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Effects of Volatile Anesthetics on Store-operated Ca^{2+} Influx in Airway Smooth Muscle

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Background: **In airway smooth muscle (ASM), volatile anesthetics deplete sarcoplasmic reticulum (SR) Ca^{2+} stores by increasing Ca^{2+} "leak." Accordingly, SR replenishment becomes dependent on Ca^{2+} influx. Depletion of SR Ca^{2+} stores triggers Ca^{2+} influx *via* specific plasma membrane channels, store-operated Ca^{2+} channels (SOCC). We hypothesized that anesthetics inhibit SOCC triggered by increased SR Ca^{2+} "leak," preventing SR replenishment and enhancing ASM relaxation.**

Methods: **In porcine ASM cells, SR Ca^{2+} was depleted by cyclopiazonic acid or caffeine in 0 extracellular Ca^{2+}, nifedipine and KCl (preventing Ca^{2+} influx through L-type and SOCC channels). Extracellular Ca^{2+} was rapidly introduced to selectively activate SOCC. After SOCC activation, SR was replenished and the protocol repeated in the presence of 1 or 2 minimum alveolar concentration halothane, isoflurane, or sevoflurane. In other cells, characteristics of SOCC and interactions between acetylcholine (Ach) and volatile anesthetics were examined.**

Results: **Cyclopiazonic acid produced slow SR leak, whereas the caffeine response was transient in ASM cells. Reintroduction of extracellular Ca^{2+} rapidly increased $[Ca^{2+}]_i$. This influx was insensitive to nifedipine, SKF-96365, and KBR-7943, inhibited by Ni^{2+} and blockade of inositol 1,4,5-triphosphate-induced SR Ca^{2+} release, and enhanced by ACh. Preexposure to 1 or 2 minimum alveolar concentration halothane completely inhibited Ca^{2+} influx when extracellular Ca^{2+} was reintroduced, whereas isoflurane and sevoflurane produced less inhibition. Only halothane and isoflurane inhibited ACh-induced augmentation of Ca^{2+} influx.**

Conclusion: **Volatile anesthetics inhibit a Ni^{2+}/La^{3+}-sensitive store-operated Ca^{2+} influx mechanism in porcine ASM cells, which likely helps maintain anesthetic-induced bronchodilation.**

VOLATILE anesthetic-induced bronchodilation involves a reduction in intracellular Ca^{2+} ($[Ca^{2+}]_i$) that is key to development and maintenance of force in airway smooth muscle (ASM) cells.[1–5] These effects of anesthetics are partly attributable to an inhibition of Ca^{2+} influx *via* L-type Ca^{2+} channels.[5–7] We[1,2] and others[8] have shown that anesthetics also deplete Ca^{2+} stores of the sarcoplasmic reticulum (SR) by increasing Ca^{2+} "leakage." Such anesthetic-induced SR Ca^{2+} leak occurs *via* both inositol 1,4,5-trisphosphate or ryanodine receptor (RyR) channels.[2]

$[Ca^{2+}]_i$ regulation in ASM is mediated by both extracellular Ca^{2+} influx and SR Ca^{2+} release. Both inositol 1,4,5-trisphosphate receptor[9] and RyR[10] channels play important roles in ASM Ca^{2+} regulation. Ca^{2+} influx occurs through both voltage-gated[11] and receptor-gated channels.[12] There is now considerable evidence from a variety of cell types that controlled Ca^{2+} influx through store-operated Ca^{2+} channels (SOCC; also termed capacitative Ca^{2+} entry) occurs in response to SR Ca^{2+} depletion, thus allowing for replenishment of intracellular Ca^{2+} stores.[13–16] Such influx does not occur through either L-type or receptor operated channels.[13]

There is now considerable evidence for SOCC in vascular[13,17–20] and other smooth muscle types.[21] Previous studies suggested that SOCC-mediated Ca^{2+} influx is restricted to the replenishment of inositol 1,4,5-trisphosphate-sensitive Ca^{2+} stores.[13,22] Recent studies in tissues other than ASM have suggested that Ca^{2+} release through RyR channels may also trigger SOCC.[21,23] The role of SOCC in ASM had been suggested by experiments in guinea pigs based on comparisons of contractions induced by agonists versus inhibitors of the SR Ca^{2+} adenosine triphosphatase.[24] Furthermore, SR Ca^{2+} adenosine triphosphatase inhibitors have been shown to increase $[Ca^{2+}]_i$ in human bronchioles and bovine ASM, suggesting the involvement of SOCC.[25] However, characterization of SOCC-mediated Ca^{2+} influx in ASM is relatively lacking.

Volatile anesthetic-induced SR Ca^{2+} leak (and eventual depletion) may be expected to trigger SOCC-mediated Ca^{2+} influx in ASM cells. However, we hypothesize that volatile anesthetics will also inhibit such influx, thus accentuating the state of SR depletion.

Materials and Methods

Cell Preparation

Details of the ASM cell preparation technique have been previously described.[10,26] Porcine tracheae were obtained from a local abattoir. The smooth muscle layer was excised, minced in Hanks' balanced salt solution (HBSS) with 10 mM HEPES (pH 7.4; Invitrogen, Carlsbad, CA), and then incubated first for 2 h with 20 U/ml papain and 2000 U/ml DNase (Worthington Biochemical Corp., Lakewood, NJ) and subsequently for 1 h at 37°C with 1 mg/ml type intravenous collagenase (Worthington). The sample was triturated, centrifuged, and resuspended in minimum essential medium with 10% fetal calf serum. Isolated cells were plated on collagen-coated glass coverslips, and incubated for 1 h in 5% CO_2 at 37°C.

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Real-Time Confocal Imaging

Details of the confocal imaging technique for ASM cells have also been previously published.[10,26] Briefly, cells attached to coverslips were loaded with 5 μM fluo-3 AM (Molecular Probes, Eugene, OR) for 30–45 min at 37°C in HBSS, washed, mounted on an open slide chamber (RC-25F, Warner Instruments, Hamden, CT), and perfused with HBSS at room temperature. An Odyssey XL real-time confocal system (Noran Instruments, Middleton, WI) with an Ar-Kr laser (488 nm line) and a Nikon 40×/1.3 oil-immersion objective lens was used to visualize ASM cells. Using manufacturer-provided software, regions of interest encompassing single cells were outlined for measurements of $[Ca^{2+}]_i$. Empirical calibration of fluo-3 fluorescence concentrations for $[Ca^{2+}]_i$ based on exposure to known Ca^{2+} concentrations was performed as described previously.[26]

Administration of Volatile Anesthetics

As previously described,[2] calibrated online vaporizers were used to add halothane (Wyeth-Ayerst Laboratories, Philadelphia, PA), isoflurane, and sevoflurane (Abbott Laboratories, Deerfield, IL) to the aerating gas mixture. Aqueous anesthetic concentrations equivalent to 1 and 2 adult porcine minimum alveolar concentration (MAC) at room temperature (25°C) were determined for halothane and isoflurane by gas chromatography and electron capture detector (Hewlett-Packard 5880A; Hewlett-Packard, Sunnyvale, CA) and for sevoflurane by a flame ionization detector. Concentrations were halothane 1 MAC 0.35 ± 0.09 mM, 2 MAC 0.53 ± 0.10 mM, isoflurane 1 MAC 0.4 ± 0.09 mM, 2 MAC 0.6 ± 0.11 mM, and sevoflurane 1 MAC 0.50 ± 0.10 mM, 2 MAC 0.68 ± 0.10 mM.

Store-Operated Ca^{2+} Influx

Baseline Ca^{2+} concentrations were measured while ASM cells were perfused with HBSS. Extracellular Ca^{2+} ($[Ca^{2+}]_o$) was then removed by exposure to zero-Ca^{2+} HBSS (5 mM EGTA). In the continued absence of $[Ca^{2+}]_o$, cells were also exposed to 1 μM nifedipine and 10 mM KCl to ensure that L-type Ca^{2+} channels were not activated during the protocol. Cells were then rapidly exposed to 1 μM cyclopiazonic acid (CPA) in zero-Ca^{2+} HBSS. This technique has been frequently used to induce passive SR depletion by inhibiting SR Ca^{2+} adenosine triphosphatase with continued SR Ca^{2+} leak, presumably from both inositol 1,4,5-trisphosphate- and ryanodine-sensitive SR stores.[13,16] Continued SR Ca^{2+} leak resulted in increased $[Ca^{2+}]_i$ concentrations that typically reached a plateau concentration or started to trend down (reflecting increasing Ca^{2+} efflux *via* the plasma membrane). At this point, 2.5 mM $[Ca^{2+}]_o$ was rapidly reintroduced (in the continued presence of CPA). The observed $[Ca^{2+}]_i$ response was then characterized using various pharmacological manipulations.

In separate sets of ASM cells, we performed additional studies using caffeine for selective depletion of ryanodine-sensitive Ca^{2+} stores and the assessment of any interactions between Ca^{2+} release *via* RyR channels and SOCC. The protocol involved replacing CPA in the above experiments with 5 mM caffeine. As with the CPA protocols, pharmacological manipulations were used to characterized SOCC.

Effect of Volatile Anesthetics on Store-Operated Ca^{2+} Influx

SOCC in ASM cells was first established by performing a control protocol using CPA or caffeine in the presence of nifedipine and KCl (as above). Once SOCC-mediated Ca^{2+} influx was observed, the cells were washed for 15–20 min with HBSS to remove CPA (or caffeine) and to replenish SR Ca^{2+} stores. $[Ca^{2+}]_o$ was then removed and the cells exposed to either CPA or caffeine. Once a $[Ca^{2+}]_i$ response was observed (slow development of plateau with CPA or a transient with caffeine), the cells were exposed for 1 min to 1 or 2 MAC halothane, isoflurane, or sevoflurane in the continued presence of CPA or caffeine. This technique ensured that the anesthetics did not influence the SR Ca^{2+} release process itself, but were present in sufficient concentration before activation of SOCC. In the continued presence of CPA (or caffeine), and anesthetic, $[Ca^{2+}]_o$ was rapidly reintroduced and the $[Ca^{2+}]_i$ response was recorded. In control experiments, the SOCC protocol was performed twice without anesthetic, with an intervening wash in HBSS.

Interactions Between Acetylcholine and Volatile Anesthetic Effects on Store-Operated Ca^{2+} Influx

As above, SOCC in ASM cells was first established by performing a control protocol using CPA or caffeine in the presence of nifedipine and KCl. Cells were then washed in HBSS. $[Ca^{2+}]_o$ was removed, nifedipine and KCl were added, and SR Ca^{2+} release was induced by CPA or caffeine. In the continued presence of CPA or caffeine, the cells were exposed to 10 nM, 100 nM, or 1 μM acetylcholine (Ach) for 2 min. This ensured activation of muscarinic receptors occurred after SR Ca^{2+} release had already occurred. Activation of muscarinic receptors occurred with both protocols; however, in the case of CPA, SR Ca^{2+} depletion is passive, whereas for caffeine, complete release through RyR channels occurs, although inositol 1,4,5-trisphosphate-induced release is dependent on ACh concentration. In the continued presence of CPA (or caffeine) and ACh, $[Ca^{2+}]_o$ was rapidly reintroduced to activated SOCC. Cells were then washed for a second time, and $[Ca^{2+}]_o$ removed (with addition of nifedipine and KCl). After CPA or caffeine exposure, ACh was added as above and after 1 min, cells were exposed to 1 or 2 MAC halothane, isoflurane, or sevoflurane for an additional 1 min. This ensured that muscarinic activation occurred before anesthetic exposure and that confounding effects of anesthetic interactions with

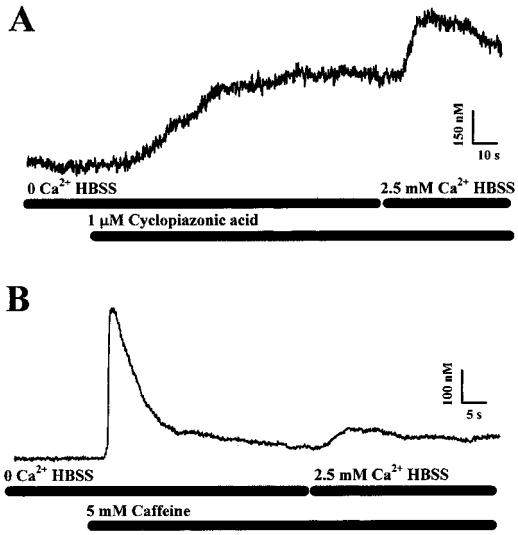

Fig. 1. Ca^{2+} influx *via* store operated Ca^{2+} channels in porcine airway smooth muscle cells. The sarcoplasmic reticulum was depleted either by cyclopiazonic acid or caffeine in the absence of extracellular Ca^{2+} ($[Ca^{2+}]_o$). Subsequently, $[Ca^{2+}]_o$ was reintroduced in the continued presence of cyclopiazonic acid or caffeine. HBSS = Hanks' balanced salt solution.

the receptor itself were avoided. $[Ca^{2+}]_o$ was then rapidly reintroduced. In control protocols, after a control SOCC activation, the ACh protocol was performed twice, without anesthetic.

Statistical Analysis

For each ASM cell, comparisons before and after exposure to a drug were made using paired Student *t* test. Repeated measures analysis of variance was used for multiple comparisons with *post hoc* Bonferroni and Sheffé F tests. All experimental protocols were not performed in the same cells. Results were replicated in at least 3–5 cells obtained from each of five animals (paired comparisons within cells, independent testing across cells). Statistical significance was tested at $P < 0.05$. Values are reported as mean ± SD.

Results

Establishment of Store-Operated Ca^{2+} Influx

At the beginning of experiments, baseline $[Ca^{2+}]_i$ concentrations of ASM cells ranged from 70 to 100 nM (78 ± 10 nM; n = 128). Neither removal of $[Ca^{2+}]_o$ nor addition of nifedipine and KCl significantly affected $[Ca^{2+}]_i$ concentrations. In the absence of $[Ca^{2+}]_o$, 1 μM CPA resulted in increased $[Ca^{2+}]_i$ concentrations that reached a plateau (228 to 518 nM; n = 65). Subsequent rapid reintroduction of $[Ca^{2+}]_o$ resulted in a further, sustained increase of $[Ca^{2+}]_i$ (fig. 1A; 340 to 660 nM; $P < 0.05$ when compared to the first plateau). In contrast to CPA, exposure to 5 mM caffeine in the absence of $[Ca^{2+}]_o$ resulted in a transient $[Ca^{2+}]_i$ response (fig. 1B; n = 63). Reintroduction of $[Ca^{2+}]_o$ resulted in a relatively sustained increase in $[Ca^{2+}]_i$ ranging from 110 to 386 nM (compared to a peak caffeine response ranging from 455 to 890 nM, the influx response was ~25% of the caffeine peak). The influx observed with reintroduction of $[Ca^{2+}]_o$ after caffeine exposure was significantly smaller than that after CPA exposure ($P < 0.05$).

Table 1. Characteristics of SOCC-Mediated Ca^{2+} Influx in ASM

Drug	Ca^{2+} Influx (% peak CPA response)	Ca^{2+} Influx (% peak caffeine response)
Control	39.4 ± 8.3	25.1 ± 4.9
1 μM Ni^{2+}	13.3 ± 2.6*	9.7 ± 1.2*
1 μM La^{2+}	8.6 ± 2.6*	7.2 ± 2.0*
10 μM SKF96365	9.9 ± 5.7*	6.3 ± 3.4*
10 μM KBR7943	38.6 ± 8.0	23.7 ± 4.8
10 nM ACh	38.5 ± 10.2	26.0 ± 5.7
100 nM ACh	51.2 ± 6.9*	31.1 ± 3.2*
1 μM ACh	65.2 ± 5.2*	38.6 ± 3.5*

Values are means ± SE.

* Significant difference from control.

ACh = acetylcholine; CPA = cyclopiazoric acid; SOCC = store-operated Ca^{2+} channel.

Characterization of Store-Operated Ca^{2+} Influx

It has been previously reported that SOCC-mediated Ca^{2+} influx to be inhibited by Ni^{2+} and La^{3+}, at least in tissues other than ASM. After control activation of SOCC with CPA (n = 10) or caffeine (n = 10), and HBSS wash, ASM cells were exposed to nifedipine, KCl and 1 μM $NiCl_2$ or $LaCl_3$ in HBSS. $[Ca^{2+}]_o$ was then removed and, in the continued presence of nifedipine, KCl and Ni^{2+} (or La^{3+}), cells were reexposed to CPA or caffeine, with subsequent reintroduction of $[Ca^{2+}]_o$. In the presence of either 1 μM Ni^{2+} or La^{3+}, reintroduction of $[Ca^{2+}]_o$ did not significantly increase $[Ca^{2+}]_i$ (Table 1; $P < 0.05$ compared to control) indicating that SOCC-mediated Ca^{2+} influx in ASM cells is sensitive to both ions.

To determine whether the observed Ca^{2+} influx was mediated by mechanisms other than SOCC (because L-type Ca^{2+} channels were already inhibited by nifedipine and clamped membrane potential), inhibitors of store-operated Ca^{2+} influx (SKF-96365; 10 μM) and Na^+/Ca^{2+} exchange (KBR-7943; 10 μM) were tested. Although the bidirectional plasma membrane Na^+/Ca^{2+} exchanger may not play a major role in most smooth muscle types, expression of this protein has been found in ASM.[27] When operating in the "reverse" or influx mode, the exchanger can bring Ca^{2+} intracellularly, in exchange for Na^+. After control SOCC activation with CPA (n = 11) or caffeine (n = 11) and HBSS wash, ASM cells were exposed to nifedipine, KCl, and 10 μM SKF-96365 or KBR7943 in HBSS. The SOCC protocol was then repeated in the continued presence of these agents. In 90% of cells, SKF-96365 inhibited SOCC-mediated

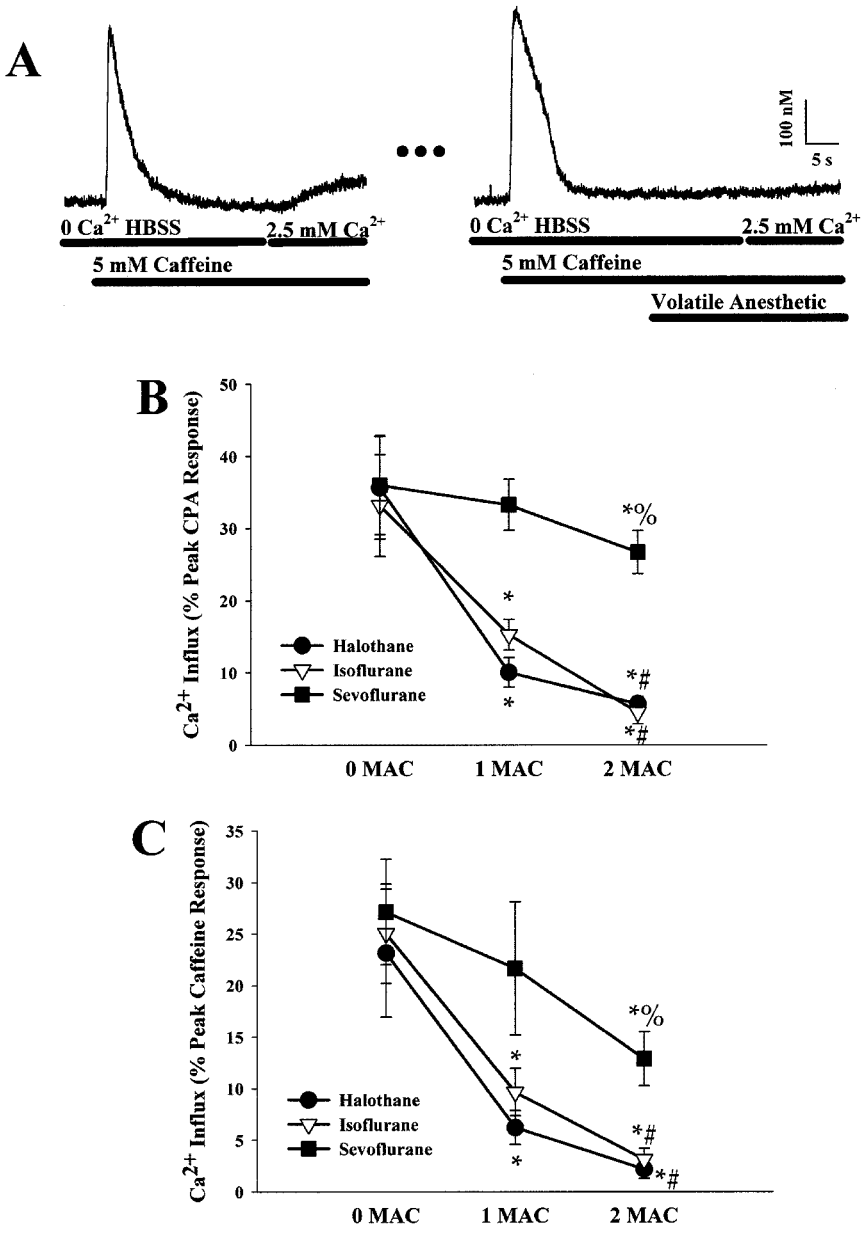

Fig. 2. Effect of volatile anesthetics on store operated Ca^{2+} channel (SOCC)-mediated Ca^{2+} influx. After a control verification of SOCC-mediated Ca^{2+} influx (*A*; caffeine protocol shown), airway smooth muscle (ASM) cells were washed (*dotted lines*) and reexposed to cyclopiazonic acid (CPA) or caffeine. Cells were then exposed to 1 or 2 minimum alveolar concentration (MAC) volatile anesthetic. Preexposure to 1 or 2 MAC halothane or isoflurane resulted in significant reduction of Ca^{2+} influx on reintroduction of $[Ca^{2+}]_o$ after SR Ca^{2+} depletion by CPA (*B*) or caffeine (*C*). In contrast, only 2 MAC sevoflurane decreased influx in either protocol. Halothane and isoflurane were comparable in their inhibitory effect. * = significant difference from control; # = significant difference between 1 and 2 MAC; % = significant difference from halothane ($P < 0.05$); 0 MAC = control response.

Ca^{2+} influx with both CPA and caffeine protocols (Table 1; $P < 0.05$ compared with controls). In contrast, the presence of KBR-7943 had no effect on the $[Ca^{2+}]_i$ response upon reintroduction of $[Ca^{2+}]_o$ (Table 1), demonstrating that Na^+/Ca^{2+} exchange did not substantially contribute to the observed Ca^{2+} influx.

Effect of ACh on Store-Operated Ca^{2+} Influx

Compared with controls where no ACh was present, preexposure of ASM cells to either 100 nM or 1 μM ACh significant increased Ca^{2+} influx with reintroduction of $[Ca^{2+}]_o$ for both CPA and caffeine protocols ($P < 0.05$; Table 1). In contrast, in other controls where the ACh protocol was repeated, there was ~5–7% decrease in the Ca^{2+} influx upon repetition (not shown).

Effect of Volatile Anesthetics on Store-Operated Ca^{2+} Influx

In ASM cells where SOCC-mediated Ca^{2+} influx was first established, repetition of the protocol (CPA or caffeine) resulted in a 5–8% decrease in the observed Ca^{2+} influx (rundown control). In contrast, exposure to halothane, isoflurane, or sevoflurane all resulted in significant decreases in the extent of Ca^{2+} influx after reintroduction of $[Ca^{2+}]_o$ ($P < 0.05$ compared to control for each anesthetic at 1 or 2 MAC, both same cell and rundown control; fig. 2). For halothane and isoflurane, the effects at 1 MAC were significantly less than at 2 MAC, whereas for sevoflurane, the effects were comparable between 1 and 2 MAC. Among the three anesthetics, sevoflurane caused the least inhibition. At 2 MAC, halothane and isoflurane produced comparable inhibition (fig. 2). Be-

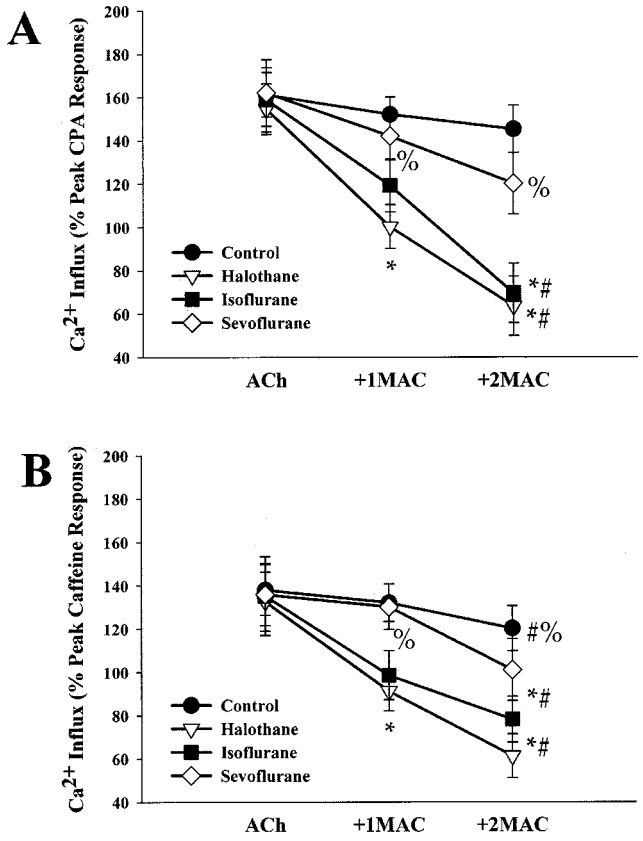

Fig. 3. Interaction between ACh and volatile anesthetic effects on store operated Ca^{2+} channel (SOCC)-mediated Ca^{2+} influx. Preexposure to 1 μM ACh introduced after cyclopiazonic acid (CPA) or caffeine exposure was complete significantly enhanced SOCC-mediated Ca^{2+} influx. However, 1 or 2 minimum alveolar concentration (MAC) halothane or isoflurane (but not sevoflurane) significantly blunted ACh enhancement of Ca^{2+} influx after SR Ca^{2+} depletion by either CPA (panel A) or caffeine (panel B). Halothane and isoflurane were comparable in their inhibitory effect. * = significant difference from control; # = significant difference between 1 and 2 MAC; % = significant difference from halothane ($P < 0.05$).

tween the CPA and caffeine protocols, for each volatile anesthetic, the effect on Ca^{2+} influx was significantly greater for CPA compared to caffeine ($P < 0.05$).

Interaction Between ACh and Volatile Anesthetic Effects on Store-Operated Ca^{2+} Influx

In ASM cells in which both SOCC-mediated Ca^{2+} influx and an ACh-induced augmentation of influx were first established using the CPA or caffeine protocols, exposure to halothane and isoflurane, but not sevoflurane, resulted in significant decreases in the Ca^{2+} influx after reintroduction of $[Ca^{2+}]_o$ ($P < 0.05$ compared to both same cell and ACh rundown control for 1 or 2 MAC; fig. 3). Compared to anesthetic effects on Ca^{2+} influx in the absence of ACh, anesthetic inhibition of ACh-induced augmentation of influx was significantly greater ($P < 0.05$). For halothane and isoflurane, the effects at 1 MAC were significantly less than at 2 MAC, with halothane causing greater inhibition. For both halothane and isoflurane, the effect on ACh augmentation of Ca^{2+} influx was significantly greater for the CPA protocol ($P < 0.05$).

Discussion

Volatile anesthetic effects on ASM have been previously shown to involve inhibition of Ca^{2+} influx *via* L-type Ca^{2+} channels[5–7] as well as increased SR Ca^{2+} leak *via* both inositol 1,4,5-trisphosphate and RyR channels.[1,2,8] Such a leak may be expected to trigger SOCC-mediated Ca^{2+} influx in ASM cells, thus providing a mechanism for replenishment of depleted SR Ca^{2+} stores. However, in support of our hypothesis, the results of this study demonstrate that volatile anesthetics also inhibit such store-operated Ca^{2+} influx, thus accentuating the state of SR depletion. The results further demonstrate that volatile anesthetics, especially halothane and isoflurane, interfere with ACh-induced enhancement of SOCC-mediated Ca^{2+} influx, potentially maintaining bronchodilation even during agonist stimulation.

$[Ca^{2+}]_i$ Regulation in ASM

Regulation of $[Ca^{2+}]_i$ in ASM is mediated by both extracellular Ca^{2+} influx and SR Ca^{2+} release *via* both inositol 1,4,5-trisphosphate receptor[9] and RyR[10] channels. Previous studies have demonstrated that Ca^{2+} influx in ASM occurs through both voltage-gated[11] and receptor-gated channels.[12,28–30] Agonists such as ACh are known to produce SR Ca^{2+} release *via* both inositol 1,4,5-trisphosphate and RyR channels.[10] Indeed, it is thought that the initial $[Ca^{2+}]_i$ response to ACh stimulation is SR Ca^{2+} release, whereas maintenance of ASM $[Ca^{2+}]_i$ and thus muscle tone is thought to involve sustained Ca^{2+} influx. However, we have previously shown that the $[Ca^{2+}]_i$ response to ASM is oscillatory, mediated by repetitive SR Ca^{2+} release and reuptake, where initiation of oscillations is dependent on Ca^{2+} release through inositol 1,4,5-trisphosphate receptor channels, but sustenance of oscillations occurs through Ca^{2+}-induced Ca^{2+} release mechanisms *via* RyR channels.[10,26] The amplitude of $[Ca^{2+}]_i$ oscillations represent SR Ca^{2+} content, and frequency depends on sensitivity for Ca^{2+} release *via* RyR channels. These studies also showed that Ca^{2+} influx serves to maintain and replenish SR Ca^{2+} because oscillations were not sustained in the absence of extracellular Ca^{2+} or in the presence of inhibitors of Ca^{2+} influx.[10,26] Our previous study on SOCC[31] as well as the current report emphasize the importance of this SR- and $[Ca^{2+}]_o$-dependent mechanism in maintaining the repletion state of the SR in ASM.

Effect of Volatile Anesthetics on $[Ca^{2+}]_i$ in ASM

Volatile anesthetics have been shown to target several $[Ca^{2+}]_i$ regulatory mechanisms in ASM at both SR and

the plasma membrane. In terms of SR Ca^{2+} release, we and others have shown that volatile anesthetics deplete SR Ca^{2+} by decreasing SR Ca^{2+} content (store) *via* increased "leak" through both inositol 1,4,5-trisphosphate and RyR channels.[2,32–35] We have previously demonstrated that clinically relevant concentrations of halothane decrease both amplitude and frequency of ACh-induced $[Ca^{2+}]_i$ oscillations. We interpreted these findings as a decrease in SR Ca^{2+} content and reduced sensitivity for Ca^{2+} induced Ca^{2+} release by volatile anesthetics.[35] These data collectively demonstrate that volatile anesthetics deplete SR Ca^{2+} stores, thus preventing an increase of $[Ca^{2+}]_i$ in response to agonist stimulation. Anesthetic-induced depletion of SR Ca^{2+} then raises the question of whether Ca^{2+} influx *via* SOCC (if present) is then activated.

Given the existence of voltage-gated and receptor-operated Ca^{2+} influx mechanisms in ASM, there is already considerable evidence in the literature that the decrease in $[Ca^{2+}]_i$ by volatile anesthetics involves inhibition of Ca^{2+} influx[3,5] through voltage-gated L-type Ca^{2+} channels.[5,6] These studies underline the inhibitory effect of volatile anesthetics on plasma membrane Ca^{2+} regulatory proteins. Accordingly, the results of the current study demonstrating inhibition of SOCC in the plasma membrane are consistent. Functionally, such inhibition only emphasizes the accentuation of anesthetic effects on $[Ca^{2+}]_i$ in ASM because prevention of influx would only maintain the SR in a state of depletion, thus keeping $[Ca^{2+}]_i$ low even with agonist stimulation.

SOCC-Mediated Ca^{2+} Influx and Volatile Anesthetics Effects

In addition to previously established modes of Ca^{2+} influx, *i.e.*, voltage-gated[11] and receptor-gated channels,[12,28–30] in a recent study, we[31] found that Ca^{2+} influx in ASM occurs in response to SR Ca^{2+} depletion. Such a mechanism for influx in response to the state of SR Ca^{2+} stores was initially reported in parotid acinar cells and vascular smooth muscle in which influx was found to be dependent on $[Ca^{2+}]_o$ but not L-type or receptor operated channels.[13,36] Other studies have demonstrated that SR Ca^{2+} adenosine triphosphatease inhibitors (such as thapsigargin, similar to CPA) increase Ca^{2+} influx or smooth muscle tone.[20,37–40] In these studies, whereas $[Ca^{2+}]_o$ is necessary for maintenance of muscle tone or $[Ca^{2+}]_i$, Ca^{2+}-channel blockers do not consistently prevent influx, suggesting that both voltage-gated Ca^{2+} channels and SOCC contribute to smooth muscle Ca^{2+} regulation and tone. Studies to date suggest that SOCC may play a significant role in tonic muscles such as blood vessels.[13,17,18] Only a few studies, including our own, have reported the existence of SOCC in ASM cells.[31,41]

Although the channels that represent SOCC have not been definitively identified, members of the tryptophan family have been implicated in several studies, and mammalian tryptophan genes have been identified.[15,42] Some of these isoforms have been shown to be involved in SOCC.[15,43] We have previously verified the expression of several tryptophan C isoforms in porcine ASM,[31] although other studies have reported tryptophan C expression in guinea pig ASM.[44] Therefore, it is possible that SOCC in porcine ASM is mediated *via* tryptophan C proteins.

There are scarce data on anesthetic effects on SOCC (or capacitative Ca^{2+} influx per some authors). Horibe *et al.*[45] found that propofol inhibits capacitative Ca^{2+} entry in pulmonary artery smooth muscle. Tas *et al.*[46] found that in primary human endothelial cells, isoflurane inhibits histamine induced Ca^{2+} influx *via* SOCC. However, this group also found that enflurane enhances Ca^{2+} influx in rat glioma cells.[47] Pochet *et al.*[48] reported that trichloroethanol, the active metabolite of chloral hydrate, inhibits SOCC-mediated Ca^{2+} influx in submandibular acinar cells. The current study is the first to report the effects of anesthetics on SOCC in airway smooth muscle.

Studies from several cell types have now shown that SOCC does not appear to be activated by changes in membrane potential, thus making this mechanism different from L-type Ca^{2+} channels.[13] In accordance, in our previous study, we found that changes in membrane potential (induced by altering extracellular K^+ concentration) did not affect SOCC-mediated Ca^{2+} influx in ASM.[31] Furthermore, Ca^{2+} influx after SR Ca^{2+} depletion by CPA was found to be insensitive to nifedipine, confirming that L-type Ca^{2+} channels do not mediate the observed influx. However, the existence of L-type Ca^{2+} channels in ASM has already been established,[11,49] as has the inhibitory effect of volatile anesthetics.[5,6] Certainly, volatile anesthetic inhibition of L-type channels would also prevent SR repletion, and blunt the $[Ca^{2+}]_i$ response to ACh. Therefore, in the current study, we used nifedipine to block the confounding effect of this mechanism on the observed influx and volatile anesthetic effects. Addition of KCl to the extracellular medium also helped maintain membrane potential at a value where L-type Ca^{2+} channels were unlikely to be activated.

Consistent with our previous study, and with studies in other smooth muscle types, we found that SOCC is blocked by Ni^{2+}[17,19,21,31,41] and La^{3+}.[17,21,31] Although both ions are capable of nonspecifically inhibiting influx *via* several mechanisms, inhibition of influx by μM concentrations of either ion strongly indicate that the observed influx is mediated *via* SOCC.

Previous studies in tissues other than ASM have found that SOCC-mediated Ca^{2+} influx occurs after depletion of inositol 1,4,5-trisphosphate-sensitive SR Ca^{2+} stores.[13,22] In our previous study, we found that SOCC in ASM is triggered after SR Ca^{2+} release *via* inositol 1,4,5-trisphosphate receptor channels.[31] It has been previ-

ously demonstrated that volatile anesthetics markedly increase inositol 1,4,5-trisphosphate concentrations in ASM cells, even in the absence of agonist stimulation[2] (but see Yamakage *et al.*[8]). Accordingly, anesthetic exposure should enhance SOCC-mediated Ca^{2+} influx. However, we[31] and others[14] have found that inositol 1,4,5-trisphosphate by itself does not trigger SOCC-mediated Ca^{2+} influx even in the presence of agonist stimulation, and it is SR Ca^{2+} release *via* inositol 1,4,5-trisphosphate receptor channels that is important. In this regard, previous studies have demonstrated that volatile anesthetics enhance SR Ca^{2+} leak *via* inositol 1,4,5-trisphosphate receptor channels.[2,32–35] This should lead to enhanced SOCC-mediated Ca^{2+} influx in the presence of anesthetics. However, the current study demonstrates that despite this potential enhancement, volatile anesthetics can inhibit Ca^{2+} influx.

In addition to triggering of SOCC after release *via* inositol 1,4,5-trisphosphate receptor channels, there are now data in skeletal muscle[50,51] and in ASM[31] demonstrating SOCC activation in response to depletion of caffeine-sensitive SR Ca^{2+} stores and subsequent introduction of $[Ca^{2+}]_o$. Again, it has been previously demonstrated that volatile anesthetics increase SR Ca^{2+} leak *via* RyR channels,[2,52,53] suggesting that in the presence of anesthetics, SOCC-mediated influx may be enhanced. However, as with inositol 1,4,5-trisphosphate receptor channels, the data from the current study (*i.e.*, the caffeine protocol), demonstrates that volatile anesthetics overwhelm any potential enhancement of influx resulting from depletion of caffeine-sensitive Ca^{2+} stores.

In ASM, exposure to ACh appears to enhance SOCC-mediated influx. However, it is not entirely clear whether agonist stimulation modulates SOCC because influx is confounded by the presence of mechanisms for Ca^{2+} influx that are neither voltage-gated nor mediated by SOCC, *i.e.*, G-protein coupled receptor-operated mechanisms.[41,54,55] Whether such a mechanism is involved in ASM is also not clear.[41] In our previous study,[31] we found that selective activation of the non-SOCC component by preventing SR Ca^{2+} depletion resulted in significantly less enhancement of Ca^{2+} influx by ACh compared with when SR depletion was present, suggesting the existence of both SOCC and noncapacitative Ca^{2+} influx in ASM. Regardless, the data at least suggests that ACh does modulate SOCC in ASM.

Muscarinic modulation of SOCC has the potential for at least partial inhibition of anesthetic blockade of SOCC; however, it is possible that anesthetic effects on SOCC more likely help maintain bronchodilation, even during agonist stimulation. Furthermore, volatile anesthetics also inhibit muscarinic receptor activation.[56,57] Accordingly, even in the presence of ACh, volatile anesthetics likely maintain their inhibition of SOCC.

Significance of SOCC and Volatile Anesthetic Effects

Increased $[Ca^{2+}]_i$ is key to force generation in ASM. SOCC-mediated Ca^{2+} influx has been shown to produce ASM contraction.[25] Accordingly, anesthetic inhibition of SOCC has the potential to prevent SR Ca^{2+} repletion and agonist-induced bronchoconstriction. Volatile anesthetics are known to affect other mechanisms that regulate both $[Ca^{2+}]_i$ (*e.g.*, SR and voltage-gated Ca^{2+} channels) and force (*e.g.*, Ca^{2+} sensitivity of force generation[58]), albeit with different relative potencies (*e.g.*, sevoflurane has a much smaller effect on influx, but is more potent than isoflurane in decreasing Ca^{2+} sensitivity). Nonetheless, anesthetic inhibition of SOCC may significantly contribute to the overall bronchodilatory effect of these agents by decreasing overall $[Ca^{2+}]_i$ availability and thus help to maintain bronchodilation.

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