, there are potentially many other ways in which the frequency of the Ca^{2+} oscillations can control cellular processes; in the presence of RyR sensitization, recurrent exposure to agonist is likely to stimulate the ASMCs in ways other than simple contraction.

Unfortunately, however, we were unable to test this second prediction. Although caffeine is known to increase the sensitivity of RyR to luminal Ca^{2+}, it also inhibits the IP_3R. It is, thus, not possible to test the effects of RyR sensitization in the absence of other confounding effects. Experimental results are shown in Figures E7–E9. Addition of MCh and caffeine results in a plateau of increased Ca^{2+}, but this plateau has superimposed slow oscillations, a result that does not agree with our model predictions. It is possible to modify our model to include the potential effects of caffeine inhibition of the IP_3R, and simulations with such a modified model display much better agreement with the experimental results shown in Figures E7–E9. However, such modifications are speculative and outside the scope of the present study. It is left to future work to develop, more rigorously, a model of caffeine inhibition of IP_3R.

Given the impossibility of testing the effect of RyR sensitization on agonist-induced Ca^{2+} oscillations with caffeine, due to its inhibitory effect on the IP_3R, we have looked for other drugs that could possibly sensitize RyR without inhibiting IP_3R. We found that pentifylline induces slow Ca^{2+} oscillations similar to those induced by caffeine (Figures E10 and E11), but it is not clear whether pentifylline does so via luminal sensitization of RyR (which is necessary to prevent RyR inhibition during agonist-induced Ca^{2+} oscillations) or via another mechanism.

However, like caffeine, pentifylline impairs agonist-induced Ca^{2+} oscillations (Figures E12 and E13); this could be due to an inhibition of IP_3R, similar to that induced by caffeine, or possibly to the absence of luminal RyR sensitization. We have also tried the drug, chlorocresol (25), but it did not induce Ca^{2+} oscillations.

Limitations of the Model

In this work, we have modeled ASMC Ca^{2+} dynamics in a deterministic and homogenous manner, although Ca^{2+} channels operate stochastically, and are not distributed homogeneously on the SR membrane. The extent to which this stochasticity translates into "macroscopic" (cell-level) Ca^{2+} dynamics depends on the cell type, the Ca^{2+} channels involved, and the level of stimulation (e.g., agonist concentration). The spatial distribution of Ca^{2+} channels on the SR membrane could also be modified in disease. In ASMCs, as well as in other cell types, the distribution of the occurrence of Ca^{2+} spikes during agonist-induced Ca^{2+} oscillations is well described by a Poisson process, characterized by a linear dependence between the SD and the mean of the interspike interval (26). However, the main properties of these stochastic Ca^{2+} oscillations can be predicted by a deterministic mathematical model (27). Hence, the stochastic nature of the process does not preclude the value of a deterministic description of Ca^{2+} dynamics as used in this study.

The mathematical model used here was originally designed to account for agonist-induced Ca^{2+}oscillations and SOCE in ASMCs of human lung slices at room temperature. These Ca^{2+} oscillations are much slower than Ca^{2+} oscillations in mice (10/min versus 30/min). The model is therefore qualitative in this respect. Similarly, the RyR-mediated Ca^{2+} oscillations have a profile that is not fully reproduced by the model. Some differences in the Ca^{2+} profile of the model and the data are likely the result of the affnity of Oregon Green BAPTA-1-AM (OGB) that was used to report changes in Ca^{2+} ($K_d \sim 0.2$ μM). This reporter is saturated at $[Ca^{2+}]$ above 1 μM. Hence, peak amplitudes of Ca^{2+} oscillations are unlikely to be reproduced in the fluorescent signal. Similarly, the kinetics of OGB may not be fast enough to track the rapid changes in $[Ca^{2+}]_i$. This could explain the elevated fluorescent baseline observed during agonist-induced Ca^{2+} oscillations, which is not apparent during RyR-mediated Ca^{2+} oscillations.

However, our model accounts for a key feature of Ca^{2+} oscillation frequencies in ASMCs; that is, IP_3R-mediated Ca^{2+} oscillations are faster than RyR-mediated Ca^{2+} oscillations. This is because IP_3R gating is mainly governed by inhibition of the receptor by $[Ca^{2+}]_i$, whereas RyR gating is essentially governed by the level of Ca^{2+} store depletion. Because the refilling of the SR with Ca^{2+} occurs on a longer timescale than recovery of IP_3R from inhibition, RyR-mediated oscillations have a lower frequency than IP_3R-mediated oscillations. The frequencies of these two types of oscillations are closer in human ASMCs (10/min [IP_3R] versus 3/min [RyR]) than in mouse ASMC (30/min [IP_3R] versus 3/min [RyR]), which suggests that the effect of RyR sensitization on agonist-induced Ca^{2+} oscillations could be larger in human ASMCs (because oscillations of closer frequency are more likely to interact).

Scope of the Experimental Results

As mentioned previously, we have used mouse ASMCs in this study. These exhibit faster IP_3R-mediated Ca^{2+} oscillations than human ASMCs upon agonist stimulation, but both species exhibit maximum contraction (~55% in both cases) at the highest end of the frequency spectrum (e.g., figure 6 in Ref. 28). Hence, the two species respond differently to a given Ca^{2+} oscillation frequency. Our predictions are, however, independent of this quantitative difference, because, in both cases, we expect RyR sensitization–induced Ca^{2+} oscillations to enhance agonist-induced Ca^{2+} oscillations; the size of the effect will, of course, depend on the species, but the prediction is qualitatively the same.

The key observation, that increased RyR sensitivity can underlie unexpected complications in ASMC Ca^{2+} signaling and downstream physiology, suggests RyR as a target for therapeutic drugs. However, the exact nature of such a drug is diffcult to predict, as the mechanism of luminal Ca^{2+} sensitivity is not well understood. Compounds that could prevent Ca^{2+} binding to RyR would serve to reduce its Ca^{2+} sensitivity. This might take the form of a Ca^{2+} buffer within the SR; the protein, calsequestrin, serves such a process in the SR. Because the SR Ca^{2+} content is a key parameter, drugs reducing SERCA pump activity or SOCE may also counteract increased RyR sensitivity by helping to maintain reduced SR Ca^{2+} content. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

References

1. Perez JF, Sanderson MJ. The frequency of calcium oscillations induced by 5-HT, ACH, and KCl determine the contraction of smooth muscle cells of intrapulmonary bronchioles. *J Gen Physiol* 2005;125:535–553.
2. Ressmeyer AR, Bai Y, Delmotte P, Uy KF, Thistlethwaite P, Fraire A, Sato O, Ikebe M, Sanderson MJ. Human airway contraction and formoterol-induced relaxation is determined by Ca^{2+} oscillations and Ca^{2+} sensitivity. *Am J Respir Cell Mol Biol* 2010;43:179–191.
3. Bai Y, Sanderson MJ. The contribution of Ca^{2+} signaling and Ca^{2+} sensitivity to the regulation of airway smooth muscle contraction is different in rats and mice. *Am J Physiol Lung Cell Mol Physiol* 2009; 296:L947–L958.
4. Wang IY, Bai Y, Sanderson MJ, Sneyd J. A mathematical analysis of agonist- and KCl-induced Ca^{2+} oscillations in mouse airway smooth muscle cells. *Biophys J* 2010;98:1170–1181.
5. Trian T, Benard G, Begueret H, Rossignol R, Girodet PO, Ghosh D, Ousova O, Vernejoux JM, Marthan R, Tunon-de-Lara JM, *et al*. Bronchial smooth muscle remodeling involves calcium-dependent enhanced mitochondrial biogenesis in asthma. *J Exp Med* 2007;204: 3173–3181.
6. White TA, Xue A, Chini EN, Thompson M, Sieck GC, Wylam ME. Role of transient receptor potential C3 in TNF-α–enhanced calcium influx in human airway myocytes. *Am J Respir Cell Mol Biol* 2006;35:243–251.
7. Moynihan B, Tolloczko B, Michoud MC, Tamaoka M, Ferraro P, Martin JG. MAP kinases mediate interleukin-13 effects on calcium signaling in human airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 2008;295:L171–L177.
8. Sieck GC, White TA, Thompson MA, Pabelick CM, Wylam ME, Prakash YS. Regulation of store-operated Ca^{2+} entry by CD38 in human airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 2008;294: L378–L385.
9. Mahn K, Hirst SJ, Ying S, Holt MR, Lavender P, Ojo OO, Siew L, Simcock DE, McVicker CG, Kanabar V, *et al*. Diminished sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) expression contributes to airway remodelling in bronchial asthma. *Proc Natl Acad Sci USA* 2009;106:10775–10780.
10. Sathish V, Thompson MA, Bailey JP, Pabelick CM, Prakash YS, Sieck GC. Effect of proinflammatory cytokines on regulation of sarcoplasmic reticulum Ca^{2+} reuptake in human airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 2009;297:L26–L34.
11. Sathish V, Delmotte PF, Thompson MA, Pabelick CM, Sieck GC, Prakash YS. Sodium–calcium exchange in intracellular calcium handling of human airway smooth muscle. *PLoS One* 2011;6: e23662.
12. Deshpande DA, Dogan S, Walseth TF, Miller SM, Amrani Y, Panettieri RA, Kannan MS. Modulation of calcium signaling by interleukin-13 in human airway smooth muscle: role of CD38/cyclic adenosine diphosphate ribose pathway. *Am J Respir Cell Mol Biol* 2004;31: 36–42.
13. Prakash YS, Kannan MS, Walseth TF, Sieck GC. Role of cyclic ADP-ribose in the regulation of $[Ca^{2+}]_i$ in porcine tracheal smooth muscle. *Am J Physiol* 1998;274:C1653–C1660.
14. Jude J, Dileepan M, Panettieri R, Walseth TF, Kannan MS. Altered CD38/cyclic ADP-ribose signaling contributes to the asthmatic phenotype. *J Allergy (Cairo)* 2012;2012:289468.
15. Kong H, Jones PP, Koop A, Zhang L, Duff HJ, Chen SRW. Caffeine induces Ca^{2+} release by reducing the threshold for luminal Ca^{2+} activation of the ryanodine receptor. *Biochem J* 2008;414:441–452.
16. Porta M, Zima AV, Nani A, Diaz-Sylvester PL, Copello JA, Ramos-Franco J, Blatter LA, Fill M. Single ryanodine receptor channel basis of caffeine's action on Ca^{2+} sparks. *Biophys J* 2011;100:931–938.
17. Croisier H, Tan X, Perez-Zoghbi JF, Sanderson MJ, Sneyd J, Brook BS. Activation of store-operated calcium entry in airway smooth muscle cells: insight from a mathematical model. *PLoS One* 2013;8:e69598.
18. Parker I, Ivorra I. Caffeine inhibits inositol trisphosphate-mediated liberation of intracellular calcium in *Xenopus* oocytes. *J Physiol* 1991; 433:229–240.
19. Bezprozvanny I, Bezprozvannaya S, Ehrlich BE. Caffeine-induced inhibition of inositol(1,4,5)-trisphosphate-gated calcium channels from cerebellum. *Mol Biol Cell* 1994;5:97–103.
20. Li W, Llopis J, Whitney M, Zlokarnik G, Tsien RY. Cell-permeant caged InsP3 ester shows that Ca^{2+} spike frequency can optimize gene expression. *Nature* 1998;392:936–941.
21. Dolmetsch RE, Xu K, Lewis RS. Calcium oscillations increase the efficiency and specificity of gene expression. *Nature* 1998;392: 933–936.
22. Berridge MJ. The AM and FM of calcium signalling. *Nature* 1997;386: 759–760.
23. Sweeney D, Hollins F, Gomez E, Saunders R, Challiss RAJ, Brightling CE. $[Ca^{2+}]_i$ oscillations in ASM: relationship with persistent airflow obstruction in asthma. *Respirology* 2014;19:763–766.
24. Roux E, Guibert C, Savineau JP, Marthan R. $[Ca^{2+}]_i$ oscillations induced by muscarinic stimulation in airway smooth muscle cells: receptor subtypes and correlation with the mechanical activity. *Br J Pharmacol* 1997;120:1294–1301.
25. Al-Mousa F, Michelangeli F. Commonly used ryanodine receptor activator, 4-chloro-*m*-cresol (4CmC), is also an inhibitor of SERCA Ca^{2+} pumps. *Pharmacol Rep* 2009;61:838–842.
26. Skupin A, Kettenmann H, Winkler U, Wartenberg M, Sauer H, Tovey SC, Taylor CW, Falcke M. How does intracellular Ca^{2+} oscillate: by chance or by the clock? *Biophys J* 2008;94:2404–2411.
27. Cao P, Tan X, Donovan G, Sanderson MJ, Sneyd J. A deterministic model predicts the properties of stochastic calcium oscillations in airway smooth muscle cells. *PLoS Comput Biol* 2014;10:e1003783.
28. Lauzon AM, Bates JHT, Donovan G, Tawhai M, Sneyd J, Sanderson MJ. A multi-scale approach to airway hyperresponsiveness: from molecule to organ. *Front Physiol* 2012;3:191.