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Store-operated Ca^{2+} Influx in Airway Smooth Muscle

Interactions between Volatile Anesthetic and Cyclic Nucleotide Effects

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Background: **Volatile anesthetics produce bronchodilation in part by depleting sarcoplasmic reticulum Ca^{2+} stores in airway smooth muscle (ASM). Other bronchodilatory drugs are known to act *via* cyclic nucleotides (cyclic adenosine 3′,5′-cyclic monophosphate, cyclic guanosine 3′,5′-cyclic monophosphate). Intracellular Ca^{2+} regulation in ASM involves plasma membrane Ca^{2+} influx, including that triggered by sarcoplasmic reticulum Ca^{2+} depletion (store-operated Ca^{2+} entry [SOCE]). The authors hypothesized that anesthetics and bronchodilatory agents interact in inhibiting SOCE, thus enhancing ASM relaxation.**

Methods: **In enzymatically dissociated porcine ASM cells imaged using fluorescence microscopy, sarcoplasmic reticulum Ca^{2+} was depleted by 1 μM cyclopiazonic acid in 0 extracellular Ca^{2+}, nifedipine, and potassium chloride (preventing Ca^{2+} influx through L-type channels and SOCE). Extracellular Ca^{2+} was rapidly reintroduced to selectively activate SOCE in the presence or absence of 1 minimum alveolar concentration (MAC) halothane, isoflurane, or sevoflurane. Anesthetic interference with SOCE regulation by cyclic nucleotides was examined by activating SOCE in the presence of (1) 1 μM acetylcholine, (2) 100 μM dibutryl cyclic adenosine 3′,5′-cyclic monophosphate, or (3) 100 μM 8-bromo-cyclic guanosine 3′,5′-cyclic monophosphate.**

Results: **SOCE was enhanced by acetylcholine, whereas volatile anesthetics and both cyclic nucleotides partially inhibited Ca^{2+} influx. Preexposure to 1 or 2 MAC anesthetic (halothane > isoflurane > sevoflurane) inhibited SOCE. Only halothane and isoflurane inhibited acetylcholine-induced augmentation of Ca^{2+} influx, and significantly potentiated cyclic nucleotide inhibition such that no influx was observed in the presence of anesthetics and cyclic nucleotides.**

Conclusions: **These data indicate that volatile anesthetics prevent sarcoplasmic reticulum refilling by inhibiting SOCE and enhancing cyclic nucleotide blunting of Ca^{2+} influx in ASM. Such interactions likely result in substantial airway relaxation in the presence of both anesthetics and bronchodilatory agents such as β agonists or nitric oxide.**

THE goal of clinical therapy in airway diseases such as asthma and allergy is to prevent excessive bronchoconstriction, in both the acute and the chronic setting, and to restore a balance between bronchoconstriction and bronchodilation. Indeed, patients with reactive airway disease frequently require perioperative administration of bronchodilatory agents such as β agonists. Furthermore, volatile anesthetics are potent bronchodilators, sometimes used as rescue therapy in persistent bronchoconstriction in status asthmaticus.

Anesthetic-induced bronchodilation involves, at least in part, a reduction in intracellular Ca^{2+} ($[Ca^{2+}]_i$) in airway smooth muscle (ASM) cells.[1–4] Elevation of $[Ca^{2+}]_i$ by bronchoconstrictors such as acetylcholine and histamine involves both Ca^{2+} release from sarcoplasmic reticulum (SR) stores and plasma membrane Ca^{2+} influx. In ASM, Ca^{2+} influx can occur *via* voltage-gated[5] and receptor-gated[6,7] channels, as well as in response to SR Ca^{2+} depletion[8,9] (store-operated Ca^{2+} entry [SOCE]). There is now considerable evidence from a variety of cell types including different smooth muscles for SOCE,[10–17] and our previous results suggest that SOCE is an important regulator of $[Ca^{2+}]_i$ in ASM. Anesthetics are known to deplete SR Ca^{2+} stores by increasing Ca^{2+} "leakage."[1,2,18] Accordingly, anesthetic-induced SR depletion would be expected to enhance SOCE. However, in a recent study, we demonstrated that clinically relevant concentrations of volatile anesthetics also inhibit SOCE in ASM.[8]

The effects of a variety of endogenous substances as well as bronchodilatory drugs such as β agonists are mediated *via* the cyclic nucleotide cyclic adenosine 3′,5′-cyclic monophosphate (cAMP). In comparison, the second messenger cyclic guanosine 3′,5′-cyclic monophosphate (cGMP), which mediates the effects of nitric oxide, is known to produce vasodilation but may not be an effective bronchodilation. Nonetheless, there is recent research evidence that cGMP is also an important second messenger involved in ASM relaxation.[19,20] We previously demonstrated in ASM that β_2 agonists, as well as nitric oxide donors,[21] inhibit the SR Ca^{2+} response (which would be in contrast to the leakage induced by volatile anesthetics). Cyclic nucleotides also influence Ca^{2+} influx *via* L-type Ca^{2+} channels.[22,23] Recently, we found that cAMP and, to a lesser extent, cGMP inhibit Ca^{2+} influx *via* SOCE,[24] consistent with effects in vascular smooth muscle.[25]

Volatile anesthetics and bronchodilating drugs are administered concomitantly in the operative setting. Therefore, it would be of interest to determine whether SOCE is differently modulated in the presence of both anesthetics and drugs affecting cyclic nucleotides. In this

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regard, anesthetics and cyclic nucleotides differently affect SR Ca^{2+} and thereby the state of SR depletion. Because SOCE is regulated by SR Ca^{2+} depletion, we hypothesized that anesthetics and drugs affecting cyclic nucleotides would have contrasting effects (inhibitory *vs.* stimulatory) on SOCE.

Materials and Methods

Cell Preparation

As described in several of our previous studies (*e.g.*, Kannan *et al.*,[26] 1996), porcine ASM cells were enzymatically dissociated in Earle's balanced salt solution (10 mM HEPES, pH 7.4; Invitrogen, Carlsbad, CA) using papain, DNase, and type IV collagenase (Worthington Biochemical Corp., Lakewood, NJ). Isolated cells were plated on collagen-coated glass coverslips, loaded with 5 μM fura-2 AM (Molecular Probes, Eugene, OR) for 30–45 min at room temperature in Hanks balanced salt solution (HBSS), washed, mounted on an open slide chamber (RC-25F; Warner Instruments, Hamden, CT), and imaged at room temperature. Cells were visualized using a MetaFluor real-time fluorescence imaging system (Universal Imaging, Downingtown, PA) on a Nikon Diaphot inverted microscope (Fryer Instruments, Edina, MN). Image pairs (excitation 340 and 380 nm) were obtained once every second, and 340/380 nm fluorescence ratiometric data were calibrated for Ca^{2+} levels using previously published techniques.[27]

Real-time Fluorescence Ca^{2+} Imaging

Cells attached to coverslips were loaded with 5 μM fura-2 AM for 30–45 min at 37°C in HBSS, washed, mounted on an open slide chamber (RC-25F), and perfused with HBSS at room temperature. Fura-2–loaded cells were visualized using a MetaFluor real-time fluorescence imaging system on a Nikon Diaphot inverted microscope (40×/1.3 NA lens). Pairs of images for excitation wavelengths 340 and 380 nm and common emission of 510 nm were obtained once every second, and the ratio of emissions for 340 *versus* 380 nm was calculated. The ratios were calibrated for $[Ca^{2+}]_i$ levels using previously published techniques.[9,27] The calibration technique relies on calculation of the ratios at minimal $[Ca^{2+}]_i$ concentration and with maximum $[Ca^{2+}]_i$ obtained with a Ca^{2+} ionophore. We have previously calculated the actual calibration constant in freshly dissociated porcine ASM cells.

Administration of Volatile Anesthetics

Perfusion media were bubbled with volatile anesthetics using calibrated vaporizers (halothane: Wyeth-Ayerst Laboratories, Philadelphia, PA; isoflurane and sevoflurane: Abbott Laboratories, Deerfield, IL).[2] Aqueous anesthetic concentrations equivalent to 1 adult porcine minimum alveolar concentration (MAC) at room temperature (25°C) were determined by gas chromatography and electron capture detector (halothane, isoflurane: Hewlett-Packard 5880A; Hewlett-Packard, Sunnyvale, CA) or flame ionization detector (sevoflurane). Concentrations were 1 MAC 0.32 ± 0.07 mM halothane, 1 MAC 0.38 ± 0.08 mM isoflurane, and 1 MAC 0.48 ± 0.11 mM sevoflurane.

Store-operated Ca^{2+} Entry

Store-operated Ca^{2+} entry was evaluated using previously described protocols.[8,9,24] Briefly, ASM cells were washed with 0 Ca^{2+} HBSS (5 mM EGTA) for 5 min to remove extracellular Ca^{2+} ($[Ca^{2+}]_o$). In the continued absence of $[Ca^{2+}]_o$, 1 μM nifedipine and 10 mM KCl were added to inhibit L-type Ca^{2+} channels and "clamp" the membrane potential (KCl) respectively, and the SR was passively depleted by 1 μM cyclopiazonic acid (CPA; inhibitor of the SR Ca^{2+} adenosine triphosphatase). As reported in our previous studies, CPA exposure caused a gradual elevation of $[Ca^{2+}]_i$ (reflecting progressive SR Ca^{2+} depletion) and an eventual plateau level (or occasionally a gradual trend down reflecting Ca^{2+} efflux mechanisms).[8,9] At this point, 2.5 mM $[Ca^{2+}]_o$ was rapidly reintroduced in the continued presence of CPA, nifedipine, and KCl to trigger SOCE.

Effect of Volatile Anesthetics on SOCE

The protocol for examining volatile anesthetic effects on SOCE has also been previously described.[9] Cells in which SOCE was first demonstrated using CPA (control response) were washed for 15–20 min with HBSS to remove CPA and to replenish SR Ca^{2+} stores. $[Ca^{2+}]_o$ was then removed, and the cells were reexposed to CPA in the presence of nifedipine and KCl. When the $[Ca^{2+}]_i$ response representing SR Ca^{2+} depletion was observed, cells were exposed for 1 min to 1 MAC halothane, isoflurane, or sevoflurane in the continued presence of CPA, nifedipine, and KCl, thus ensuring that the anesthetics did not influence SR Ca^{2+} release itself. In the continued presence of CPA and anesthetic, SOCE was then triggered by reintroducing $[Ca^{2+}]_o$. In control experiments, the SOCE protocol was performed twice without anesthetic, with an intervening wash in HBSS.

Volatile Anesthetic Effects on Acetylcholine Enhancement of SOCE

Acetylcholine was used as an ASM agonist to examine interactions with volatile anesthetics. Ca^{2+} influx *via* SOCE was first established (see "Store-operated Ca^{2+} Entry"). Cells were then washed with HBSS for 15–20 min, and the protocol was repeated with the introduction of 1 μM acetylcholine for 2 min before reintroduction of $[Ca^{2+}]_o$ (but after CPA exposure, ensuring that activation of muscarinic receptors after SR Ca^{2+} release had already occurred). Cells were then washed for a second time, and the SOCE protocol was then per-

formed with the introduction of both acetylcholine and 1 MAC halothane, isoflurane, or sevoflurane before activation of SOCE by reexposure to $[Ca^{2+}]_o$. Volatile anesthetics are known to interfere with muscarinic receptor stimulation.[28] Acetylcholine was introduced first, followed by anesthetic for 1 min (in the continued presence of acetylcholine), ensuring that muscarinic activation occurred before anesthetic exposure. If anesthetics were introduced first, it would have been difficult to distinguish between anesthetic effects on SOCE *versus* on muscarinic receptor activation itself.

Volatile Anesthetic Interactions with Cyclic Nucleotide Modulation of SOCE

We have previously reported the inhibitory effects of cyclic nucleotides on SOCE in ASM.[24] In this study, we first performed the SOCE protocol in the presence of drugs that increase cAMP or cGMP to verify SOCE inhibition. The same cells were then washed with HBSS, and the SOCE protocol was repeated in the combined presence of these drugs as well as volatile anesthetics. Briefly, after SOCE was first established by performing a control protocol using CPA, ASM cells were washed for 15–20 min with HBSS, $[Ca^{2+}]_o$ was then removed, nifedipine and KCl were added, and the cells were reexposed to CPA (in the continued presence of nifedipine and KCl). When an $[Ca^{2+}]_i$ response was observed (thus ensuring SR Ca^{2+} depletion first), cells were exposed for 5 min to either 100 μM dibutryl cAMP (dbcAMP; a membrane permeable cAMP analog) or 100 μM 8-Br-cGMP (cell-permeable cGMP analog). $[Ca^{2+}]_o$ was then rapidly reintroduced to reactivate SOCE. Cells were then thoroughly washed in HBSS for up to 15 min to ensure sufficient time for washout of dbcAMP or 8-Br-cGMP as well as refilling of SR Ca^{2+} stores (in pilot control experiments where the SOCE protocol in the presence of these drugs was performed twice in the same cells, we found that 15 min was sufficient to result in less than 10% difference in amplitudes of two subsequent SOCE responses). After the second wash, extracellular Ca^{2+} was removed, and cells were exposed to CPA, nifedipine, and KCl. Subsequently, cells were first exposed to the same drug as in the previous SOCE activation (dbcAMP or 8-Br-cGMP) and then to 1 MAC halothane, isoflurane, or sevoflurane. $[Ca^{2+}]_o$ was then introduced to activate SOCE.

Statistical Analysis

Statistical comparisons were performed for the same ASM cell before and after exposure to a drug and/or anesthetic using the paired Student *t* test. Comparisons involved absolute values or amplitudes of responses. Where appropriate, multiple comparisons (*e.g.*, across anesthetics) were performed using analysis of variance and Bonferroni corrections. All experimental protocols were not performed in all cells. Results were replicated

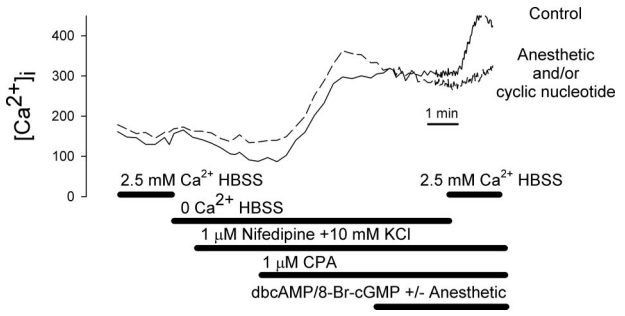

Fig. 1. Store-operated Ca^{2+} entry in airway smooth muscle. After a wash in Hanks balanced salt solution (HBSS), extracellular Ca^{2+} ($[Ca^{2+}]_o$) was removed, and in the presence of nifedipine and KCl (to inhibit Ca^{2+} influx *via* voltage-gated channels), the sarcoplasmic reticulum Ca^{2+} adenosine triphosphatase inhibitor cyclopiazonic acid (CPA) was used to deplete Ca^{2+} stores as described previously.[8,9] $[Ca^{2+}]_o$ was then reintroduced to activate store-operated Ca^{2+} entry. To examine the effect of volatile anesthetics and/or cell-permeant agents that increase cAMP (dibutryl cAMP [dbcAMP]) or cGMP (8-Br-cGMP), these agents were introduced after sarcoplasmic reticulum depletion and during reintroduction of $[Ca^{2+}]_o$. $[Ca^{2+}]_i$ = intracellular Ca^{2+} concentration.

in at least five cells obtained from at least four animals (paired comparisons within cells, independent testing across cells). Statistical significance was tested at the $P < 0.05$ level. Values are reported as mean ± SE.

Results

Store-operated Ca^{2+} Influx in ASM

Baseline $[Ca^{2+}]_i$ levels in ASM cells ranged from 85 to 125 nM (93 ± 8 nM; n = 74) and decreased slightly or did not change significantly with initial removal of $[Ca^{2+}]_o$, addition of nifedipine, and addition of KCl. Under these conditions, 1 μM CPA elevated $[Ca^{2+}]_i$, resulting in a plateau (fig. 1). In some cells, there was a gradual decrement in $[Ca^{2+}]_i$ after the plateau, presumably reflecting unopposed efflux mechanisms such as plasma membrane Ca^{2+} adenosine triphosphatase. Rapid reintroduction of $[Ca^{2+}]_o$ resulted in a sustained elevation of $[Ca^{2+}]_i$ ($P < 0.05$ compared with the first plateau) reflecting SOCE (as reported in our previous studies). We have previously characterized SOCE in ASM, including pharmacologic inhibition *versus* activation, as well as time dependence,[8,9] and therefore, these studies were not repeated here.

Effect of Volatile Anesthetics on SOCE

In control ASM cells, SOCE varied less than 10% with repeated activation (rundown control). In the absence of $[Ca^{2+}]_o$, exposure to 1 MAC halothane, isoflurane, or sevoflurane did not significantly alter the plateau $[Ca^{2+}]_i$ level in the presence of CPA. However, with reintroduction of $[Ca^{2+}]_o$ in the presence of any of the three anesthetics, SOCE was significantly diminished compared with the previous control activation in the same cell ($P < 0.05$ for each anesthetic, compared with both same cell and rundown control; fig. 2). Halothane pro-

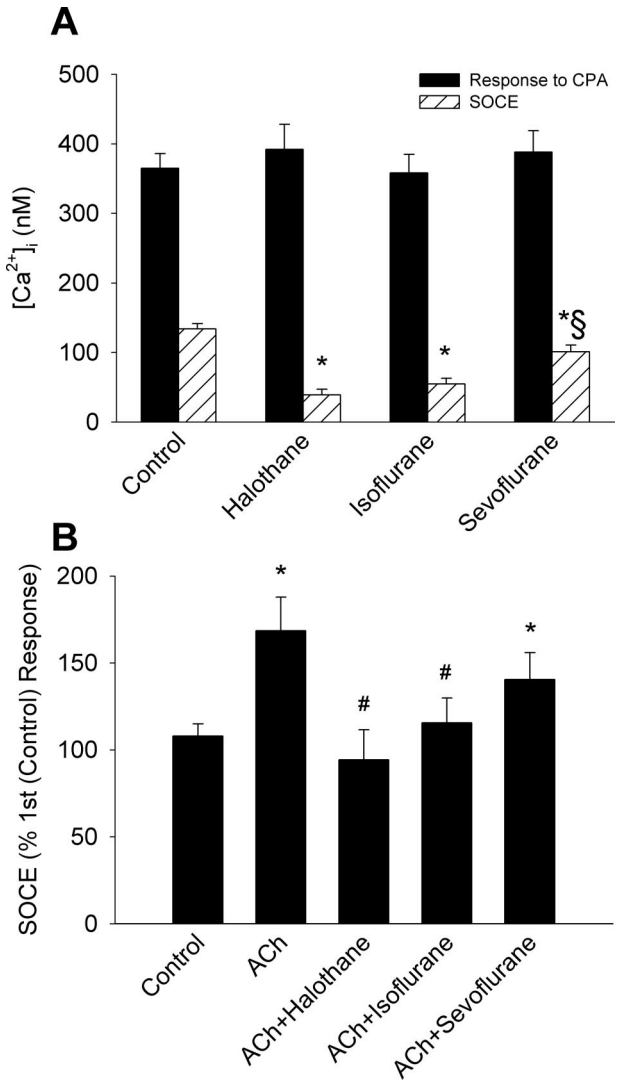

Fig. 2. Effect of volatile anesthetics on store-operated Ca^{2+} entry (SOCE). Anesthetics did not significantly affect the intracellular calcium ($[Ca^{2+}]_i$) response to cyclopiazonic acid (CPA) (*A*), whereas 1 minimum alveolar concentration halothane, isoflurane, and sevoflurane all significantly inhibited SOCE. Sevoflurane was significantly less potent compared with halothane or isoflurane in inhibiting SOCE. With 1 μM acetylcholine (ACh) stimulation (during CPA exposure), SOCE was significantly enhanced (*B*). Halothane and isoflurane reversed such SOCE enhancement, but not sevoflurane. * Significant difference from control ($P < 0.05$); § significant difference from halothane; # significant difference from acetylcholine. Values are mean ± SE.

duced significantly greater inhibition, compared with isoflurane or sevoflurane, the latter causing the least inhibition.

Volatile Anesthetic Effects on Acetylcholine Enhancement of SOCE

In cells where 1 μM acetylcholine produced augmentation of SOCE after CPA exposure, exposure to halothane and isoflurane, but not sevoflurane, significantly decreased Ca^{2+} influx after reintroduction of $[Ca^{2+}]_o$ ($P < 0.05$ for both same cell and acetylcholine rundown control; fig. 2). The inhibition of SOCE by volatile anesthetics was significantly greater in the presence of acetylcholine than in its absence (*i.e.*, volatile anesthetics alone; $P < 0.05$).

Cyclic Nucleotide Effects on Store-operated Ca^{2+} Influx

In absence of $[Ca^{2+}]_o$, exposure to either cyclic nucleotide did not significantly alter the $[Ca^{2+}]_i$ response to CPA (data not shown). However, in the presence of either 100 μM dbcAMP (fig. 3) or 8-Br-cGMP (fig. 4), subsequent reintroduction of $[Ca^{2+}]_o$ resulted in a significantly smaller SOCE compared with control in the same cells ($P < 0.05$; figs. 3 and 4).

Volatile Anesthetic Interactions with Cyclic Nucleotide Modulation of SOCE

In the presence of dbcAMP, exposure to 1 MAC halothane resulted in almost complete inhibition of SOCE when $[Ca^{2+}]_o$ was reintroduced in the combined presence of both dbcAMP and halothane ($P < 0.05$ compared with control; fig. 3). Indeed, only 10% of the cells showed any SOCE under these conditions. Compared with the effects of halothane alone or dbcAMP alone, SOCE inhibition in the presence of both agents was significantly greater ($P < 0.05$ for both halothane and dbcAMP). As with dbcAMP, in the presence of 8-Br-cGMP, 1 MAC halothane produced significantly greater inhibition of SOCE compared with control, halothane alone, or 8-Br-cGMP alone ($P < 0.05$; fig. 4).

Similar to halothane, 1 MAC isoflurane–induced inhibition of SOCE in the presence of dbcAMP or 8-Br-cGMP was significantly greater than that by isoflurane, dbcAMP, or 8-Br-cGMP alone ($P < 0.05$; figs. 3 and 4). Overall, isoflurane effects were smaller than those of halothane. In contrast to halothane or isoflurane, sevoflurane did not produce any greater inhibition of SOCE in the presence of dbcAMP (fig. 3). However, with 8-Br-cGMP, significantly greater inhibition was observed ($P < 0.05$; fig. 4).

Discussion

Both cyclic nucleotides (cAMP and cGMP)[19,20] and volatile anesthetics[1,2,29,30] relax ASM, by interference with mechanisms that would normally elevate $[Ca^{2+}]_i$.[29,31–33] Anesthetics have been previously shown to increase SR Ca^{2+} leak *via* both inositol 1,4,5-triphosphate and ryanodine channels.[1,2,18] Although such a leak would be expected to trigger SOCE, our studies show that volatile anesthetics also inhibit SOCE, thus accentuating the state of SR depletion. As in our previous study, we found that cyclic nucleotides also inhibit SOCE. Given that SR Ca^{2+} release is an important contributor to total $[Ca^{2+}]_i$ in ASM, SOCE inhibition would only lead to

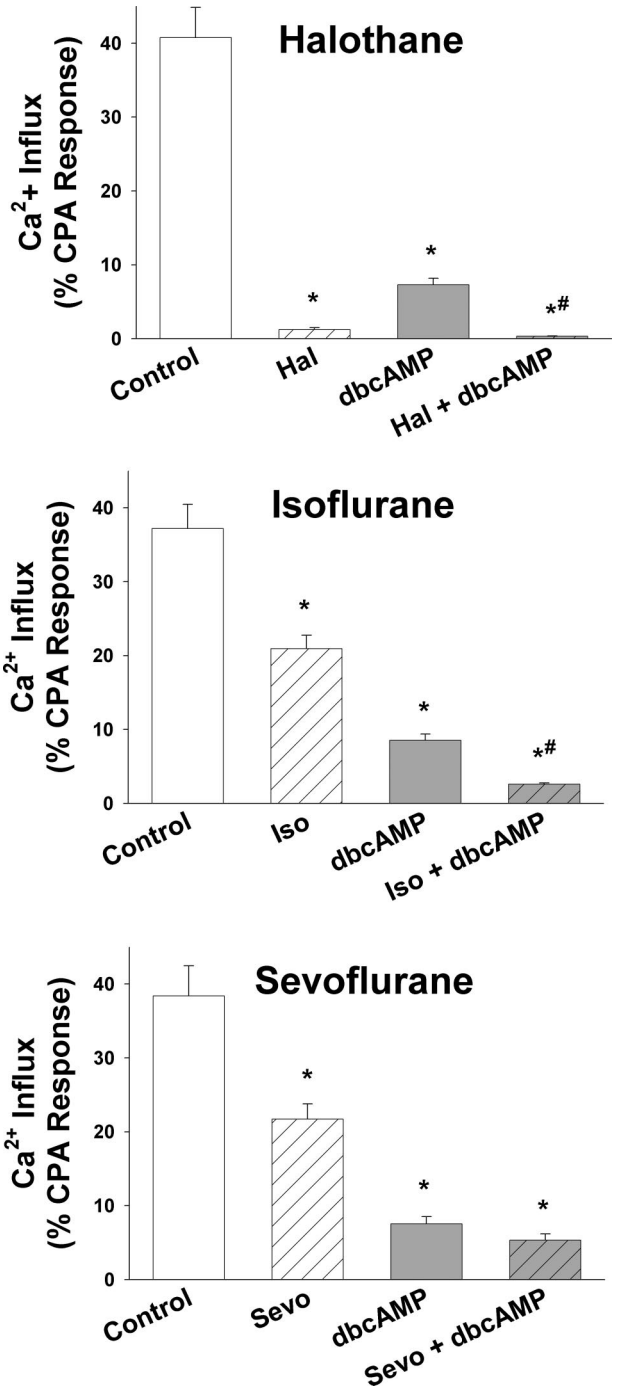

Fig. 3. Interactions between volatile anesthetics and cyclic adenosine 3′,5′-cyclic monophosphate (cAMP). After a control verification of store-operated Ca^{2+} entry, airway smooth muscle cells were washed and reexposed to cyclopiazonic acid (CPA), followed by 100 μM dibutryl cAMP (dbcAMP) before reactivation of store-operated Ca^{2+} entry. Cells were then washed, and the protocol was repeated for a third time with dbcAMP (rundown control). In other cells, 1 minimal alveolar concentration halothane (Hal), isoflurane (Iso), or sevoflurane (Sevo) was added. Store-operated Ca^{2+} entry was significantly decreased by dbcAMP. In the presence of halothane and isoflurane, store-operated Ca^{2+} entry inhibition was much greater than that with anesthetic or dbcAMP alone. * Significant difference from control; # significant difference from both anesthetic and dbcAMP ($P < 0.05$).

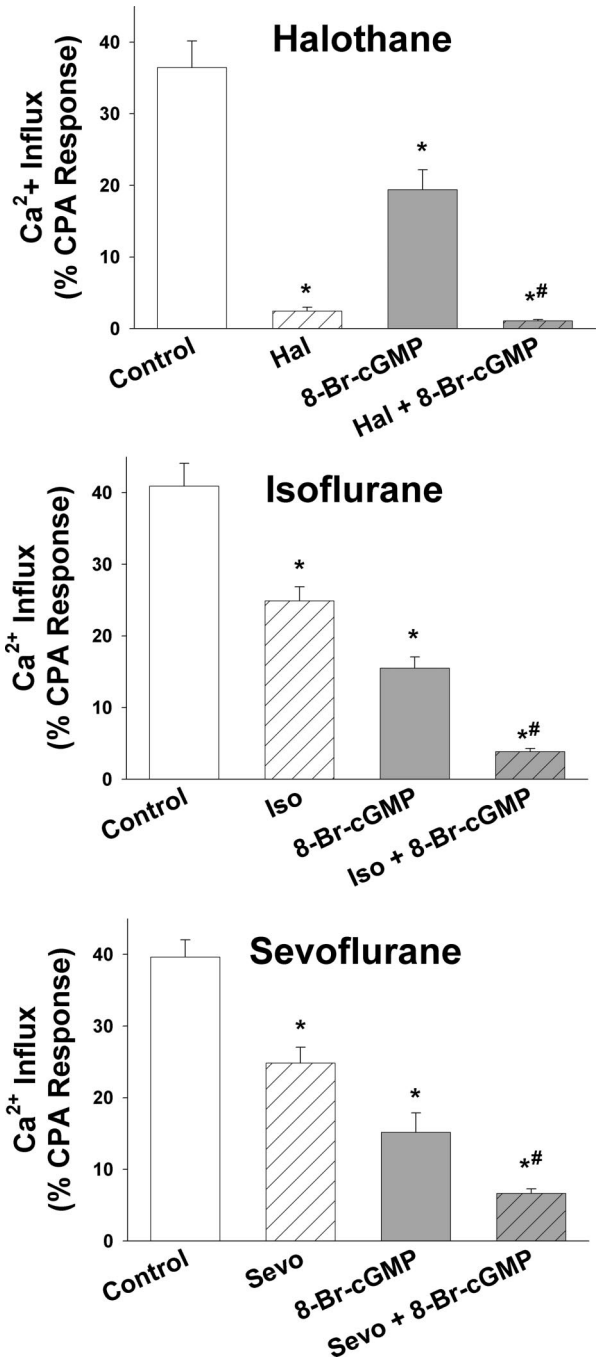

Fig. 4. Interactions between volatile anesthetics and cyclic guanosine 3′,5′-cyclic monophosphate (cGMP). As with fig. 3, store-operated Ca^{2+} entry was triggered three times (with intervening washes). However, 100 μM 8-Br-cGMP was used instead of dibutryl cAMP (dbcAMP). Store-operated Ca^{2+} entry was also significantly decreased by 8-Br-cGMP. In the presence of halothane (Hal), isoflurane (Iso), or sevoflurane (Sevo), store-operated Ca^{2+} entry inhibition was much greater than that with anesthetic or 8-Br-cGMP alone. * Significant difference from control; # significant difference from both anesthetic and 8-Br-cGMP ($P < 0.05$). CPA = cyclopiazonic acid.

less available SR Ca^{2+} release during agonist stimulation and thus to enhanced relaxation of ASM. The data presented in the current study demonstrate that in the simultaneous presence of both anesthetics and agents

that elevate cyclic nucleotides, there is an additive effect (disproving our hypothesis of antagonism), resulting in almost complete SOCE inhibition, especially with halothane. These data suggest that the combined effects of anesthetics and cyclic nucleotides may be a potent mechanism for inducing bronchodilation.

Effect of Volatile Anesthetics on SOCE in ASM

$[Ca^{2+}]_i$ regulation in ASM involves both extracellular Ca^{2+} influx and SR Ca^{2+} release[34,35] channels. Ca^{2+} influx occurs through voltage-gated,[5] receptor-gated,[6,7,36,37] and store-operated[8,9] channels. SOCE has now been reported in a multitude of cell types, including vascular smooth muscle,[14,38] where muscle tone is increased with SOCE activation, indicating a physiologic role.[12–14] In previous studies, we have shown that Ca^{2+} influx serves to maintain and replenish SR Ca^{2+} stores. Accordingly, SOCE represents an important SR- and $[Ca^{2+}]_o$-dependent mechanism for maintenance and repletion of SR in ASM, and thus modulating the $[Ca^{2+}]_i$ (and force) response of ASM to agonist.

Volatile anesthetics are known to target several ASM $[Ca^{2+}]_i$ regulatory mechanisms. For example, anesthetics inhibit Ca^{2+} influx[3,29] through voltage-gated L-type Ca^{2+} channels.[29,39] We and others have shown that anesthetics deplete SR Ca^{2+} *via* increased "leak" through both inositol 1,4,5-triphosphate and ryanodine channels.[1,2,40–42] Anesthetic-induced SR Ca^{2+} depletion would be expected to enhance SOCE. However, in a recent study, we found that clinically relevant concentrations of volatile anesthetics inhibit SOCE.[8] The results of the current study (repeated to establish one set of "controls" for examining interactions with cyclic nucleotides) are completely consistent with our previous studies. SOCE inhibition has been in addition reported in pulmonary artery smooth muscle with propofol[43] and histamine-induced SOCE in human endothelial cells with isoflurane.[44] Functionally, SOCE inhibition underlines accentuation of anesthetic effects on $[Ca^{2+}]_i$, maintaining the SR in a state of depletion and keeping $[Ca^{2+}]_i$ low even with agonist stimulation.

As with previous studies, we found that exposure to acetylcholine enhances SOCE. Muscarinic receptor stimulation induces SR Ca^{2+} release and consequently SR depletion, thus enhancing SOCE. Furthermore, SOCE has been linked to agonist receptor interactions in other cell types.[45] Accordingly, receptor activation (and potentially G-protein involvement) may modulate SOCE. Such muscarinic modulation has the potential for at least partial reversal of anesthetic blockade of SOCE. However, it is possible that anesthetic effects on SOCE maintain bronchodilation, even during agonist stimulation. Furthermore, anesthetics are known to inhibit muscarinic receptor activation.[46] Accordingly, even in the presence of agonists such as acetylcholine, volatile anesthetics likely maintain their inhibition of SOCE.

Comparing the effects across volatile anesthetics, halothane and isoflurane seemed to have similar inhibitory effects on SOCE, whereas sevoflurane demonstrated the least effect on SOCE. Such differences are consistent with our previous work on SOCE.[8] In addition, sevoflurane had no effect on SOCE during muscarinic receptor stimulation. Differences in the chemical structure between sevoflurane *versus* halothane/isoflurane may be the reason for these findings. On the other hand, the effects of isoflurane and sevoflurane on SOCE in the presence of either cyclic nucleotide were comparable. Whether different anesthetic agents produce different conformational changes in proteins mediating SOCE or alter other mechanisms that regulate SOCE remains to be determined.

Effect of Cyclic Nucleotides on SOCE in ASM

Cyclic nucleotides are known to produce smooth muscle relaxation.[19,20] cAMP, activated by β-adrenoreceptor agonists (*e.g.*, albuterol, isoproterenol), is coupled to adenylate cyclase, which in turn activates protein kinase A. cAMP-mediated actions in smooth muscle reflect down-regulation of $[Ca^{2+}]_i$-elevating mechanisms, such as increased inositol 1,4,5-triphosphate hydrolysis, inhibited inositol 1,4,5-triphosphate–induced SR Ca^{2+} release, increased calcium uptake by internal stores,[31] and inhibited Ca^{2+} influx *via* membrane hyperpolarization.[22,23,32] In a recent study, we found that both β-adrenoceptor activation *via* isoproterenol and direct cAMP elevation by dbcAMP significantly inhibited SOCE in ASM.[24] The results of the current study with dbcAMP are completely consistent with previous results. Similar inhibitory effects of cAMP on SOCE have been reported in vascular smooth muscle.[25,47] In our previous study, we demonstrated that cAMP effects on SOCE are largely mediated *via* protein kinase A. Given the well-recognized bronchodilatory role of β-adrenoceptor agonists, SOCE inhibition would be expected to lead to lesser available SR Ca^{2+} release during agonist stimulation and thus enhanced ASM relaxation.

In our previous study, we found that cGMP, *via* protein kinase G, also inhibits SOCE in ASM.[24] Similar inhibitory effects are suggested by studies in vascular smooth muscle[48] and endothelial cells.[49] Interestingly, we found that in comparison to cAMP, cGMP (at least *via* a nitric oxide donor) is more potent in inhibiting SOCE. However, in the current study, direct cGMP elevation with 8-Br-cGMP actually resulted in less SOCE inhibition compared with dbcAMP. Whether such differences translate to differential bronchodilatory responses of drugs acting *via* the cAMP *versus* cGMP pathways remains to be determined.

Interactions between Volatile Anesthetics and Cyclic Nucleotides in Effects on SOCE

The discussion above indicates that volatile anesthetics and cyclic nucleotides differently affect the SR, in that anesthetics in general deplete the SR of Ca^{2+} stores, whereas cyclic nucleotides (especially cAMP) enhance mechanisms that should increase SR Ca^{2+} stores overall (*e.g.*, enhanced SR Ca^{2+} reuptake, inhibited release). Accordingly, the simultaneous presence of both anesthetics and cyclic nucleotides (or agents that increase their levels) may be expected to balance the effects of acetylcholine on the SR. However, both do decrease $[Ca^{2+}]_i$ levels. More importantly, both anesthetics and cyclic nucleotides individually inhibit SOCE in a significant fashion. Furthermore, the data of the current study demonstrate that the inhibitory effect of anesthetics on SOCE in the presence of cyclic nucleotides is greater than that with either agent alone. Accordingly, regardless of whether the airway is exposed to anesthetics first (depleting SR) followed by cyclic nucleotides (attempting to refill the SR) or to cyclic nucleotides followed by anesthetics (resulting in SR depletion anyway), there is unlikely to be substantial Ca^{2+} available for SR replenishment in the face of inhibited Ca^{2+} influx. In this regard, it is interesting to note that anesthetic agents differ in their interactions with cyclic nucleotides in terms of SOCE inhibition. Halothane, as with its effects on several other $[Ca^{2+}]_i$ regulatory mechanisms, displayed the strongest interactions with both cAMP and cGMP in inhibiting SOCE. However, given the already potent bronchodilatory effect of halothane, it is unlikely that the more pronounced inhibition in the presence of cAMP (and to a lesser extent in cGMP) is of much clinical significance. On the other hand, it is interesting to note that the combination of isoflurane with either cAMP or cGMP results in a significantly greater SOCE inhibition. On the other hand, sevoflurane displayed no significant interaction with cAMP, and thus the combination of the two may not be of much added benefit compared with each agent alone. Interestingly, both isoflurane and sevoflurane had significant interactions with cGMP in inhibiting SOCE. Although cGMP-elevating agents such as nitric oxide or nitrates are not commonly used clinically for bronchodilation, such interactions provide insight into potential alternative therapies in refractory airway hyperreactivity. In this regard, an important finding in both vascular smooth muscle[33] and in our recent study[24] in ASM has been that cAMP and cGMP cross-activate protein kinases A and G, resulting in common effects on smooth muscle. Such cross-activation is of significant interest to clinical therapy given the potential for pathways activated by nitric oxide donors or atrial natriuretic peptide to enhance effects of β-adrenoceptor agonists in the treatment of bronchoconstriction. Furthermore, drugs that activate one pathway may still be effective in decreasing $[Ca^{2+}]_i$ in ASM, even when the other pathway is inhibited by other drugs.

In summary, our study demonstrates that the combination of cyclic nucleotides and volatile anesthetics leads to greater inhibition of SOCE in ASM than each agent alone. Even under conditions of muscarinic receptor stimulation with acetylcholine, volatile anesthetics inhibit SOCE. Overall, halothane has the most profound effect on SOCE, and sevoflurane has the least. These findings suggest that a combination of cyclic nucleotides and anesthetics may be beneficial in the treatment of persistent bronchoconstriction in disease states such as asthma.

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