Effects of cyclopiazonic acid on cytosolic calcium in bovine airway smooth muscle cells

MICHAEL F. ETHIER, HIROSHI YAMAGUCHI,† AND J. MARK MADISON

Departments of Medicine and Physiology, University of Massachusetts Medical School, Worcester, Massachusetts 01655

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Ethier, Michael F., Hiroshi Yamaguchi, and J. Mark Madison. Effects of cyclopiazonic acid on cytosolic calcium in bovine airway smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 281: L126–L133, 2001.—In many cells, inhibition of sarcoplasmic reticulum (SR) Ca\(^{2+}\)-ATPase activity induces a steady-state increase in cytosolic calcium concentration ([Ca\(^{2+}\)]\(_i\)) that is sustained by calcium influx. The goal was to characterize the response to inhibition of SR Ca\(^{2+}\)-ATPase activity in bovine airway smooth muscle cells. Cells were dispersed from bovine tracheals and loaded with fura 2-AM (0.5 \(\mu\)M) for imaging of single cells. Cyclopiazonic acid (CPA; 5 \(\mu\)M) inhibited refilling of both caffeine- and carbachol-sensitive calcium stores. In the presence of extracellular calcium, CPA caused a transient increase in [Ca\(^{2+}\)]\(_i\), from 166 ± 11 to 671 ± 100 nM, and then [Ca\(^{2+}\)]\(_i\), decreased to a sustained level (CPA plateau; 236 ± 19 nM) significantly above basal. The CPA plateau spontaneously declined toward basal levels after 10 min and was attenuated by discharging intracellular calcium stores. When CPA was applied during sustained stimulation with caffeine or carbachol, decreases in [Ca\(^{2+}\)]\(_i\) were observed. We concluded that the CPA plateau depended on the presence of SR calcium and that SR Ca\(^{2+}\)-ATPase activity contributed to sustained increases in [Ca\(^{2+}\)]\(_i\) during stimulation with caffeine and, to a lesser extent, carbachol.

Calcium-mobilizing agonists cause a transient increase in cytosolic calcium concentration ([Ca\(^{2+}\)]\(_i\)) that is followed by a small, sustained elevation in [Ca\(^{2+}\)]\(_i\). Calcium influx supporting the sustained responses to CPA and the source of calcium supporting the sustained responses both have fundamental implications for models of calcium regulation. Therefore, the goal of the current study was to assess the mechanisms underlying sustained responses to CPA in bovine airway smooth muscle cells.

†Deceased 9 February 1999.

Address for reprint requests and other correspondence: J. M. Madison, Pulmonary, Allergy, and Critical Care Medicine, Dept. of Medicine, UMass Medical School, 55 Lake Ave., N., Worcester, MA 01655 (E-mail: MadisonM@ummc.org).

IN MANY CELLS, including airway smooth muscle, calcium-mobilizing agonists cause a transient increase in cytosolic calcium concentration ([Ca\(^{2+}\)]\(_i\)) that is followed by a small, sustained elevation in [Ca\(^{2+}\)]\(_i\). Calcium influx supporting the sustained elevation in [Ca\(^{2+}\)]\(_i\) is mediated by voltage- and ligand-gated channels (17, 28). One model of calcium regulation (capacitative model) emphasizes that the filling state of the sarcoplasmic reticulum (SR) is an important determinant of these sustained increases in [Ca\(^{2+}\)]\(_i\) in many cells (19, 20). According to this model, when the SR is depleted of calcium, an as yet unidentified signal (21) triggers an increase in calcium influx through store-operated calcium channels to increase [Ca\(^{2+}\)]\(_i\) until the SR is refilled with calcium. An alternative model (superficial buffer barrier model) emphasizes that the SR, located at the periphery of the cell cytosol, serves as a barrier to calcium influx and that the presence of agonist renders this barrier permeable (6, 7, 27). A recent study of canine airway smooth muscle supports the superficial buffer barrier model with evidence that the SR functionally segregates the cytosol into distinct compartments at the periphery of the cell where calcium is regulated independently of the deep cytosol (10).

Important evidence that supports both the capacitative and superficial buffer barrier models of calcium regulation has been the observation that inhibitors of SR Ca\(^{2+}\)-ATPase activity, such as the irreversible inhibitor thapsigargin or the reversible inhibitor cyclopiazonic acid (CPA; see Ref. 22) cause sustained steady-state increases in [Ca\(^{2+}\)]\(_i\). In many cell types, including vascular smooth muscle, inhibitors of SR Ca\(^{2+}\)-ATPase activity typically cause a rapid increase in [Ca\(^{2+}\)]\(_i\) as a result of an unopposed leak of calcium from the SR, and this peak increase in [Ca\(^{2+}\)]\(_i\) is then followed by a large, sustained elevation, or plateau, in [Ca\(^{2+}\)]\(_i\) (6, 19, 21). These sustained increases in [Ca\(^{2+}\)]\(_i\) are not seen in the absence of extracellular calcium, persist indefinitely, and are not significantly affected by discharging intracellular calcium stores (6, 24). However, in preliminary experiments with bovine airway smooth muscle cells, we found that CPA caused only a small, sustained increase in [Ca\(^{2+}\)]\(_i\). Similar small responses to inhibitors of SR Ca\(^{2+}\)-ATPase activity previously have been reported in some (12, 23, 25, 26, 28), but not all (1, 10), airway smooth muscle cells, and this suggests that there may be species- or preparation-dependent differences in the regulation of [Ca\(^{2+}\)]\(_i\) among airway smooth muscle cells. The presence of sustained responses to CPA and the source of calcium supporting the sustained responses both have fundamental implications for models of calcium regulation. Therefore, the goal of the current study was to assess the mechanisms underlying sustained responses to CPA in bovine airway smooth muscle cells.

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METHODS

Cell isolation. Smooth muscle cells were dispersed from bovine tracheal (15). Approximately 250 mg of minced tissue were placed in a Coulter counter vial containing a magnetic stirring bar and 2.5 ml of physiological salt solution [PSS (in mM): 118 NaCl, 4.7 KCl, 1.2 KH2PO4, 1.2 MgSO4, 25.6 NaHCO3, 11.1 glucose, and 2.5 CaCl2] modified to have no added CaCl2 and containing collagenase D (6 mg) and elastase (grade II; 3 mg). The minces were incubated at 37°C with constant stirring for 12 min and then were transferred to another vial, and the incubation was repeated. The supernatant was monitored repeatedly by microscopy, and the incubation was terminated when cells began to be released from the tissue. The partially digested mince then was transferred to 2 ml of PSS modified to contain 0.1 mM CaCl2 and was incubated for 3 min with constant stirring. The cells released during this and one subsequent identical incubation were used for studies. The cells were loaded with 0.5 μM fura 2-AM in the presence of pluronic F-127 (0.004%) for 60 min at room temperature and then were introduced into a superfusion chamber (0.3 ml volume) having a bottom cover glass. After cells adhered for 10 min, PSS superfused the chamber at 1 ml/min at 37°C.

[Ca2+]i measurement. Fura 2 loaded into cells was excited by computer-controlled 337- and 380-nm ultraviolet light generated by a nitrogen laser and a nitrogen-pumped dye laser, respectively (Laser Science, Franklin, MA; see Refs. 13 and 15). Each laser alternately fired short pulses at 30 Hz, and these alternating pulses of light were guided by a bifurcated quartz fiber to a neutral-density filter at the epiport of the microscope and then focused on cells through a ×40 objective lens (Nikon). The fluorescent signals emitted by the fura 2 were passed back through the objective to a 455-nm dichroic mirror, a 475-nm barrier filter (Omega Optics, Brattleboro, VT), and an image intensifier (Xybion Electronic Systems, San Diego, CA) and were captured by a Philips-based frame transfer charge-coupled device camera (CCTV, New York, NY). The analog signals from the camera were digitized and stored in an imaging board, and digital outputs from this board were transferred to a personal computer with software by Recognition Technology (Framingham, MA).

As described previously (15), to measure [Ca2+]i, in cells loaded with fura 2, background from a cell-free region of the cover glass was subtracted before data acquisition, and then an 11 × 11 pixel area was selected over each cell. With the low concentration of fura 2-AM used for loading, detected fluorescence mainly reflected changes in [Ca2+]i, and the equilibrium dissociation constant describing calcium binding to fura 2. Average values for Rmin, Rmax, and \( K_D \) are the equilibrium dissociation constant describing calcium binding to fura 2. Average values for Rmax, Rmin, and \( K_D \) were 0.74, 12.4, and 8.3, respectively. Based on an in situ determination, a \( K_D \) value of 386 nM was used in converting fluorescence ratios to [Ca2+]i, (13). This in situ \( K_D \) value is higher than values commonly obtained in vitro (9) and could potentially cause an overestimation of absolute [Ca2+]i levels. However, this possible overestimation would not affect the general pattern of results.

Protocols. Cell chambers were perfused with PSS at 1 ml/min at 37°C for at least 30 min before the start of experiments. In all experiments, a four-way valve allowed changing of perfusates without disturbing recordings. In experiments to assess the effects of CPA alone on [Ca2+]i, cells perfused by PSS were recorded from to establish a stable baseline, and then the perfusate was changed to PSS containing CPA (5 μM) as the recording continued for up to 25 min. In preliminary experiments, the effects of a higher concentration of CPA (10 μM) on [Ca2+]i were usually, but not always, reversible with washing. Therefore, 5 μM CPA was used routinely in these studies. In some experiments, thapsigargin was substituted for CPA as indicated. In experiments to assess the effects of CPA on respiration of caffeine- and carbachol-sensitive stores, cells were stimulated by caffeine or carbachol for 2 min (S1) and then perfused with either PSS (time-matched controls) or PSS containing CPA (5 μM) for 10 min before being stimulated a second time with either caffeine or carbachol (S2). The peaks of the calcium transients were compared by calculating an S2-to-S1 ratio as an index of intracellular store refilling (15, 16). In experiments to assess the effects of depleting caffeine- and carbachol-sensitive calcium stores on the sustained response to CPA, the response to CPA (5 μM) was recorded for 200 s, and then the cells were perfused by either CPA (5 μM) alone (time-matched control), CPA (5 μM) plus caffeine (10 μM), or CPA (5 μM) plus carbachol (10 μM) while recordings continued. Finally, in experiments designed to assess the effects of CPA on sustained responses to carbachol or caffeine, cells were stimulated with vehicle (time-matched control), caffeine (10 μM), or carbachol (10 μM) for 200 s, and then CPA (5 μM) was added in the continued presence of vehicle, caffeine, or carbachol, respectively. For experiments with CPA, after recordings from any single cell, the cell chamber was perfused with PSS at 2.5 mM CaCl2 at 37°C for at least 30 min before selection of another cell. Responses were not different for the first and last cells studied in the chambers.

Data analysis. All data are expressed as means ± SE, and n indicates the number of cells studied. Means were compared by Student’s t-test or ANOVA with Newman-Keuls follow-up testing for multiple comparisons. P < 0.05 was considered significant.

Reagents. Collagenase and elastase were obtained from Boehringer Mannheim (Indianapolis, IN). Fura 2-AM and pluronic F-127 were obtained from Molecular Probes (Eugene, OR). CPA was obtained from Calbiochem (La Jolla, CA). The Rp diastereomer of adenosine 3',5'-cyclic monophosphoioate (Rp-cAMPS) triethylamine was obtained from Research Biochemicals International (Natick, MA). All other reagents were obtained from Sigma (St. Louis, MO).

RESULTS

Effect of CPA on [Ca2+]i. CPA (5 μM) caused a transient increase in [Ca2+]i from 166 ± 11 to 671 ± 100 nM (P < 0.05, n = 55). The initial increase was variable, ranging from 23 to 3,168 nM. However, with less variability, [Ca2+]i then decreased, within 200 s, to a plateau level of 236 ± 19 nM, and this was sustained at 400 s (Fig. 1). Although the CPA plateau was small in magnitude (~70 nM above basal), it was significantly above basal and was observed in 51 of 55 cells. The small magnitude of the CPA plateau could not be attributed to the concentration of CPA used (5 μM). Higher concentrations of CPA and thapsigargin did not
increase the magnitude of the CPA plateau. In seven cells, 30 μM CPA caused plateau levels of [Ca\(^{2+}\)]\(_i\) (205 ± 29 nM) that were similar to that observed with 5 μM CPA. Also, in seven cells, thapsigargin (0.3 μM) caused a sustained increase in [Ca\(^{2+}\)]\(_i\) that was small in magnitude (217 ± 26 nM, with basal [Ca\(^{2+}\)]\(_i\) being 177 ± 17 nM in these cells, \(P < 0.05\)). Finally, when four cells were treated with CPA (5 μM) and then thapsigargin (0.3 μM) as well, the thapsigargin caused no additional increase in the CPA plateau (13 ± 15 nM decrease, not significant).

Prior evidence suggests that caffeine-sensitive stores may be resistant to the effects of CPA compared with agonist-sensitive stores (4, 14). However, the fact that the CPA plateau was small, only 70 nM above basal level, could not be attributed to CPA (5 μM) ineffectively inhibiting the refilling of caffeine-sensitive stores. There was a functional overlap between carbachol- and caffeine-sensitive stores in this cell type; that is, discharging stores with carbachol (10 μM) decreased a subsequent peak response to caffeine (10 mM; S\(_2\)-to-S\(_1\) ratio 0.11 ± 0.07, \(P < 0.05, n = 3\)), and the reverse experiment decreased responses to carbachol (S\(_2\)-to-S\(_1\) ratio 0.08 ± 0.04, \(P < 0.05, n = 3\)). Also, with the use of the S\(_2\)-to-S\(_1\) ratio as an index of refilling (see METHODS), CPA (5 μM) did, in fact, have an inhibitory effect on refilling of both carbachol- and caffeine-sensitive stores (Table 1). That CPA inhibited these S\(_2\)-to-S\(_1\) ratios by inhibiting SR Ca\(^{2+}\)-ATPase activity was supported by thapsigargin also inhibiting S\(_2\)-to-S\(_1\) ratios. This inhibition of refilling of caffeine-sensitive stores by both CPA and thapsigargin also was evident when S\(_2\)-to-S\(_1\) ratios were calculated using the areas under the transients rather than using peak responses.

In 11 experiments, cells with a basal [Ca\(^{2+}\)]\(_i\) level of 197 ± 26 nM were perfused for 2 min with PSS containing no added calcium, and [Ca\(^{2+}\)]\(_i\), decreased to a new baseline of 118 ± 13 nM. Upon addition of CPA (5 μM), [Ca\(^{2+}\)]\(_i\) increased to 230 ± 43 nM (\(P < 0.05\)) and then decreased to 138 ± 22 nM within 5 min. However, responses were not consistent among cells. Even after 5 min of exposure to CPA, the majority of cells (8 of 11) had [Ca\(^{2+}\)]\(_i\) levels (153 ± 29 nM) that were above baseline.

**Discharging intracellular stores during stimulation with CPA.** Dependence of the CPA plateau on SR calcium stores was assessed. The CPA plateau was not sustained indefinitely, even in the presence of extracellular calcium. In six experiments, after 10 min of stimulation with CPA, [Ca\(^{2+}\)]\(_i\) slowly began to decrease to basal levels (Fig. 2). When cells were stimulated with caffeine (10 mM) during exposure to CPA, the magnitude of the CPA plateau predicted the peak response to caffeine. That is, whenever the magnitude of the CPA plateau declined to values <5% of the peak response to CPA, the transients in response to caffeine were significantly decreased (Fig. 2, inset).

The finite duration of the CPA plateau and the fact that the magnitude of the CPA plateau predicted the magnitude of caffeine-stimulated calcium transients both suggested that intracellular stores, containing a finite amount of calcium, might be the source of the calcium supporting the CPA plateau. To assess this, in 19 cells, caffeine-sensitive stores were discharged during stimulation with CPA. For these experiments, CPA (5 μM) caused a sustained increase in [Ca\(^{2+}\)]\(_i\) from

### Table 1. Effect of CPA on refilling of intracellular stores

<table>
<thead>
<tr>
<th>Initial Stimulus (S(_1))</th>
<th>Refilling Conditions</th>
<th>Second Stimulus (S(_2))</th>
<th>S(_2)-to-S(_1) Ratio</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caffeine</td>
<td>Calcium (2.5 mM) present</td>
<td>Caffeine</td>
<td>0.89 ± 0.15</td>
<td>5</td>
</tr>
<tr>
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<td>Caffeine</td>
<td>0.18 ± 0.07*</td>
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<tr>
<td>Caffeine</td>
<td>Calcium (2.5 mM) + CPA</td>
<td>Caffeine</td>
<td>0.06 ± 0.02*</td>
<td>6</td>
</tr>
<tr>
<td>Caffeine</td>
<td>Calcium (2.5 mM) + thapsigargin</td>
<td>Caffeine</td>
<td>0.03 ± 0.03*</td>
<td>6</td>
</tr>
<tr>
<td>Carbachol</td>
<td>Calcium (2.5 mM) present</td>
<td>Carbachol</td>
<td>0.75 ± 0.07</td>
<td>12</td>
</tr>
<tr>
<td>Carbachol</td>
<td>No calcium (0 mM)</td>
<td>Carbachol</td>
<td>0.25 ± 0.10*</td>
<td>7</td>
</tr>
<tr>
<td>Carbachol</td>
<td>Calcium (2.5 mM) + CPA</td>
<td>Carbachol</td>
<td>0.55 ± 0.02*</td>
<td>8</td>
</tr>
</tbody>
</table>

Data for S\(_2\)-to-S\(_1\) ratio are means ± SE; \(n\), no. of cells studied. CPA, cyclopiazonic acid. *\(P < 0.05\) compared with calcium (2.5 mM) present.
175 ± 18 (basal) to 224 ± 22 (CPA plateau) nM. Next, when these cells were perfused with caffeine (10 mM) in the continued presence of CPA, there was an initial calcium transient followed by a decrease in $[Ca^{2+}]_i$ ($P < 0.05$) to 163 ± 23 nM, a value not distinguishable from basal. In nine other experiments, the inhibitory effect of caffeine on the CPA plateau could not be reversed by 6 min of washing. In those experiments, caffeine decreased the CPA plateau from 248 ± 47 to 178 ± 37 nM. When caffeine was washed from the chambers, $[Ca^{2+}]_i$ increased to 187 ± 41 nM (basal = 183 ± 39 nM). In other experiments, the protein kinase A antagonist Rp-cAMPS (30 μM) did not inhibit this effect of caffeine on the CPA plateau. In the presence of Rp-cAMPS, caffeine decreased $[Ca^{2+}]_i$ from 209 ± 21 nM (CPA plateau) to 130 ± 15 nM ($n = 9$, $P < 0.05$), a level similar to basal (125 ± 11 nM) in these nine experiments.

Discharging intracellular stores before stimulation with CPA. If the CPA plateau were maintained by continued release of calcium from the SR rather than by calcium influx from the extracellular space, then there should be no CPA plateau when stores are discharged before application of CPA. In 17 experiments, cells with a basal $[Ca^{2+}]_i$ level of 142 ± 13 nM were perfused with caffeine (10 mM), and the response was recorded. Caffeine caused a large, transient increase in $[Ca^{2+}]_i$ (2.3 ± 0.8 μM) that rapidly decreased to a sustained level of 183 ± 25 nM (caffeine plateau), and this was significantly above basal. When CPA was added in the continued presence of caffeine, the peak response to CPA was attenuated (250 ± 40 nM; Fig. 4), and a sustained elevation in $[Ca^{2+}]_i$ in response to the CPA was not detectable (Fig. 5). In fact, the opposite occurred, with $[Ca^{2+}]_i$ decreasing to 141 ± 17 nM after the peak response to CPA (Fig. 5). That is, CPA decreased the caffeine plateau. A similar pattern was observed with thapsigargin. Thapsigargin (0.3 μM) maximally decreased the caffeine plateau by 51 ± 5 nM ($P < 0.05$), and the sustained level of $[Ca^{2+}]_i$ after...
thapsigargin was 130 ± 11 nM, a value similar to basal for these five cells (147 ± 17 nM).

Additional experiments were conducted substituting carbachol (10 μM) for caffeine. For 17 cells with a basal [Ca\(^{2+}\)]\(_i\) level of 167 ± 23 nM, carbachol caused a large, transient increase in [Ca\(^{2+}\)]\(_i\) (2.0 ± 0.3 μM) that rapidly decreased to a sustained level of 246 ± 31 nM (carbachol plateau). The application of CPA in the continued presence of carbachol elicited a complex response (Fig. 6). In 13 of 17 cells, [Ca\(^{2+}\)]\(_i\) rapidly (within 30 s) and transiently decreased (47 ± 8 nM) in response to CPA. Such rapid decreases in [Ca\(^{2+}\)]\(_i\) in response to CPA were uncommonly observed when CPA was added to cells during a caffeine plateau (4 of 17) and were rarely observed when CPA alone was added to cells (3 of 55 cells; \(P < 0.05, \chi^2\)). However, these inhibitory effects of CPA on the carbachol plateau were transient. That is, after the initial rapid decrease in [Ca\(^{2+}\)]\(_i\), there typically followed a small increase in [Ca\(^{2+}\)]\(_i\) (Fig. 4), and then [Ca\(^{2+}\)]\(_i\) plateaued at levels not significantly different from the carbachol plateau (Fig. 6).

**DISCUSSION**

For bovine airway smooth muscle cells, CPA inhibited refilling of both caffeine- and agonist-sensitive intracellular calcium stores but did not cause a large, sustained, steady-state increase in [Ca\(^{2+}\)]\(_i\). Instead, CPA caused only a small CPA plateau; importantly, it was not sustained indefinitely, and its magnitude decreased when intracellular calcium stores were depleted. That the effects of CPA depended on inhibition...
of SR Ca\(^{2+}\)-ATPase activity rather than on a nonspecific effect was supported by the observation that thapsigargin also caused only a small plateau elevation in [Ca\(^{2+}\)]_i, that decreased when intracellular calcium stores were depleted. We concluded that the CPA plateau depended on the presence of calcium in intracellular stores. Implications of these findings are that neither the inhibition of SR Ca\(^{2+}\)-ATPase activity nor the depletion of SR calcium stores causes large, sustained increases in [Ca\(^{2+}\)]_i in these cells. An additional conclusion was that, in the presence of caffeine or carbachol, small, but significant, decreases in [Ca\(^{2+}\)]_i could be observed in response to CPA. Similar decreases were observed when thapsigargin was applied in the presence of caffeine. These unexpected findings suggest that SR Ca\(^{2+}\)-ATPase activity plays a role in promoting increases in [Ca\(^{2+}\)]_i during sustained stimulation with caffeine and, to a lesser extent, with carbachol.

According to both the capacitative and the superficial buffer barrier models of calcium regulation, inhibition of SR Ca\(^{2+}\)-ATPase activity with CPA or thapsigargin should cause a sustained increase in [Ca\(^{2+}\)]_i (6–8, 19, 20). Consistent with both models, CPA did cause a sustained increase in [Ca\(^{2+}\)]_i in bovine airway smooth muscle cells. Notably, however, this CPA plateau was small (70 nM above basal), being ~15% of the peak response to CPA. Our finding a relatively small CPA plateau agrees with many studies (12, 23, 26, 28) for airway smooth muscle cells, with at least one possible exception being cultured human airway smooth muscle cells (1). In human cells, thapsigargin caused a sustained ~200 nM increase in [Ca\(^{2+}\)]_i. In the current study, it caused an ~40 nM increase.

To explain the small magnitude of the CPA plateau, we considered previously published evidence showing that CPA might not inhibit calcium uptake by stores specifically sensitive to caffeine (4, 14). Two findings argue against this for bovine airway smooth muscle cells. First, CPA did have a significant inhibitory effect on refilling of either agonist- or caffeine-sensitive stores (Table 1), and this was consistent with canine airway smooth muscle cells (10). Second, as in canine airway smooth muscle (10, 11), there was functional overlap between caffeine- and agonist-sensitive calcium stores, and this makes it less likely that refilling of these stores could be differentially sensitive to CPA.

We also considered that partial, rather than maximal, inhibition of SR Ca\(^{2+}\)-ATPases could explain the small magnitude of the CPA plateau. However, higher concentrations of CPA (30 μM) did not increase the magnitude of the small CPA plateau. This is consistent with previous studies that have suggested that bovine trachealis is sensitive to CPA, with 5 μM CPA being nearly maximally effective (2). Moreover, adding thapsigargin to cells already exposed to CPA (5 μM) did not further increase the magnitude of the small CPA plateau. Therefore, the small magnitude of the CPA plateau was not augmented by attempts to further inhibit SR Ca\(^{2+}\)-ATPase activity with higher concentrations of CPA or thapsigargin.

Even though the CPA plateau was small, it was observed in nearly all cells. Therefore, we attempted to assess the calcium source supporting it by observing the response to CPA in the absence of extracellular calcium. Notably, in the majority of cells, even after 5 min of exposure to CPA, [Ca\(^{2+}\)]_i levels were above baseline. Although this apparent increase above baseline was not statistically significant for all cells, the finding did raise the possibility that the calcium source for the CPA plateau might be more complex than simple dependence on extracellular calcium. It is also important to acknowledge that there is an inherent problem in this type of experiment because the removal of extracellular calcium by itself caused a significant decrease in [Ca\(^{2+}\)]_i in these cells. This created an interpretive problem because a change in baseline [Ca\(^{2+}\)]_i alone could theoretically affect SR calcium release (18). That this might have occurred in these cells was suggested by the observation that the peak increase in [Ca\(^{2+}\)]_i, in response to CPA also was attenuated by the absence of extracellular calcium. Therefore, our finding some evidence of a small CPA plateau, even in the absence of extracellular calcium, was potentially significant. It suggested that the small CPA plateau observed in the presence of extracellular calcium might not be a result of calcium influx from the extracellular space.

Consequently, we looked for evidence that the SR might be the source of calcium for the CPA plateau, and three findings supported that possibility. First, the increase in [Ca\(^{2+}\)]_i, in response to CPA was not sustained indefinitely, being undetectable after 10 min (Fig. 2). This finding was different from that reported for vascular smooth muscle (6) and was consistent with a finite source of calcium, such as the SR, supporting the CPA plateau. Second, the magnitude of the CPA plateau positively correlated with the magnitude of caffeine-stimulated calcium transients (Fig. 2). This suggested that the CPA plateau was smallest when SR stores were most depleted. Third, the CPA plateau present at 5 min could be abolished by discharging caffeine-sensitive calcium stores. That is, application of caffeine during stimulation with CPA caused [Ca\(^{2+}\)]_i to decrease to values indistinguishable from basal. A similar pattern was observed when thapsigargin was substituted for CPA. These findings contrast with vascular smooth muscle cells, where caffeine has only a small effect on the large CPA plateau typically seen in that cell type (6). The effect caffeine had on the CPA plateau in the current study was likely a result of discharge of intracellular stores rather than caffeine-stimulated cAMP accumulation, since the same effect of caffeine was observed in the presence of Rp-cAMPS and the effect of caffeine could not be reversed by washing.

Therefore, based on all of our findings, we concluded that the small plateaus in [Ca\(^{2+}\)]_i, present after ~5 min of treatment with CPA or thapsigargin were dependent on the presence of SR calcium. Two possibilities could explain the apparent dependence of the CPA plateau on SR calcium in these cells. First, a certain minimal
amount of SR calcium might be required to maintain calcium influx across the plasma membrane. Second, and more likely, the SR could be the source of the calcium sustaining the small CPA plateau. Calcium content of SR stores depends on a dynamic balance between uptake and release rates. When uptake is slowed by CPA, there follows, for a period of time, a net increase in calcium release as SR calcium content gradually achieves a new equilibrium level. We suggest that it is this net increase in calcium release that accounts for the CPA plateau in these cells. The CPA plateau gradually decreases to basal levels after ~20 min because SR calcium content has gradually decreased to a new equilibrium over this time interval. When caffeine is applied while the CPA plateau is still detectable, the plateau is immediately abolished because the SR calcium source sustaining it has been suddenly depleted. Our findings have important implications for calcium regulation in these cells. Either there is no large increase in calcium influx to the deep cytosol associated with store depletion or, if there is, it is well counterbalanced by efflux mechanisms insensitive to CPA or thapsigargin, probably plasma membrane Ca2+-ATPases (12).

Both caffeine and carbachol caused rapid transient increases in [Ca2+]i that were followed by sustained increases in [Ca2+]i. When CPA was applied during stimulation with caffeine, not only was there no further increase in [Ca2+]i, in response to CPA but [Ca2+]i actually decreased. Smaller, transient decreases in [Ca2+]i were observed when CPA was applied during stimulation with carbachol. Because similar decreases in [Ca2+]i were observed when thapsigargin was applied during stimulation with caffeine and because decreases in [Ca2+]i were not seen in control cells exposed to CPA alone, it is unlikely that the decreases in [Ca2+]i caused by CPA were a nonspecific effect. Decreases in [Ca2+]i in response to CPA or thapsigargin have not been previously described and are potentially important because they suggest that, during sustained stimulation with caffeine or carbachol, SR Ca2+-ATPase activity has a role in promoting, rather than inhibiting, the movement of Ca2+ into the cytosol.

Growing evidence suggests that there are cytoplasmic compartments lying between the peripheral SR and the plasma membrane (5) where calcium concentrations are regulated independently of the core cytosol (3, 10), are poorly detected by loading with low concentrations of fura 2 (29), and contribute to SR refilling (15). Influx of calcium in segregated calcium compartments at the cell periphery could explain why CPA caused small decreases in [Ca2+]i during sustained stimulation with either caffeine or carbachol. For bovine airway smooth muscle cells, we propose that, during sustained stimulation with caffeine or carbachol, there is calcium influx from the extracellular space into these restricted regions of cytosol lying between the plasma membrane and the SR. Possibly, some portion of this calcium influx is regulated by the filling state of the SR. Because the SR has been depleted of calcium by stimulation with caffeine or carbachol, SR Ca2+-ATPase activity pumps Ca2+ from these restricted cytosolic compartments into the SR. Because caffeine or carbachol is still present, the calcium pumped into the SR is immediately released, some of it toward the deep cytosol. When CPA is applied during sustained stimulation with caffeine or carbachol, the SR Ca2+-ATPases stop pumping Ca2+ from the restricted cytosolic compartments into the SR and, therefore, less Ca2+ reaches the deep cytosol. Instead, this Ca2+ is removed from the restricted cytosolic compartment by plasma membrane efflux mechanisms (12). This model is consistent with that aspect of the superficial buffer barrier hypothesis where calcium entering the cell during agonist stimulation is, in part, pumped into the SR before being released into the deep cytosol (8). Our findings suggest that delivery of Ca2+ to the deep cytosol by SR Ca2+-ATPase activity is a major calcium influx pathway sustaining the caffeine plateau. During sustained stimulation with carbachol, this pathway appears to be minor relative to other calcium entry pathways (e.g., receptor-operated channels; see Ref. 17).

In summary, CPA caused only a small, plateau increase in [Ca2+]i that depended on the presence of SR calcium. For freshly dispersed bovine airway smooth muscle cells, the findings do not support the concept that a depleted SR stimulates calcium influx to cause a large increase in [Ca2+]i in the deep cytosol, and they do not support the concept that inhibition of SR Ca2+-ATPase activity rapidly causes a large sustained increase in [Ca2+]i in the deep cytosol because of unopposed calcium influx. However, other findings do support the concept that SR Ca2+-ATPase activity may be important to the delivery of Ca2+ into the deep cytosol during sustained stimulation with caffeine and, to a minor extent, with carbachol. In aggregate, the findings are consistent with models featuring the presence of calcium efflux pathways that can compensate well during inhibition of SR Ca2+-ATPase activity and the presence of functionally segregated calcium compartments lying between the SR and plasma membrane.

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REFERENCES

INCREASES IN [Ca$^{2+}$], IN RESPONSE TO CYCLOPIAZONIC ACID


