Cyclopiazonic acid activates a Ca\(^{2+}\)-permeable, nonselective cation conductance in porcine and bovine tracheal smooth muscle

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It is well established that agonist-induced contraction of airway smooth muscle involves an elevation of intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) that is mediated in part by release of internally sequestered Ca\(^{2+}\) from the sarcoplasmic reticulum (SR) and influx of calcium through Ca\(^{2+}\)-permeable channels on the plasma membrane (24, 30). Previous studies have shown that agonist-induced [Ca\(^{2+}\)]\(_i\) responses are biphasic, with the initial transient rise in [Ca\(^{2+}\)]\(_i\), largely being due to inositol 1,4,5-trisphosphate-dependent release of Ca\(^{2+}\) from the SR, whereas the latter sustained portion of the [Ca\(^{2+}\)]\(_i\) response is thought to be mediated by Ca\(^{2+}\) influx via plasma membrane channels that, in airway smooth muscle, are thought to involve voltage-gated Ca\(^{2+}\) channels (VGCCs) (4, 30) and nonselective cation channels (6, 24, 37). Interestingly, there is increasing evidence from many cell types that store depletion, whether induced actively via agonist stimulation or passively through inhibition of the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA), results in the activation of a unique “store-operated” Ca\(^{2+}\) influx pathway distinct from the VGCCs (3, 7, 8, 13, 25).

In some cells, this store-operated Ca\(^{2+}\) influx, otherwise referred to as capacitative Ca\(^{2+}\) entry (CCE), appears to be mediated by Ca\(^{2+}\)-permeable nonselective cation channels (1, 2, 8, 33). Although the molecular identity of these channels is still a topic of current investigation (and controversy), possible candidates include members of the transient receptor potential family (TRP) that in airway smooth muscle are thought to include TRP-canonical (TRPC) isoforms 1, 3, and 6 (3, 5, 27, 28). CCE may aid in refilling of the internal Ca\(^{2+}\) store (20), contribute to contraction (17, 21, 25, 33), and serve as a signal for smooth muscle proliferation (8, 17, 33). Interestingly, airway smooth muscle contraction, proliferation, and migration are features often associated with respiratory diseases including asthma and acute respiratory distress syndrome; as such, many have begun to explore the contribution of these voltage-independent Ca\(^{2+}\) channels in the pathophysiology underlying these airways diseases (18). In contrast to the relative wealth of data regarding CCE in vascular tissues including rabbit portal vein (1) and rat (21, 25) and human (8) pulmonary artery, relatively little is known about the electrophysiological properties of these channels in airway smooth muscle (33). Much of the work to date in the airway field has involved indirect assessments of channel activity by measuring Ca\(^{2+}\) influx by fluorometric techniques (3, 13, 29, 33) or utilizing airway smooth muscle contractility as an index of Ca\(^{2+}\) entry mediated by capacitative Ca\(^{2+}\) entry channels (13, 33).

Consequently, the purpose of this study was to examine the electrophysiological characteristics and changes in [Ca\(^{2+}\)]\(_i\), associated with a cyclopiazonic acid (CPA)-activated membrane current (IC\(_{\text{CPA}}\)) in porcine and bovine airway smooth muscle using patch-clamp and fluorometric techniques. Here we demonstrate that CPA activates a Gd\(^{3+}\)- and La\(^{3+}\)-sensitive nonselective cation conductance that is likely responsible for capacitative Ca\(^{2+}\) entry in porcine and bovine tracheal smooth muscle.

**MATERIALS AND METHODS**

*Cell isolation. All experimental procedures were approved by the McMaster University Animal Care Committee and conform to the guidelines set out by the Canadian Council on Animal Care. Tracheae from market hogs (60–90 kg) and commercial cattle (136–454 kg) were obtained at a local abattoir and transported in...*
ice-cold Krebs solution (see Solutions and chemicals). Airway smooth muscle was dissected free of epithelium, connective tissue, and cartilage and maintained in Krebs solution at 4°C for up to 24 h. Tracheal smooth muscle strips were digested in modified Hanks’ balanced salt solution (with NaHCO₃, without CaCl₂ and MgSO₄) containing collagenase (Sigma blend type F, 2 mg/ml) and elastase (type IV, 250 μg/ml). After a 30-min incubation period at 37°C, papain (30 μg/ml) and (−1)-4-dithiothreitol (750 μg/ml) were also added and incubated an additional 20–30 min. Cells were gently triturated using a wide-bore pipette, then centrifuged to form a loose pellet. Supernatant was removed and cells were resuspended in standard Ringer’s solution (see Solutions and chemicals).  

[Ca²⁺], fluorometry. Isolated tracheal myocytes (see Cell isolation) were incubated with fluo-4 AM (2 μM, containing 0.1% pluronic F-127) for 30 min at 37°C. Cells were then placed in a Plexiglas recording chamber and superfused with Ringer’s solution for a period of 30 min before experimentation to allow for complete dye deesterification. CPA and nifedipine were delivered via the bathing solution while caffeine was delivered via a micropipette driven by a pressure ejection system (Picospritzer II, General Valve, Fairfield, NJ). Confocal microscopy was performed at room temperature (21–23°C) using a custom-built apparatus (see Ref. 31) on the basis of an inverted Nikon Eclipse TE2000–4 microscope using a ×40 S Fluor objective. Briefly, 488-nm illumination from a photodiode laser was scanned across an isolated cell in x- and y-planes by using two oscillating mirrors. The emitted fluorescence (~514 nm) was detected by a photomultiplier; the signal was then digitized and images were generated (1 frame/s, 480 × 400 pixels); these were stored in TIF stacks of several hundred frames on a local hard drive using image-acquisition software (Image Savant 4.0; IO Industries, London, ON, Canada). Image files were then imported into Scion (Scion; free download: www.scioncorp.com) for subsequent analysis, by using a custom-written macro.

Patch-clamp recordings. After digestion of tracheal smooth muscle strips (see Cell isolation), several drops of cell suspension were added to the bottom of a recording chamber (1.5-ml volume). Cells were allowed to settle and adhere and then were superfused with standard Ringer’s solution at room temperature (21–23°C). Electrophysiological responses were tested in cells that were phase dense and appeared relaxed. Average cell size was 55.6 ± 4 μm (n = 22) and 43.3 ± 7 μm (n = 4) for bovine and porcine tracheal smooth muscle, respectively.

Whole cell currents were recorded using the nystatin perforated patch configuration of the standard patch-clamp technique (11). Pipettes with tip resistances of 3–5 MΩ, when filled with sterile filtered (0.2 μm) electrode solution (see Solutions and chemicals), were fashioned from borosilicate glass using a P-87 Flaming/Brown micropipette puller (Sutter Instrument, Novato, CA). Liquid junction potentials were nullled once the recording electrode entered the bath and electrophysiological recordings commenced as series resistance dropped below 30 MΩ (typically within 10–15 min of seal formation); 70–80% compensation was routinely employed. Command potentials were generated by pClamp 6 software (Clampex 6.0.2, Axon Instruments, Foster City, CA). Whole cell currents were filtered at a bandwidth of 1 kHz (~3 dB, low pass 4-pole Bessel filter, Axopatch-1D patch clamp amplifier), sampled at 2.5 kHz, digitized (DigiData 1200 A/D converter, Axon Instruments) and stored on a local hard drive (Dell OptiPlex G1, pentium II, Dell, Mississauga, Canada). Subsequent current analysis was conducted with pClamp 6 software (Clampfit 6.0.2, Axon Instruments).

SERCA function was inhibited by CPA added directly to the bathing solution, and the consequent development of membrane currents was monitored at a holding potential of −60 mV (to obtain a continuous record of electrophysiological events) or during voltage steps (200-ms duration; 15-s intervals) to −60 mV from a holding potential of 0 mV. Current-voltage relationships were examined using voltage ramps (+80 to −100 mV, 2-s duration) or voltage steps (−80 to +50 mV, 200-ms duration, 10-mV increments) delivered from a holding potential of 0 mV (previously shown to inactivate voltage-dependent Ca²⁺ channels) (14). Ramps were applied at 5-s intervals, and each record represents the average of 4 consecutive ramps.

Liquid junction potentials (LJP) between the electrode and bathing solutions were measured using a 3 M KCl salt bridge. LJPVs were −3.8 ± 0.5 and −11 ± 0.3 mV for Ringer’s and Na⁺-free Ringer’s, respectively (four independent measurements taken for each). Posterior corrections were applied to measurements of reversal potentials reported in the text. Corrections were of opposite polarity to measured liquid junction potentials as determined by the equation Vm = Vcmd − LJP; where Vm is the membrane potential of the cell and Vcmd is the command potential delivered by the patch-clamp amplifier. Membrane recordings illustrated in the figures were not corrected for LJPs.

Solutions and chemicals. All drugs and reagents were obtained from Sigma Chemical (Louisville, MO). Krebs buffer utilized for the transport and dissection of tissues consisted of (in mM) 116 NaCl, 4.6 KCl, 2.5 CaCl₂, 13 NaHPO₄, 1.2 MgSO₄, 23 NaHCO₃, 11 d-glucose, and 10⁻⁵ M indomethacin (to prevent formation of endogenous prostaglandins), bubbled with 95% O₂-5% CO₂ to maintain a pH of 7.4. Ringer’s buffer, utilized for patch-clamp and Ca²⁺-fluorometry experiments, consisted of (in mM) 130 NaCl, 5 KCl, 1 CaCl₂, 1 MgCl₂, 20 HEPEs, 10 d-glucose, and 0.1 niflumic acid; pH 7.4 with NaOH (~300 mg/H₂O). Ca²⁺-free Ringer’s was prepared by eliminating CaCl₂ and adding 10 mM EGTA. Na⁺-free Ringer’s solution, in which Na⁺ was replaced by the membrane impermeable cation N-methyl-D-glucamine (NMDG) consisted of (in mM): 140 NMDG, 5 KCl, 1 CaCl₂, 1 MgCl₂, 20 HEPEs, 10 d-glucose and 0.1 niflumic acid; pH 7.4 with HCl (~310 mg/H₂O). The composition of the intracellular electrode solution was (in mM) 130 CsCl, 5 MgCl₂, 1 CaCl₂, 10 HEPEs, and 5 EGTA; pH 7.2 with CsOH (~275 mg/H₂O). These pharmacological and ionic conditions eliminated currents through Ca²⁺-dependent Cl⁻ and K⁺ channels.

Nystatin (30 mg/ml) was prepared in dimethyl sulfoxide (DMSO) for storage up to 5 days and diluted to a final concentration of 300 μg/ml in electrode solution daily. Gd³⁺, La³⁺, and nifedipine were delivered via external bathing solutions. CPA (10⁻² M stock), fluo-4 AM (10⁻² M stock), and nifedipine (10⁻¹ M stock) were prepared in DMSO and stored at −70°C until use (nifedipine was stored up to 5 days); final concentration of DMSO in bath was ≤0.001%.

Data analysis. Average fluorescence intensities from regions of interest (30 × 30 pixels) defined in central nonnuclear regions of single tracheal myocytes were calculated for each frame and plotted against time. Fluorescence values were expressed as a fluorescence ratio (F/F₀), where F₀ is the initial fluorescence level.

Current records were obtained immediately before (control) and during drug application, with each cell acting as its own control. Where indicated, traces recorded before application of CPA were used to subtract baseline currents offline. Current records were filtered offline at 250 Hz (low-pass Gaussian filter) using pClamp6 software (Clampfit 6.0.2, Axon Instruments) for subsequent analysis and presentation. In studies where channel inhibitors were utilized, data are reported as percent inhibition of CPA-evoked current before subtraction of control (baseline) currents.

All data are reported as means ± SE; n values indicate number of animals tested. Comparisons were made using a paired, two-tailed Student’s t-test (unless otherwise noted), with P values <0.05 considered significant.

RESULTS

Inhibition of SERCA pump by CPA induces capacitative Ca²⁺ entry. Ca²⁺-fluorometry was performed on bovine tracheal myocytes treated with CPA to investigate the relationship between internal Ca²⁺ store depletion and Ca²⁺ influx (capacitative Ca²⁺ entry).
Under resting conditions (in the presence of external Ca\(^{2+}\) and absence of CPA), a 10-s application of 10 mM caffeine (an agonist of ryanodine receptors on the SR) caused a transient rise in F/F\(_o\) with an average magnitude of 2.1 ± 0.3 (n = 11, P < 0.05) (Fig. 1A). Cells were allowed to recover for a period of 2–5 min, before experiments were started. Subsequent removal of external Ca\(^{2+}\) caused F/F\(_o\) in the majority of cells (13/20) to decrease (e.g., Fig. 1B), reaching a stable level of 0.86 ± 0.04 (n = 20, P < 0.05) over a 10- to 20-min period; interestingly, 7 of 20 cells examined did not experience a drop in F/F\(_o\) upon removal of extracellular Ca\(^{2+}\) (e.g., Fig. 1A). Application of CPA (to induce passive SR Ca\(^{2+}\) depletion) led to a transient rise in F/F\(_o\) (early phase) that was followed by a sustained decrease (late phase) back to levels existing before CPA-EVOKED CURRENT IN AIRWAY SMOOTH MUSCLE

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application of CPA ($n=10$, $P<0.05$) (Fig. 1, B and C, left). Subsequent reintroduction of extracellular Ca$^{2+}$ resulted in a rapid and often sustained increase in F/F$_{0}$ ($n=10$, $P<0.05$ vs. F/F$_{0}$ before reintroduction of Ca$^{2+}$) (Fig. 1, B and C, left). In a separate group of cells, reintroduction of external Ca$^{2+}$ after a 10- to 20-min exposure to zero-Ca$^{2+}$ Ringer’s in the absence of CPA did not result in a significant elevation in F/F$_{0}$ ($n=5$, $P>0.05$ vs. F/F$_{0}$ before reintroduction of Ca$^{2+}$), as exemplified in Fig. 1A. Taken together, these observations suggest that passive depletion of SR Ca$^{2+}$ in airway smooth muscle results in the activation of a Ca$^{2+}$ influx pathway.

To determine whether VGCCs play a role in mediating the Ca$^{2+}$ influx observed in cells treated with CPA, we repeated the above experiments in the presence of 1 mM nifedipine, a putatively selective inhibitor of L-type VGCCs. When cells were bathed in Ringer’s solution containing 1 mM Ca$^{2+}$, the addition of nifedipine had no significant effect on F/F$_{0}$ ($n=5$, $P>0.05$) (Fig. 1C, right). However, subsequent removal of Ca$^{2+}$ from the bathing medium and addition of CPA (late phase) each caused a significant reduction in F/F$_{0}$ ($n=5$, $P<0.05$ for each condition) (Fig. 1C, right). Reintroduction of external Ca$^{2+}$ in cells pretreated with CPA resulted in a significant increase in F/F$_{0}$ to $1.2 \pm 0.05$ ($n=5$, $P<0.05$ vs. F/F$_{0}$ before reintroduction of Ca$^{2+}$) that was resistant to treatment with 1 μM nifedipine, suggesting that L-type VGCCs do not mediate Ca$^{2+}$ influx in response to CPA.

**CPA activates a nonselective cation conductance.** Whole-cell currents were studied by using an internal pipette solution containing 130 mM Cs$^{+}$ (to eliminate K$^{+}$ currents) and 100 μM niflumic acid (to inhibit Ca$^{2+}$-dependent Cl$^{-}$ currents), in combination with an external bathing solution containing 130 mM Na$^{+}$ (standard Ringer’s). In bovine tracheal smooth muscle cells voltage clamped at −60 mV, baseline membrane currents were negligible ($−1.6 \pm 0.3$ pA/pF, $n=22$, Fig. 2, A and B). However, application of 10 μM CPA resulted in the activation of a large inward current (Fig. 2A) with a magnitude of $−7.6 \pm 1.6$ pA/pF ($n=22$, $P<0.05$ vs. baseline, Fig. 2B). $I_{CPA}$ activated rapidly, reaching 10% of peak magnitude within

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**Fig. 2.** CPA activates an inward current at −60 mV in porcine and bovine tracheal smooth muscle cells. A: representative trace of a CPA-evoked current in a bovine tracheal myocyte voltage-clamped at −60 mV. Dotted line represents zero current level. Vertical and horizontal scale bars indicate 20 pA and 1 min, respectively. Gap in data (30-s; [CPA] = 10 μM. B: mean current at −60 mV holding potential before (open bars) and after (solid bars) exposure to CPA in bovine (BTSM; $n=22$) and porcine (PTSM; $n=4$) tracheal smooth muscle cells. *Significantly different from baseline. C: time course of CPA-activated membrane current ($I_{CPA}$) in a cell held at 0 mV and stepped to −60 mV at 15-s intervals.
4.9 ± 0.9 min (n = 15) and becoming fully developed 10.1 ± 1.8 min (n = 15) after introduction of CPA (Fig. 2C). In porcine tissues, membrane currents were increased from −2.0 ± 0.9 pA/pF at baseline to −7.3 ± 0.6 (n = 4, P < 0.001) after treatment with CPA (Fig. 2B).

The steady-state current-voltage relationship of \( I_{\text{CPA}} \) was examined using voltage steps (−80 to +50 mV, 200-ms duration, 10-mV increments) delivered from a holding potential of 0 mV; difference currents were obtained by subtracting baseline currents measured before drug application (Fig. 3A). In both porcine and bovine tracheal smooth muscle cells, \( I_{\text{CPA}} \) exhibited a linear current-voltage relationship with mean reversal potential (\( E_{\text{rev}} \)) of 9.7 ± 0.6 mV (n = 4) and 4.5 ± 1.3 mV (n = 22), respectively (Fig. 3B). Under these experimental conditions, i.e., 130 mM extracellular Na\(^+\), blockade of Ca\(^{2+}\)-dependent Cl\(^-\) channels with 100 \( \mu \)M niflumic acid, and elimination of K\(^+\) currents by replacement with intracellular Cs\(^+\), the observed \( E_{\text{rev}} \) is consistent with the involvement of a nonselective cation conductance (1, 2, 6).

Effect of extracellular Na\(^+\) replacement on CPA-activated current. Given our hypothesis that \( I_{\text{CPA}} \) may be a nonselective cation conductance, we examined the effects of replacing extracellular Na\(^+\) with the impermeable cation NMDG. In bovine tracheal myocytes, replacement of extracellular Na\(^+\) resulted in a marked decrease in the magnitude of \( I_{\text{CPA}} \) measured at −60 mV, which was also accompanied by a decrease in the noise level associated with the current (Fig. 4A). Overall, \( I_{\text{CPA}} \) was significantly reduced from −1.3 ± 0.4 pA/pF to −0.1 ± 0.4 pA/pF in the absence of external Na\(^+\) (n = 6, P < 0.05). On the other hand, the outward component of \( I_{\text{CPA}} \) (measured at positive membrane potentials) was largely unaffected or slightly augmented in the absence of extracellular Na\(^+\) (Fig. 4B). In addition to a reduction in the magnitude of the inward component of \( I_{\text{CPA}} \), there was a concomitant shift in the reversal potential toward more negative potentials (Fig. 4B). Overall, \( E_{\text{rev}} \) for \( I_{\text{CPA}} \) was shifted from 9.3 ± 4 mV to −24.8 ± 16 mV after replacement of Na\(^+\) with NMDG (n = 4–6, P < 0.05, one-way ANOVA). Figure 4C illustrates that the Na\(^+\)-dependent portion of \( I_{\text{CPA}} \), i.e., the difference current obtained from Fig. 4B, is inward at potentials below +20 mV, as would be expected if Na\(^+\) were a major charge carrier for this conductance.

Pharmacological profile of CPA-activated current. Store-operated currents and CCE are sensitive to a variety of inor-

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**Fig. 3.** Current-voltage relationship of \( I_{\text{CPA}} \) in porcine and bovine tracheal smooth muscle. **A:** representative trace of currents evoked by voltage pulses delivered from a holding potential of 0 mV (see inset) in a porcine tracheal smooth muscle cell before (left) and during (middle) exposure to CPA (10 \( \mu \)M). **Right:** difference current (\( I_{\text{CPA}} \)) obtained by subtracting baseline (control) currents from those recorded in the presence of CPA. Vertical and horizontal scale bars indicate 200 pA and 100 ms, respectively. **B:** mean CPA-sensitive current (\( I_{\text{CPA}} \)) from porcine (left, n = 4) and bovine (right, n = 22) tracheal smooth muscle obtained using voltage protocol in A.
ganic cations including Ni$^{2+}$, Cd$^{2+}$, Gd$^{3+}$, and La$^{3+}$ (3, 7, 8, 21, 25, 29, 33). Consequently, we tested the sensitivity of $I_{\text{CPA}}$ to Gd$^{3+}$ and La$^{3+}$ in bovine tracheal smooth muscle cells. In addition, we also studied the effects of nifedipine, an inhibitor of L-type VGCCs (14), which is thought to discriminate well between Ca$^{2+}$ entry mediated by VGCCs as opposed to CCE mediated by nonselective cation channels (3, 7, 25). The results of these interventions on the CPA-evoked current in bovine tracheal smooth muscle cells are summarized in Table 1.

Application of 10 μM Gd$^{3+}$, which inhibits CCE in rabbit cerebral arteriolar myocytes (7) but which has not been tested in airway smooth muscle cells, caused a marked reduction in $I_{\text{CPA}}$ at both positive and negative potentials (Fig. 5A). Application of 100 μM Gd$^{3+}$ did not cause any further reduction in $I_{\text{CPA}}$. In addition, there was no apparent shift in $E_{\text{rev}}$ for $I_{\text{CPA}}$ at either concentration of Gd$^{3+}$ tested ($n = 5$ for each Gd$^{3+}$ concentration, $P > 0.05$ for both). Although others have previously reported that Gd$^{3+}$ inhibits CCE in rabbit cerebral arteriolar myocytes with an IC$_{50}$ of 101 nM (7), we found our preparation to be remarkably resistant to 100 nM Gd$^{3+}$ (data not shown).

Similarly, La$^{3+}$, a widely utilized inhibitor of store-operated channels and CCE in many tissues (3, 7, 21, 29) with highly inhibitory effects in the micromolar range, reversibly inhibited $I_{\text{CPA}}$ in a concentration-dependent fashion (Fig. 5B and Table 1). Again, there was no apparent shift in $E_{\text{rev}}$ for $I_{\text{CPA}}$ ($n = 4$ and 5 for 1 and 10 μM La$^{3+}$, respectively, $P > 0.05$ for both concentrations of La$^{3+}$).

Next we examined the effects of nifedipine, which has been shown to be ineffective in reducing CCE and CPA-evoked currents in several tissues (3, 7, 13). Interestingly, 1 μM nifedipine (a concentration reported to be highly effective in abolishing L-type Ca$^{2+}$ currents in airway smooth muscle) (14) caused only a minor suppression of the CPA-evoked current (Fig. 5C, Table 1).

**DISCUSSION**

The intracellular Ca$^{2+}$ store plays a central role in agonist-mediated contraction of airway smooth muscle (4, 12, 19), yet the mechanisms underlying Ca$^{2+}$ homeostasis in this tissue are
Fig. 5. Pharmacology of CPA-evoked current in bovine tracheal smooth muscle. A: representative traces of currents evoked by voltage pulses (see inset), demonstrating inhibition of CPA-activated current by 10 μM Gd⁴⁺. [CPA] = 10 μM. B: representative traces of currents evoked by voltage ramps (right), illustrating concentration-dependent effect of La⁴⁺ on CPA-activated current. C: mean Gd⁴⁺-sensitive (10 μM, n = 5) and La⁴⁺-sensitive (10 μM, n = 5) portion of I_{CPA} using voltage protocol in A. D: representative traces of currents evoked by voltage stepping 1 cell to −60 mV from a holding potential of 0 mV (see inset), illustrating that I_{CPA} is largely insensitive to 1 μM nifedipine. Vertical and horizontal scale bars indicate 200 pA and 100 ms, respectively.
still poorly understood. In the present study, we examined a membrane current activated by the SERCA inhibitor CPA. In addition, we examined the pharmacology and ionic permeability of this membrane conductance to better characterize the properties of the channel responsible for mediating capacitative Ca\(^{2+}\) entry in porcine and bovine tracheal smooth muscle cells.

**Baseline Ca\(^{2+}\) entry.** In the present study, fluorometric techniques were utilized to monitor changes in [Ca\(^{2+}\)]\(_i\) in response to changing external Ca\(^{2+}\) concentration and passive internal Ca\(^{2+}\) store depletion utilizing CPA. We found that eliminating extracellular Ca\(^{2+}\) alone generally caused a reduction in F/F\(_o\), suggesting that Ca\(^{2+}\) influx pathway(s) in airway smooth muscle may operate even under resting conditions (Fig. 1B). However, this baseline Ca\(^{2+}\) influx does not seem to involve VGCCs because application of 1 \(\mu\)M nifedipine had no effect on F/F\(_o\), (Fig. 1C, right). Others have likewise reported a similar lack of effect on baseline Ca\(^{2+}\) fluorescence levels when nifedipine and other Ca\(^{2+}\) channel blockers (e.g., Gd\(^{3+}\) and Ni\(^{2+}\)) were utilized (7, 8).

**CPA induces capacitative Ca\(^{2+}\) entry.** Application of CPA in itself resulted in a moderate rise in F/F\(_o\), a well-documented phenomenon (3, 8, 16, 17, 21, 25, 29, 33) that likely represents an unmasking of Ca\(^{2+}\) “leak” from the SR as CPA inhibits SERCA activity. This rise in F/F\(_o\) was short lived and was accompanied by a sustained reduction in F/F\(_o\) as internal store Ca\(^{2+}\) content continued to decline under the influence of CPA and Ca\(^{2+}\) was extruded from the cytoplasm by plasmalemmal Ca\(^{2+}\)-ATPases. Reintroduction of external Ca\(^{2+}\) resulted in a rapid and, in most cases, sustained elevation of F/F\(_o\) that was insensitive to nifedipine (Fig. 1C). These results parallel those previously reported by others (3, 7, 13) and suggest that CPA induces CCE in bovine tracheal myocytes, which is not mediated by L-type VGCCs.

In human pulmonary artery smooth muscle cells, store depletion induces a rise in [Ca\(^{2+}\)]\(_i\) via the reverse mode operation of the Na\(^+\)/Ca\(^{2+}\) exchanger (39). Although we did not test this possibility directly, we feel it is unlikely to contribute to I\(_{CPA}\) or CCE in airway smooth muscle. First, we have previously shown that Na\(^+\)/Ca\(^{2+}\) exchange contributes very little to regulation of [Ca\(^{2+}\)]\(_i\) in airway smooth muscle, neither under normal conditions nor those that strongly favor reverse mode operation of the Na\(^+\)/Ca\(^{2+}\) exchanger (i.e., removal of extracellular Na\(^+\)) (16). Second, a more recent report ruled out a role for Na\(^+\)/Ca\(^{2+}\) exchange in CCE in porcine airway smooth muscle on the basis that Ca\(^{2+}\) influx after store depletion with caffeine was insensitive to KBR-7943, a selective inhibitor of Na\(^+\)/Ca\(^{2+}\) exchange (29).

**Time course of CPA-evoked current.** The CPA-evoked current began to manifest within ~5 min after application of CPA and developed completely within the next ~5 min (Fig. 2B). CPA-evoked currents in isolated rabbit portal vein myocytes (1), human (8) and rat (25) pulmonary arterial myocytes, as well as human and rat bronchial smooth muscle cells (33) show a similar time course of activation. In addition, others have demonstrated that store depletion in canine airway smooth muscle cells, using agonist-evoked Cu\(^{2+}\) release as an index of SR Ca\(^{2+}\) levels, may require anywhere from 15 to 25 min to develop fully (4, 15, 16).

**I\(_{CPA}\) is a nonselective cation conductance.** Utilizing patch clamp techniques, we found that CPA activates a membrane current in both porcine and bovine tracheal smooth muscle cells that exhibits a linear current-voltage relationship with a reversal potential around 0 mV (Fig. 3B). These characteristics are consistent with the involvement of a nonselective cation channel (1, 2, 6), given the ionic and pharmacological conditions in which our experiments were conducted. CCE in many cell types is mediated by a set of nonselective cation channels of the TRP superfamily (26), the mammalian homolog of the Drosophila phototransduction pathway (23). In addition to a recent report indicating that porcine airway smooth muscle express TRPC isoforms 1, 3, and 4 (3), some have demonstrated that expression of TRPC isoforms such as 1 and 6 in airway (33) and pulmonary vascular smooth muscle (8) is increased during smooth muscle proliferation and coincides with an increase in the magnitude of CPA-evoked membrane currents and CCE in these cells. Although these data give rise to the possibility that TRP proteins may mediate CCE in several smooth muscle cell types, the evidence is circumstantial and has given rise to controversy (26, 32). However, recent evidence in human pulmonary smooth muscle is shedding some light on the relationship between TRP channels, store-operated cation channels, and CCE (38); using small interfering RNA directed against TRPC4, Zhang et al. (38) demonstrate CCE amplitude is directly related to ATP-induced TRPC4 expression in human pulmonary arterial smooth muscle. Similar experiments have yet to be performed in airway smooth muscle.

**Ionic species responsible for I\(_{CPA}\).** To further investigate the nature of I\(_{CPA}\), we examined the effects of replacement of extracellular Na\(^+\). Others have shown that, under physiological conditions (i.e., high extracellular Na\(^+\) and membrane potentials of ~70 to ~60 mV), nonselective cation channels exhibit inward currents that are largely due to Na\(^+\) permeation (1, 2, 6, 34). We found that I\(_{CPA}\) exhibited a reversal potential of 5.9 ± 0.6 mV (n = 4) and 6.5 ± 1.3 mV (n = 22) in porcine and bovine tracheal smooth muscle, respectively (Fig. 3B). These characteristics of I\(_{CPA}\) are similar to those in proliferating rat and human bronchial smooth muscle cells (31). Our results further suggest that a significant portion of I\(_{CPA}\) at negative potentials is due to Na\(^+\) influx (Fig. 4). Indeed, Na\(^+\) replacement with the impermeable cation NMDG not only leads to a significant reduction in I\(_{CPA}\) at negative potentials (Fig. 4C), but also resulted in a shift in the reversal potential toward negative potentials (Fig. 4B). This feature is characteristic of nonselective cation channels (6, 15, 34) and TRPC channels (10), which are thought to mediate capacitative Ca\(^{2+}\) entry (22).

Although Na\(^+\) replacement decreased the inward component of I\(_{CPA}\), it did not abolish it; the remaining current may represent Ca\(^{2+}\) influx. In fact, Fleischmann et al. (6) examined the muscarinic activation and calcium permeation of a nonselective cation current in equine tracheal smooth muscle, demonstrating that the fraction of this current carried by Ca\(^{2+}\) under physiological conditions was estimated to be ~14% at ~60 mV. Though these observations suggest that Ca\(^{2+}\) influx mediated by nonselective cation channels may be small, there is substantial evidence that this Ca\(^{2+}\) conductance is sufficient to raise cytosolic Ca\(^{2+}\) well above basal levels (6, 8, 17, 21, 29, 33) even in the presence of VGCC blockers (3, 7, 25).

Taken together, the findings above suggest that nonselective cation channels not only function to conduct Ca\(^{2+}\) and mediate CCE in this tissue but may also serve to depolarize the plasma.
membrane, thereby activating VGCCs to further facilitate Ca\(^{2+}\) influx during instances when SR Ca\(^{2+}\) stores are mobilized (i.e., during agonist exposure).

**Pharmacology of CPA-activated current.** Next we examined the pharmacological profile of \(I_{\text{CPA}}\) in bovine tracheal smooth muscle cells using inorganic cations including Gd\(^{3+}\) and La\(^{3+}\), which have previously been reported to inhibit CPA-activated membrane currents and/or CCE in several tissues (3, 7, 21, 25, 29).

Gd\(^{3+}\) has generally been used at micromolar concentrations, and more recently in some studies at 100 nM (7). We found \(I_{\text{CPA}}\) to be highly sensitive to 10 \(\mu\)M Gd\(^{3+}\) (Fig. 5A); however, Gd\(^{3+}\) is not a definitive tool in studies of CCE function. Wilson et al. (36) report that CCE in canine renal and pulmonary arterial smooth muscle cells is resistant to 100 \(\mu\)M Gd\(^{3+}\).

Likewise, Wayman et al. (35) demonstrated that store-operated cation channels in mouse anococcygeal smooth muscle were unaffected by concentrations of Gd\(^{3+}\) up to 400 \(\mu\)M, suggesting that there is store-operated cation channel diversity (7).

Part of the discrepancy may relate to the experimental conditions used to study these currents. Halasovich et al. (9) showed that the IC\(_{50}\) for Gd\(^{3+}\) was 4 \(\mu\)M when applied to the extracellular face of the channel (TRP3), but 20 \(\mu\)M when applied to the cytosolic face. Our experimental approach (nystatin-perforated patch, whole-cell configuration) did not allow us to apply blockers to the cytosolic face of the channels.

In addition, we found \(I_{\text{CPA}}\) to be inhibited by La\(^{3+}\) in a concentration-dependent fashion (1 and 10 \(\mu\)M concentrations tested) (Fig. 5, A and B). Others have reported similar effects of La\(^{3+}\) on CCE and CPA-evoked membrane currents (3, 21, 25, 29). In particular, only two studies have reported a La\(^{3+}\)-sensitive CCE mechanism evoked by CPA in airway smooth muscle (3, 29). However, these did not measure the membrane currents per se. In addition, we found that \(I_{\text{CPA}}\) was largely insensitive to nifedipine, a property that is characteristic of CCE in smooth muscle (3, 7, 13).

We conclude that CPA activates a Ca\(^{2+}\)-permeable, nonselective cation conductance (\(I_{\text{CPA}}\)) in airway smooth muscle cells that is both Gd\(^{3+}\) and La\(^{3+}\) sensitive but largely insensitive to nifedipine. At physiological membrane potentials (i.e., -60 mV), \(I_{\text{CPA}}\) is inward and largely carried by Na\(^{+}\) with the remainder of the inward current likely representing Ca\(^{2+}\) influx. In addition, our observations suggest that this nifedipine-insensitive Ca\(^{2+}\) influx pathway may operate under resting conditions in airway smooth muscle and is likely responsible for capacitative Ca\(^{2+}\) entry observed in this tissue.

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**References**


