Effects of salbutamol on intracellular calcium oscillations in porcine airway smooth muscle

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1Departments of Anesthesiology, and Physiology and Biophysics, Mayo Clinic and Foundation, Rochester 55905; 2Department of Veterinary Pathobiology, University of Minnesota, St. Paul, Minnesota 55108; and 3Department of Pulmonary Diseases, University Hospital Nijmegen, Nijmegen, The Netherlands NL6500 HB

Prakash, Y. S., H. F. M. van der Heijden, M. S. Kannan, and G. C. Sieck. Effects of salbutamol on intracellular calcium oscillations in porcine airway smooth muscle. J. Appl. Physiol. 82(6): 1836–1843, 1997.—Relaxation of airway smooth muscle (ASM) by β-adrenoceptor agonists involves reduction of intracellular Ca2+ concentration ([Ca2+]i). In porcine ASM cells, acetylcholine induces [Ca2+]i oscillations that display frequency modulation by agonist concentration and basal [Ca2+]i. We used real-time confocal microscopy to examine the effect of salbutamol (1 nM to 1 µM), a β2-adrenoceptor agonist, on [Ca2+]i, oscillations in freshly dissociated porcine ASM cells. Salbutamol decreased the frequency of [Ca2+]i oscillations in a concentration-dependent fashion, completely inhibiting the oscillations at 1 µM. These effects were mimicked by a cell-permeant analog of adenosine 3',5'-cyclic monophosphate. The inhibitory effect of salbutamol was partially reversed by BAY K 8644. Salbutamol reduced [Ca2+]i, even when sarcoplasmic reticulum (SR) Ca2+ reuptake and Ca2+ influx were blocked. Lanthanum blockade of Ca2+ efflux attenuated the inhibitory effect of salbutamol on [Ca2+]i. The [Ca2+]i response to caffeine was unaffected by salbutamol. On the basis of these results, we conclude that β2-adrenoceptor agonists have little effect on SR Ca2+ release in ASM cells but reduce [Ca2+]i by inhibiting Ca2+ influx through voltage-gated channels and by enhancing Ca2+ efflux.

β2-adrenoceptor agonists; confocal imaging; asthma; calcium channel; calcium efflux

ELEVATION OF INTRACELLULAR Ca2+ CONCENTRATION ([Ca2+]i) plays an important role in the development and maintenance of force in airway smooth muscle (ASM) cells (27). Acetylcholine (ACh) acts as a bronchoconstrictor by elevating [Ca2+]i in ASM cells. Symptomatic treatment of bronchostiction in asthma involves the use of β2-adrenoceptor-specific agonists. Relaxation of ASM by β-adrenoceptor agonists involves reduction of intracellular Ca2+ concentration ([Ca2+]i). Studies in different smooth muscle types have demonstrated that stimulation of β-adrenoceptors may decrease inositol 1,4,5-trisphosphate (IP3)-induced Ca2+ release from the sarcoplasmic reticulum (SR) (4, 20), promote Ca2+ reuptake (5, 17), promote Ca2+ efflux (3, 6, 15, 31), and/or inhibit Ca2+ influx (4, 26). Adenosine 3',5'-cyclic monophosphate (cAMP)-dependent hyperpolarization and consequent reduction of Ca2+ influx via activation of large-conductance Ca2+-activated K+ (BKCa) channels have also been demonstrated (16, 24).

In recent studies, we (13, 23) and others (18) have reported that Ca2+ regulation in ASM cells in response to ACh involves repetitive [Ca2+]i oscillations that arise from cyclical SR Ca2+ release and reuptake. Similar [Ca2+]i oscillations have also been previously reported in vascular (2, 7, 9, 21) and gastric smooth muscle cells (19). Nuttall and Farley (22) recently reported that isoproterenol, a β-adrenoceptor agonist, modulates the frequency of ACh-induced [Ca2+]i oscillations in porcine ASM cells. These investigators proposed that the inhibition of [Ca2+]i oscillations by β-adrenoceptor agonists involved reduced Ca2+ influx. However, given the fact that β-adrenoceptor agonists also affect other Ca2+ regulatory sites, modulation of [Ca2+]i oscillations by these agonists might also involve additional mechanisms such as reduced Ca2+ release, increased reuptake, and enhanced efflux.

In the present study, we used real-time confocal imaging to examine the effect of salbutamol, a β2-adrenoceptor agonist, on the dynamic [Ca2+]i responses of porcine ASM cells to ACh. Using oscillation amplitude, risetime, fall time, and frequency as parameters, we attempted to elucidate the mechanisms by which salbutamol reduces [Ca2+]i levels.

METHODS

Cell preparation. Porcine trachea were obtained from a local abattoir. The techniques for dissociation of ASM cells have been published previously (11, 12). Briefly, the smooth muscle layer was excised, freed of epithelium, and minced thoroughly in Hanks' balanced salt solution (HBSS) buffered with 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (pH 7.4; Gibco-BRL). ASM cells were dissociated by incubating the tissue with 20 U/ml papain, 2,000 U/ml deoxyribonuclease (worthington biochemical), and 1 mg/ml type IV collagenase (worthington Biochemical). The dissociated cells were then triturated, centrifuged, and resuspended in minimum essential medium with 10% fetal calf serum. The ASm cells were plated on collagen-coated glass coverslips. Trypsin blue exclusion was used to assess cell viability (>90% of all cells).

The cells were loaded for 30–45 min at 37°C with 5 µM fluo-3 acetoxyethyl ester (Molecular Probes). The coverslip was then washed in HBSS, mounted on an open microscope slide chamber (RC-25F, Warner Instruments), and perfused at 2–3 ml/min at room temperature.

Real-time confocal imaging. Detailed descriptions of the confocal imaging technique have been recently published (23). An Odyssey XL real-time confocal system (Noran Instruments, Middleton, WI) equipped with an Ar-Kr laser (488-nm line) and mounted on the Nikon microscope was used to visualize fluo-3-loaded ASM cells. The system was controlled through a Silicon Graphics Indy workstation. A Nikon ×40/1.3 oil-immersion objective lens was used, and image size was set to 640 × 480 pixels, with a calibrated pixel area of 0.063 µm2/pixel. Only one fixed combination of laser intensity (20%...
Ca\textsuperscript{2+} oscillations were measured across a 20-min period. However, all experimental protocols were limited to <5 min of continued laser exposure. ACh with a fixed dimension of 5 x 5 pixels (1.5 µm\textsuperscript{2}) were drawn within cell boundaries. The optical section thickness for the x40 lens was set to 1 µm by controlling the confocal slit size. Therefore, [Ca\textsuperscript{2+}] measurements were obtained from a volume of 1.5 µm\textsuperscript{3}.

The confocal system is capable of acquiring 480 frames/s. In preliminary studies on fluo-3-loaded ASM cells, we determined that an acquisition rate of 30 frames/s was sufficient to measure the various parameters for [Ca\textsuperscript{2+}] oscillations without frequency aliasing over the range of the [Ca\textsuperscript{2+}] response. Accordingly, a fixed acquisition rate of 30 frames/s was used in this study. When necessary, noise reduction was achieved by acquiring the images at 60 or 120 frames/s with frame averaging.

Ca\textsuperscript{2+} calibrations. At the fixed combination of laser intensity and photomultiplier gain, fluo-3-loaded ASM cells were exposed to Ca\textsuperscript{2+} ionophore (A-23187) and fixed levels of extracellular Ca\textsuperscript{2+} ranging from 0 (HBSS with ethylene glycol-bis-(\beta- aminoethyl ether)-N,N,N',N'-tetraacetic acid) to 10 µM. The fluorescence intensities at the different Ca\textsuperscript{2+} concentrations were then measured, and a calibration curve of [Ca\textsuperscript{2+}] vs. GL was constructed. Based on these calibrations, all GL data in the experimental protocols were converted to nanomoles of Ca\textsuperscript{2+} before further analyses.

Oscillation parameters. Oscillation amplitude was defined as the difference between the peak of the oscillation and the basal [Ca\textsuperscript{2+}]. Rise time was defined from basal [Ca\textsuperscript{2+}], to the peak of an oscillation and was normalized for the amplitude of the rise in [Ca\textsuperscript{2+}]. Fall time was defined from the peak of the oscillation to the basal level of the next oscillation and was normalized for this amplitude. Oscillation frequency was measured as the inverse of the time between two oscillations.

Effect of salbutamol on [Ca\textsuperscript{2+}] response to ACh. Before ACh exposure, basal [Ca\textsuperscript{2+}] levels were not significantly different between cells and varied from 100 to 150 nM within an ROI (122 ± 13 nM; n = 112). On exposure to ACh, [Ca\textsuperscript{2+}] oscillations originated from one end of the long axis of the cell and spread toward the other end in the form of a propagating wave. Previous studies on ASM cells have shown that the 50% effective dose (ED\textsubscript{50}) for the [Ca\textsuperscript{2+}] response to ACh is ~1 µM (24). Accordingly, a fixed ACh concentration of 1 µM was used in all the protocols. An after-oscillation period in HBSS of 5 min, ASM cells were exposed to 1 µM ACh to induce [Ca\textsuperscript{2+}] oscillations. Typically, the [Ca\textsuperscript{2+}] oscillations induced by ACh displayed a biphase pattern (Fig. 1).

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After a steady state was reached, the cells were sequentially exposed to 1 nM, 10 nM, 100 nM, and 1 µM salbutamol (Glaxo-Wellcome) in the continued presence of ACh. Each exposure of salbutamol was maintained for 2 min and was followed by a wash in 1 µM ACh for 2 min. Other ASM cells were continuously exposed to 1 µM ACh for 20 min, and the [Ca\textsuperscript{2+}] response was evaluated. During periods of equilibration and washing, the cells were not exposed to the laser.

On the basis of the results of this protocol, it was determined that the maximal effect of salbutamol on the [Ca\textsuperscript{2+}] response occurred at a concentration of 1 µM. Accordingly, a fixed concentration of 1 µM salbutamol was used in subsequent protocols.

Effect of salbutamol on Ca\textsuperscript{2+} influx. ASM cells were exposed to 1 µM ACh to induce [Ca\textsuperscript{2+}] oscillations. After a steady state was reached, the cells were exposed to 100 nM BAY 8644, to promote Ca\textsuperscript{2+} influx through voltage-gated membrane channels. The effect of 1 µM salbutamol was then evaluated. Another group of ASM cells was preexposed to 10 nM charybdotoxin, a selective inhibitor of BK\textsubscript{Ca} channels. The cells were then exposed to 1 µM ACh and subsequently to 1 µM salbutamol.

Effect of salbutamol on SR Ca\textsuperscript{2+} release. ASM cells were preexposed to 1 µM salbutamol and subsequently to 5 mM caffeine to induce SR Ca\textsuperscript{2+} release from ryanodine receptor (RyR) channels (28). In a second set of experiments, ASM cells were preexposed to Ca\textsuperscript{2+}-free HBSS and to 1 nM lanthanum to block both Ca\textsuperscript{2+} influx and efflux, respectively. The cells were then exposed to 1 µM ACh and subsequently to 1 µM salbutamol.

Effect of salbutamol on Ca\textsuperscript{2+} efflux. ASM cells were exposed to 1 µM ACh to initiate [Ca\textsuperscript{2+}] oscillations. After a steady state was reached, the cells were exposed to a Ca\textsuperscript{2+}-free HBSS and to 1 µM thapsigargin to block Ca\textsuperscript{2+} influx and SR Ca\textsuperscript{2+} reuptake (27), respectively. Continued stimulation of the cells by ACh in the presence of Ca\textsuperscript{2+}-free HBSS and thapsigargin

![Fig. 1. Acetylcholine (ACh)-induced intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]) oscillation in localized region of single porcine airways smooth muscle (ASM) cell. Basal [Ca\textsuperscript{2+}] initially increased and, in concert, oscillation amplitude decreased while frequency increased. With continued ACh exposure, basal [Ca\textsuperscript{2+}] decreased, and oscillation amplitude increased while frequency decreased. Both oscillation amplitude and frequency increased to a constant steady state of 60–90 s after ACh exposure at a constant basal [Ca\textsuperscript{2+}] level.](image-url)
caused an elevation of basal \([Ca^{2+}]_i\) level. The cells were then exposed to 1 µM salbutamol to examine its effect on \(Ca^{2+}\) influx. Another group of ASM cells was preexposed to \(Ca^{2+}\)-free HBSS, thapsigargin, and lanthanum, thus blocking \(Ca^{2+}\) influx, reuptake, and efflux, respectively, after \(ACh\)-induced \([Ca^{2+}]_i\) oscillations had reached steady state. The cells were then exposed to 1 µM salbutamol.

Effect of \(8\)-bromoadenosine 3’,5’-cyclic monophosphate on \(ACh\)-induced \([Ca^{2+}]_i\) oscillations. ASM cells were exposed to 1 µM \(ACh\) to induce \([Ca^{2+}]_i\) oscillations, and after a steady state was reached, the cells were exposed to 500 µM \(8\)-bromoadenosine 3’,5’-cyclic monophosphate (8-BrcAMP), a membrane-permeant analog of cAMP.

Data analysis. Each cell was exposed to only one experimental protocol. At least five cells were chosen from each coverslip. Overall, 280 cells were analyzed. Dose-dependent data were compared by using a one-way analysis of variance with control) as the grouping variable. Other data were compared by using t-tests. Bonferroni corrections were applied for repeated comparisons. Statistical significance was tested at a 0.05 level. Data are reported as means ± SE.

RESULTS

Effect of salbutamol on \(ACh\)-induced \([Ca^{2+}]_i\) oscillations. During the steady-state phase of \(ACh\)-induced \([Ca^{2+}]_i\) oscillations, exposure of ASM cells to increasing concentrations of salbutamol resulted in a progressive decrease in basal \([Ca^{2+}]_i\) level (Fig. 2). This decrease in basal \([Ca^{2+}]_i\) level was associated with a decrease in the frequency of \([Ca^{2+}]_i\) oscillations (P < 0.05; Fig. 2, Table 1; n = 15). The fall time of \([Ca^{2+}]_i\) transients was also increased by exposure to increasing salbutamol concentrations (Table 1). In contrast, the peak-to-trough amplitude and rise time of \([Ca^{2+}]_i\) oscillations were unaffected by salbutamol at any concentration (Table 1). Increasing salbutamol concentration beyond 1 µM completely inhibited \(ACh\)-induced \([Ca^{2+}]_i\) oscillations (Fig. 2). Removal of salbutamol from the extracellular medium restored the \(ACh\)-induced \([Ca^{2+}]_i\) oscillations to presalbutamol values.

Effect of salbutamol on \(Ca^{2+}\) influx. During the steady-state phase of \(ACh\)-induced \([Ca^{2+}]_i\) oscillations, exposure of ASM cells to BAY K 8644 resulted in a gradual increase in basal \([Ca^{2+}]_i\) level (Fig. 3). This increase in basal \([Ca^{2+}]_i\) level was associated with an increase in the frequency of \([Ca^{2+}]_i\) oscillations and a decrease in oscillation amplitude (Fig. 3A; n = 14). After \([Ca^{2+}]_i\) oscillations were induced by 1 µM \(ACh\), exposing ASM cells to 1 µM salbutamol resulted in an inhibition of \([Ca^{2+}]_i\) oscillations, which could be reversed by subsequent exposure to BAY K 8644 (Fig. 3B; n = 19). Preexposure of ASM cells to charybdotoxin to inhibit BK\(_{ca}\) channels did not significantly affect basal \([Ca^{2+}]_i\) levels, nor did it affect the ability of \(ACh\) to induce \([Ca^{2+}]_i\) oscillations or the subsequent inhibitory effects of salbutamol on \([Ca^{2+}]_i\) oscillations (n = 8).

Effect of salbutamol on SR \(Ca^{2+}\) release. Exposing ASM cells to 5 mM caffeine induced a transient increase in \([Ca^{2+}]_i\) (Fig. 4A). Preexposure of ASM cells to

Table 1. Effect of salbutamol on \(ACh\)-induced \([Ca^{2+}]_i\) oscillations

<table>
<thead>
<tr>
<th>Salbutamol</th>
<th>Basal ([Ca^{2+}]_i), nM</th>
<th>Peak-to-Trough Amplitude, nM</th>
<th>RT, ms/nM</th>
<th>FT, ms/nM</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 nM (control)</td>
<td>100.0 ± 0.0</td>
<td>100.0 ± 0.0</td>
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<tr>
<td>1 nM</td>
<td>93.3 ± 4.3*</td>
<td>101.7 ± 6.7</td>
<td>96.9 ± 3.9</td>
<td>104.1 ± 1.4</td>
<td>83.1 ± 1.1*</td>
</tr>
<tr>
<td>10 nM</td>
<td>88.2 ± 6.4*</td>
<td>97.2 ± 6.5</td>
<td>88.9 ± 5.9*</td>
<td>115.6 ± 5.9*</td>
<td>77.0 ± 4.4*</td>
</tr>
<tr>
<td>100 nM</td>
<td>74.6 ± 3.8*</td>
<td>95.1 ± 6.1*</td>
<td>117.2 ± 10.9*</td>
<td>135.4 ± 7.6*</td>
<td>46.7 ± 4.8*</td>
</tr>
<tr>
<td>1 µM</td>
<td>61.2 ± 3.2*</td>
<td>Inhibition</td>
<td>Inhibition</td>
<td>Inhibition</td>
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</tr>
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</table>

Values are means ± SE expressed as %control. \([Ca^{2+}]_i\), intracellular \(Ca^{2+}\) concentration; \(ACh\), acetylcholine; RT, rise time; FT, fall time.

*Significant concentration-dependent change, P < 0.05.
1 µM salbutamol did not affect this [Ca\(^{2+}\)] response to caffeine (Fig. 4B; n = 22). When Ca\(^{2+}\) influx and efflux were both blocked by preexposing ASM cells to Ca\(^{2+}\)-free HBSS and to 1 mM lanthanum, subsequent exposure to 1 µM ACh still induced [Ca\(^{2+}\)] oscillations that were sustained (Fig. 4C; n = 16). Further exposure of these ASM cells to 1 µM salbutamol had no effect on the amplitude or frequency of ongoing [Ca\(^{2+}\)] oscillations (Fig. 4C).

Effect of salbutamol on Ca\(^{2+}\) efflux. During the steady-state phase of ACh-induced [Ca\(^{2+}\)] oscillations, exposure of ASM cells to Ca\(^{2+}\)-free HBSS and thapsigargin resulted in a gradual increase in basal [Ca\(^{2+}\)] level (Fig. 5A; n = 16). As basal [Ca\(^{2+}\)] level increased, the ACh-induced [Ca\(^{2+}\)] oscillations were eventually inhibited (Fig. 5A). Subsequent exposure to 1 µM salbutamol decreased basal [Ca\(^{2+}\)] level but did not reinitiate [Ca\(^{2+}\)] oscillations (Fig. 5A). In another group of ASM cells, ACh-induced [Ca\(^{2+}\)] oscillations were not inhibited by exposure to Ca\(^{2+}\)-free HBSS and thapsigargin when lanthanum was also present to block Ca\(^{2+}\) influx (Fig. 5B; n = 19). However, in these cells, the amplitude of ACh-induced [Ca\(^{2+}\)] oscillations progressively decreased while basal [Ca\(^{2+}\)] level increased. In contrast to the effect of salbutamol on [Ca\(^{2+}\)] level in the presence of Ca\(^{2+}\)-free HBSS and thapsigargin (Fig. 5A), salbutamol had no effect on basal [Ca\(^{2+}\)], or [Ca\(^{2+}\)] oscillations when lanthanum was also present (Fig. 5B).

Effect of 8-BrcAMP on [Ca\(^{2+}\)] oscillations. Exposing ASM cells to 500 µM 8-BrcAMP during the steady-state phase of ACh-induced [Ca\(^{2+}\)] oscillations did not affect oscillation amplitude but significantly decreased oscillation frequency (Fig. 6; P < 0.05; n = 18).

**DISCUSSION**

Exposure of ASM cells to ACh resulted in [Ca\(^{2+}\)] oscillations. Exposure to the β2-adrenoceptor agonist salbutamol decreased the frequency, but not the amplitude, of ACh-induced [Ca\(^{2+}\)] oscillations in a dose-dependent fashion, completely inhibiting the oscillations at a concentration of 1 µM. Salbutamol inhibited BAY K 8644-induced elevation of [Ca\(^{2+}\)], suggesting a blockade of Ca\(^{2+}\) influx via voltage-gated channels. These data are in complete agreement with recent reports by Nuttle and Farley (22) on the effect of isoproterenol on [Ca\(^{2+}\)] in porcine ASM cells. The fact that BAY K 8644 partially restored ACh-induced [Ca\(^{2+}\)] oscillations after their inhibition by salbutamol also suggests that the effects of salbutamol are mediated, at least in part, by inhibition of Ca\(^{2+}\) influx. Even when Ca\(^{2+}\) influx was blocked by the removal of extracellular Ca\(^{2+}\), salbutamol decreased both basal and ACh-induced [Ca\(^{2+}\)], suggesting that salbutamol also enhances Ca\(^{2+}\) efflux. This was confirmed by the fact that blockade of Ca\(^{2+}\) efflux by using lanthanum prevented reduction of ACh-induced [Ca\(^{2+}\)], by salbutamol. The lack of an effect on the [Ca\(^{2+}\)] response to caffeine suggests that salbutamol may not significantly affect SR Ca\(^{2+}\) release. The results of this study indicate that β-adrenoceptor stimulation results in altered Ca\(^{2+}\) flux across the membrane of ASM cells, reducing [Ca\(^{2+}\)], by inhibiting influx and enhancing efflux.

The [Ca\(^{2+}\)] oscillations induced by ACh in porcine ASM cells were qualitatively similar to [Ca\(^{2+}\)] oscillations observed in vascular smooth muscle (2, 7, 9), colonic smooth muscle (19), uterine smooth muscle (14), and more recent studies using porcine ASM cells (13, 18, 22, 23). [Ca\(^{2+}\)] oscillations may arise from cyclical Ca\(^{2+}\) influx, mediated via changes in membrane potential, or from cyclical SR Ca\(^{2+}\) release and reuptake. We (13, 23) and others (18, 22) have recently shown in porcine ASM cells that [Ca\(^{2+}\)] oscillations arise from cyclical Ca\(^{2+}\) release and reuptake and may involve IP\(_3\)-receptor and RyR channels. Ca\(^{2+}\) influx appears to be necessary for the maintenance of [Ca\(^{2+}\)] oscillations, most likely by replenishing SR Ca\(^{2+}\) stores depleted by agonist stimulation (13, 18, 22, 23). In our previous study (23), we demonstrated that the amplitude and frequency of ACh-induced [Ca\(^{2+}\)] oscillations in ASM cells are correlated to basal [Ca\(^{2+}\)]. With increasing basal [Ca\(^{2+}\)], the amplitude of the [Ca\(^{2+}\)] oscillations decreases, whereas oscillation frequency increases.
These relationships between basal $[\text{Ca}^{2+}]_i$ and the amplitude and frequency of $[\text{Ca}^{2+}]_i$ oscillations may be a result of the limited SR $\text{Ca}^{2+}$ pool available for release with each oscillation. Basal $[\text{Ca}^{2+}]_i$ may set the concentration gradient for release of this limited pool and, therefore, amplitude may decrease with increasing basal $[\text{Ca}^{2+}]$. With increasing basal $[\text{Ca}^{2+}]_i$, the frequency of oscillations may increase due to the enhancement of SR $\text{Ca}^{2+}$ reuptake, allowing for faster recycling of the limited $\text{Ca}^{2+}$ pool. Accordingly, various factors that influence either resting or agonist-induced basal $[\text{Ca}^{2+}]_i$ levels, such as $\text{Ca}^{2+}$ influx, efflux, and reuptake are likely to modulate the amplitude and frequency of $[\text{Ca}^{2+}]_i$ oscillations. For example, factors that decrease basal $[\text{Ca}^{2+}]_i$ would tend to increase oscillation amplitude and decrease oscillation frequency. The modulation of oscillation frequency by salbutamol suggests effects on one or more of the factors that influence basal $[\text{Ca}^{2+}]$.

Relaxation of smooth muscle by $\beta$-adrenoceptor stimulation is thought to be achieved by cyclic nucleotide-dependent and -independent activation of BK$_{Ca}$ channels (16, 24). Specific blockers of BK$_{Ca}$ channels, such as charybdotoxin, have been shown to inhibit the relaxation induced by $\beta$-adrenoceptor agonists (8, 10). Therefore, it is possible that the inhibition of $[\text{Ca}^{2+}]_i$ oscillations in ASM cells by salbutamol is achieved by enhanced open probability of BK$_{Ca}$ channels with subsequent membrane hyperpolarization and consequent inhibition of $\text{Ca}^{2+}$ influx through voltage-gated $\text{Ca}^{2+}$ channels. However, using patch-clamp techniques, Nuttle and Farley (22) demonstrated that, even when membrane potential is clamped close to the reversal potential for $\text{K}^{+}$, ACh can induce $[\text{Ca}^{2+}]_i$ oscillations that are blocked by $\beta$-adrenoceptor agonists. Therefore, it is unlikely that the modulation of oscillation frequency or the inhibition of $[\text{Ca}^{2+}]_i$ oscillations by salbutamol results from a decrease in $\text{Ca}^{2+}$ influx by a mechanism that requires a change in membrane potential. This conclusion is also supported by the results of the present study in which, even in the presence of charybdotoxin, $[\text{Ca}^{2+}]_i$ oscillations could be induced by ACh, which were then blocked by 1 $\mu$M salbutamol.

Although salbutamol may not inhibit $\text{Ca}^{2+}$ influx indirectly via changes in membrane potential, the fact that BAY K 8644 partially reversed the inhibition of $[\text{Ca}^{2+}]_i$ oscillations by salbutamol indicates that $\text{Ca}^{2+}$ influx through voltage-gated channels is nevertheless affected. This result is in agreement with the previous study by Nuttle and Farley (22), who demonstrated...
that raising extracellular Ca\textsuperscript{2+} levels and increasing the inward driving force for Ca\textsuperscript{2+} restored [Ca\textsuperscript{2+}]i oscillations that had been blocked by isoproterenol. There is currently no precedent for a direct effect of β-adrenoceptor agonists on Ca\textsuperscript{2+}-influx channels in ASM cells. However, several studies in vascular smooth muscle have demonstrated that β-adrenoceptors can modulate voltage-dependent Ca\textsuperscript{2+} channels through a cyclic nucleotide-dependent phosphorylation of the channel or an associated protein (1, 30). A similar mechanism may explain the effect of salbutamol on Ca\textsuperscript{2+} influx. In the present study, the observation that raising extracellular Ca\textsuperscript{2+} levels and increasing the inward driving force for Ca\textsuperscript{2+} restored [Ca\textsuperscript{2+}]i oscillations that had been blocked by isoproterenol. 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8-BrcAMP also modulated the frequency of \([\text{Ca}^{2+}]_i\) oscillations suggesting that cyclic nucleotides may be involved in mediating the effects of \(\beta\)-adrenoceptors.

The results of the present study clearly demonstrate that, in addition to inhibiting \(\text{Ca}^{2+}\) influx, \(\beta\)-adrenoceptor stimulation results in enhancement of \(\text{Ca}^{2+}\) efflux, which would also tend to decrease basal \([\text{Ca}^{2+}]_i\), leading to a slowing of oscillation frequency and even an inhibition of oscillations. For example, even when \(\text{Ca}^{2+}\) influx and reuptake were blocked by zero extracellular \(\text{Ca}^{2+}\) and thapsigargin, respectively, salbutamol decreased basal \([\text{Ca}^{2+}]_i\). It was not possible to study directly the effect of salbutamol on \([\text{Ca}^{2+}]_i\) oscillations under these conditions, because removal of extracellular \(\text{Ca}^{2+}\) would itself lead to an inhibition of oscillations (18, 23), and thapsigargin also leads to an elevation of \([\text{Ca}^{2+}]_i\), and inhibition of oscillations. Nonetheless, the fact that salbutamol had no effect on the stable ACh-induced oscillations, under conditions where \(\text{Ca}^{2+}\) influx and efflux were both blocked by the combination of zero extracellular \(\text{Ca}^{2+}\) and lanthanum, clearly demonstrates that salbutamol primarily affects \(\text{Ca}^{2+}\) flux across the cell membrane. Enhancement of \(\text{Ca}^{2+}\) efflux may be achieved by modulation of the \(\text{Na}^+ / \text{Ca}^{2+}\) exchanger or by enhanced activity of the plasma membrane adenosinetriphosphatase pump.

Salbutamol did not appear to have any significant effect on SR \(\text{Ca}^{2+}\) release. In the present study, we did not specifically examine SR \(\text{Ca}^{2+}\) release through both IP\(_3\) receptor and RyR channels. The data obtained by using caffeine suggest that \(\text{Ca}^{2+}\) release through RyR is unaffected. However, the fact that, when \(\text{Ca}^{2+}\) flux across the membrane was blocked by zero extracellular \(\text{Ca}^{2+}\) and lanthanum, salbutamol had no effect on ongoing ACh-induced \([\text{Ca}^{2+}]_i\) oscillations would suggest that SR \(\text{Ca}^{2+}\) release is not significantly affected by \(\beta\)-adrenoceptors. In the present study, we observed that the fall time of ACh-induced oscillations, normalized for amplitude, was prolonged by exposure to salbutamol. This would suggest a partial inhibition of \(\text{Ca}^{2+}\) reuptake. Previous studies have suggested that \(\beta\)-adrenoceptor-agonist stimulation actually increases SR \(\text{Ca}^{2+}\) reuptake via a cAMP-mediated pathway. This would lead to an increase in oscillation frequency, which was not observed. Therefore, the results of the present study on oscillation fall time must be interpreted with caution. Because \(\text{Ca}^{2+}\) reuptake is directly correlated to basal \([\text{Ca}^{2+}]_i\), the prolongation of fall time with salbutamol exposure may be related to the reduction in basal \([\text{Ca}^{2+}]_i\).

In conclusion, the results of the present study indicate that increased \(\beta\)-adrenoceptor-agonist stimulation in ASM cells inhibits \([\text{Ca}^{2+}]_i\) oscillations in a dose-dependent fashion by decreasing basal \(\text{Ca}^{2+}\) levels. This decrease in basal \([\text{Ca}^{2+}]_i\) may be achieved by modulating \(\text{Ca}^{2+}\) flux across the cell membrane.

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