Halothane Attenuates Calcium Sensitization in Airway Smooth Muscle by Inhibiting G-proteins

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Background: Halothane directly relaxes airway muscle partly by decreasing the Ca^{2+} sensitivity. In smooth muscle, receptor stimulation is thought to increase Ca^{2+} sensitivity *via* a cascade of heterotrimeric and small monomeric guanine nucleotide-binding proteins (G-proteins). Whether this model is applicable in the airway and where halothane acts in this pathway were investigated.

Methods: A β-escin–permeabilized canine tracheal smooth muscle preparation was used. Exoenzyme C3 of *Clostridium botulinum*, which inactivates Rho monomeric G-proteins, was used to evaluate the involvement of this protein in the Ca^{2+} sensitization pathway. The effects of halothane on different stimulants acting at different levels of signal transduction were compared: acetylcholine on the muscarinic receptor, aluminum fluoride (AlF_4^-) on heterotrimeric G-proteins, and guanosine 5'-O-(3-thiotriphosphate) (GTPγS) on all G-proteins.

Results: Exoenzyme C3 equally attenuated acetylcholine- and AlF_4^--induced Ca^{2+} sensitization, suggesting that these pathways are both mediated by Rho. Halothane applied before stimulation equally attenuated acetylcholine- and AlF_4^--induced Ca^{2+} sensitization. However, when added after Ca^{2+} sensitization was established, the effect of halothane was greater during Ca^{2+} sensitization induced by acetylcholine compared with AlF_4^-, which, along with the previous result, suggests that halothane may interfere with dissociation of heterotrimeric G-proteins. Halothane applied during GTPγS-induced Ca^{2+} sensitization had no significant effect on force, suggesting that halothane has no effect downstream from monomeric G-proteins.

Conclusion: Halothane inhibits increases in Ca^{2+} sensitivity of canine tracheal smooth muscle primarily by interfering with the activation of heterotrimeric G-proteins, probably by inhibiting their dissociation. (Key words: Bronchodilation; myosin light chain phosphorylation; volatile anesthetics.)

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HALOTHANE directly relaxes airway smooth muscle by decreasing the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$)[1-3] and by decreasing the amount of force produced for a particular $[Ca^{2+}]_i$ (*i.e.*, Ca^{2+} sensitivity) during membrane receptor stimulation.[1-4] Regarding the latter effect, we have shown that halothane inhibits receptor-linked pathways that increase Ca^{2+} sensitivity in response to contractile agonists such as acetylcholine.[5] Studies of airway and other types of smooth muscle have shown that receptor stimulation activates specific guanine nucleotide-binding proteins (G-proteins), ultimately increasing force. Active force in smooth muscle is regulated by the phosphorylation of the regulatory myosin light chain (rMLC). The phosphorylation state depends on the balance between the activities of a kinase and a phosphatase on the rMLC. Myosin light chain kinase activity is increased by the binding of calcium-calmodulin complexes when $[Ca^{2+}]_i$ increases in response to receptor stimulation. This increased activity increases rMLC phosphorylation. Regulatory MLC phosphorylation can also increase if myosin light chain phosphatase activity is inhibited. This latter mechanism is thought to be responsible for agonist-induced increases in Ca^{2+} sensitivity (for a review, see Somlyo and Somlyo[6]). Recent work suggests that two families of G-proteins, heterotrimeric and small monomeric G-proteins, are involved in the regulation of Ca^{2+} sensitivity (for a review, see Horowitz *et al.*[7]). It has been proposed that members of these two families form a cascade,[8] with receptor activation first producing dissociation of a receptor-coupled heterotrimeric G-protein into active subunits. One or more of these subunits then activates a monomeric G-protein, which inhibits rMLC phosphatase, thus, increasing rMLC phosphorylation and force.

Evidence from other tissues suggests that volatile anesthetics may affect the function of G-proteins.[9] If such effects are also present in airway smooth muscle, there are several potential targets in the putative second-messenger cascade that regulates Ca^{2+} sensitivity. Previously, we showed that halothane applied before stimu-

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lation does not affect Ca^{2+} sensitization caused by direct activation of all G-proteins with guanosine 5'-O-(3-thio-triphosphate) ($GTP_\gamma S$), a nonhydrolyzable form of guanosine 5'-triphosphate (GTP).[10] Interpreted in terms of the proposed cascade of G-proteins just described, this finding implies that the action of halothane is proximal to the monomeric G-proteins. However, this interpretation is limited because this model has not been evaluated in airway smooth muscle.

The purpose of the current study was to determine (1) the involvement of heterotrimeric and small monomeric G-proteins in the regulation of Ca^{2+} sensitivity in a β-escin–permeabilized canine airway smooth muscle preparation and (2) the sites in this cascade at which halothane inhibits agonist-induced Ca^{2+} sensitization. In the β-escin–permeabilized preparation, pores in the sarcolemma permit the manipulation of the intracellular environment by changing the composition of the fluid bathing the smooth muscle, such that Ca^{2+} sensitivity may be directly studied. Pharmacologic probes used to study G-protein function included aluminum fluoride (AlF_4^-), which directly activates heterotrimeric G-proteins without direct activation of the monomeric G-proteins[11]; $GTP_\gamma S$, which directly activates both heterotrimeric and monomeric G-proteins; and the exoenzyme C3 of *Clostridium botulinum*, which ribosylates through adenosine diphosphate (ADP) and inactivates the Rho family of monomeric G-proteins.[12,13]

Materials and Methods

Tissue Preparation

After approval by the Institutional Animal Care and Use Committee, mongrel dogs (weight, 20–25 kg) of either gender were anesthetized using an intravenous injection of pentobarbital (50 mg/kg) and killed by exsanguination. A 10- to 15-cm portion of extrathoracic trachea was excised and immersed in chilled physiologic salt solution composed of 110.5 mM NaCl, 25.7 mM $NaHCO_3$, 5.6 mM dextrose, 3.4 mM KCl, 2.4 mM $CaCl_2$, 1.2 mM KH_2PO_4, and 0.8 mM $MgSO_4$. The adventitia and mucosa were removed after cutting the visceral side of cartilage, then connective tissues were carefully removed during microscopic observation to make muscle strips of 0.1–0.2-mm width, ≈ 1-cm length, and 0.2–0.3-mg wet weight.

Isometric Force Measurements

Muscle strips were mounted in 0.1-ml glass cuvettes and continuously superfused at 1.2 ml/min with physiologic salt solution (37°C) aerated with 94% oxygen and 6% carbon dioxide. One end of the muscle strips was anchored with stainless steel microforceps to a stationary metal rod, and the other end was attached with stainless steel microforceps to a calibrated force transducer (model KG4; Scientific Instruments, Heidelberg, Germany). The initial gap between microforceps (*i.e.*, the initial muscle length) was set at 5 mm. During a 3-h equilibration period, the length of the muscle strips was increased after repeated isometric contractions (lasting 1 to 2 min) induced by 1 μM acetylcholine until maximal isometric force (optimal length) was reached. Each muscle strip was maintained at this optimal length for the rest of the experiment. These tissues produced maximal isometric forces of 1 to 3 mN when stimulated with 1 μM acetylcholine. After optimal length was set, subsequent experimental protocols were performed at room temperature (25°C) to minimize deterioration of the experimental preparation.[5]

Permeabilization Procedure

The muscle strips were permeabilized with β-escin[14] using a method validated for canine tracheal smooth muscle in our laboratory.[5] β-Escin creates pores in the smooth muscle cell plasma membrane, thus allowing substances of small molecular weight such as Ca^{2+} to freely diffuse across the cell membrane. Accordingly, $[Ca^{2+}]_i$ can be manipulated and controlled by changing the concentration of Ca^{2+} in the solution bathing the smooth muscle strip. Larger cellular proteins necessary for contraction are preserved. In addition, the membrane receptor–coupled mechanisms that modulate Ca^{2+} sensitivity remain intact and can be activated.[5]

Muscle strips were superfused for 20 min with relaxing solution containing 100 μM β-escin. The relaxing solution was composed of 7.5 mM MgATP, 4 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 20 mM imidazole, 1 mM dithiothreitol, 1 mM free Mg^{2+}, 1 nM free Ca^{2+}, 10 mM creatine phosphate, and 0.1 mg/ml creatine phosphokinase using the algorithm of Fabiato and Fabiato.[15] Ionic strength was kept constant at 200 mM by adjusting the concentration of potassium acetate. The pH was adjusted to 7.0 at 25°C with KOH or HCl. After the permeabilization procedure, strips were washed with relaxing solution without β-escin for 10 min. The calcium ionophore A23187 (10 μM) was added to the relaxing solution and all subsequent experimental solutions to deplete the sarcoplasmic reticulum Ca^{2+} stores and to maintain $[Ca^{2+}]_i$ at a constant level.[5,16]

Solutions of varying free-Ca^{2+} concentrations used in the subsequent experiment were also prepared using the Fabiato algorithm.[15]

Experimental Protocols

A set of four β-escin–permeabilized strips was prepared from the same dog for each experiment. All strips first were maximally contracted with 10 μM Ca^{2+}; all subsequent force measurements were normalized to these contractions. Strips were superfused with relaxing solution containing 5 mM inorganic phosphate for 10 min and then superfused with relaxing solution for 10 min to remove inorganic phosphate. Inorganic phosphate reduces the time necessary for relaxation by accelerating the rate of cross bridge detachment.[17] Thereafter, one of four individual experimental protocols was performed.

Treatment with Exoenzyme C3 followed by Stimulation with Acetylcholine or AlF_4^-. The first protocol was conducted to evaluate the effect of treatment with exoenzyme C3, which ribosylates through ADP and inactivates the monomeric G-protein Rho,[12,13] on acetylcholine- or AlF_4^--induced Ca^{2+} sensitization. Each set of four strips was divided into two pairs, one pair for acetylcholine and the other pair for AlF_4^-. One strip of each pair was incubated with 1 μg/ml exoenzyme C3 and 10 μM β-nicotinamide adenine dinucleotide in relaxing solution for 20 min.[18] The concentration and duration of exoenzyme C3 exposure were chosen on the basis of preliminary studies as those that produced a maximal effect on acetylcholine-induced Ca^{2+} sensitization (data not shown). Another strip of each pair was not exposed to exoenzyme C3 or nicotinamide adenine dinucleotide and served as a control. After washout of exoenzyme C3, all four strips were contracted with 0.3 μM Ca^{2+} for 10 min, and then one pair was stimulated with 10 μM acetylcholine and 10 μM GTP for 20 min. The other pair was stimulated with 1 mM NaF and 10 μM $AlCl_3$ (which in solution yield AlF_4^-) and 10 μM GTP for 20 min. The concentrations of NaF and $AlCl_3$ were chosen from preliminary studies as those that produced Ca^{2+} sensitization approximately equal to that produced by 10 μM acetylcholine in the absence of C3 treatment (data not shown).

Application of Halothane before Stimulation with Acetylcholine or AlF_4^-. The second protocol was conducted to evaluate the effect of incubation with halothane on acetylcholine- or AlF_4^--induced Ca^{2+} sensitization. Each set of four strips was divided into two pairs,

one pair for acetylcholine and the other pair for AlF_4^-. All four strips were contracted with 0.3 μM Ca^{2+} for 10 min, and then one pair was stimulated with 10 μM acetylcholine and 10 μM GTP, and the other pair was stimulated with 1 mM NaF, 10 μM $AlCl_3$, and 10 μM GTP for 20 min. One strip of each pair was exposed to halothane beginning 10 min before stimulation with 0.3 μM Ca^{2+}. The other strip was not exposed to halothane and served as a control.

Application of Halothane during Stimulation with Acetylcholine or AlF_4^-. The third protocol was conducted to evaluate the effect of the addition of halothane to strips stimulated with acetylcholine or AlF_4^-. Each set of four strips was divided into two pairs, one pair for acetylcholine and the other pair for AlF_4^-. All four strips were contracted with 0.3 μM Ca^{2+} for 10 min, and then one pair was stimulated with 10 μM acetylcholine and 10 μM GTP, and the other pair was stimulated with 1 mM NaF, 10 μM $AlCl_3$, and 10 μM GTP for 20 min. Thereafter, halothane was applied to one strip of each pair for 15 min. The remaining strip of each pair was not exposed to halothane and served as a time control.

Application of Halothane during Stimulation with $GTP_\gamma S$ or Acetylcholine. The fourth protocol was conducted to evaluate the effect of the application of halothane during $GTP_\gamma S$-induced Ca^{2+} sensitization. $GTP_\gamma S$, a nonhydrolyzable form of GTP, directly activates heterotrimeric and monomeric G-proteins both. Each set of four strips was divided into two pairs, one pair for $GTP_\gamma S$ and the other for acetylcholine. The acetylcholine pair was used as a control for the effect of halothane on Ca^{2+} sensitization, which allowed us to make paired comparisons. All four strips were contracted with 0.3 μM Ca^{2+} for 10 min, and then one pair was stimulated with 10 μM $GTP_\gamma S$ and the other pair with 10 μM acetylcholine and 10 μM GTP for 20 min. The concentrations of $GTP_\gamma S$ and acetylcholine were chosen based on the preliminary studies as those that produced approximately equal Ca^{2+} sensitization (data not shown). Thereafter, halothane was applied to one strip of each pair for 15 min. The remaining strip of each pair was not exposed to halothane and served as a time control.

Administration of Halothane

Halothane was delivered to solutions *via* a calibrated vaporizer. Concentrations of halothane in solutions at the cuvette were determined by gas chromatography from anaerobically obtained samples at the end of the

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protocol using an electron capture detector (model 5880A; Hewlett-Packard, Waltham, MA) according to the method of Van Dyke and Wood.[19]

Materials

Halothane was purchased from Ayerst Laboratories (New York, NY). Adenosine 5'-triphosphate disodium salt was purchased from Research Organics (Cleveland, OH). All other drugs and chemicals were purchased from Sigma Chemical (St. Louis, MO). A23187 was dissolved in dimethyl sulfoxide (0.05% final concentration). All other drugs and chemicals were prepared in distilled filtered water.

Statistical Analysis

Data are expressed as the mean $\pm$ SD; n represents the number of dogs. Forces were expressed as percentages of the maximal force induced by 10 μM Ca^{2+} determined in each strip before the experimental protocol. When halothane was applied during the contraction (the third and fourth protocols), the decrease in force produced by halothane was expressed as a percentage of relaxation from the initial force (before exposure to halothane). In these cases, the initial force was adjusted for the effect of time using the change in force of the time-matched control strip in each set according to the following formula:

$$\%\text{relaxation} = (1 - (A_2/A_1) \times (C_1/C_2)) \times 100$$

where A_1 = the force of the anesthetic-exposed strip just before exposure to anesthetic, A_2 = the force of the anesthetic-exposed strip at the end of anesthetic exposure, C_1 and C_2 = the forces of the control strip at the matched times with A_1 and A_2, respectively. Statistical assessments were made using paired t tests. $P < 0.05$ was considered significant.

Results

Treatment with Exoenzyme C3 followed by Stimulation with Acetylcholine or AlF$_4^-$

Figure 1A shows a representative recording from a strip treated with exoenzyme C3 and then stimulated first with 0.3 μM Ca^{2+} and then with 0.3 μM Ca^{2+} and AlF$_4^-$. Also shown is a control strip that was not treated with exoenzyme C3. The control contractions induced by acetylcholine or AlF$_4^-$ at 0.3 μM Ca^{2+} did not differ (fig. 1B). Exoenzyme C3 did not affect the
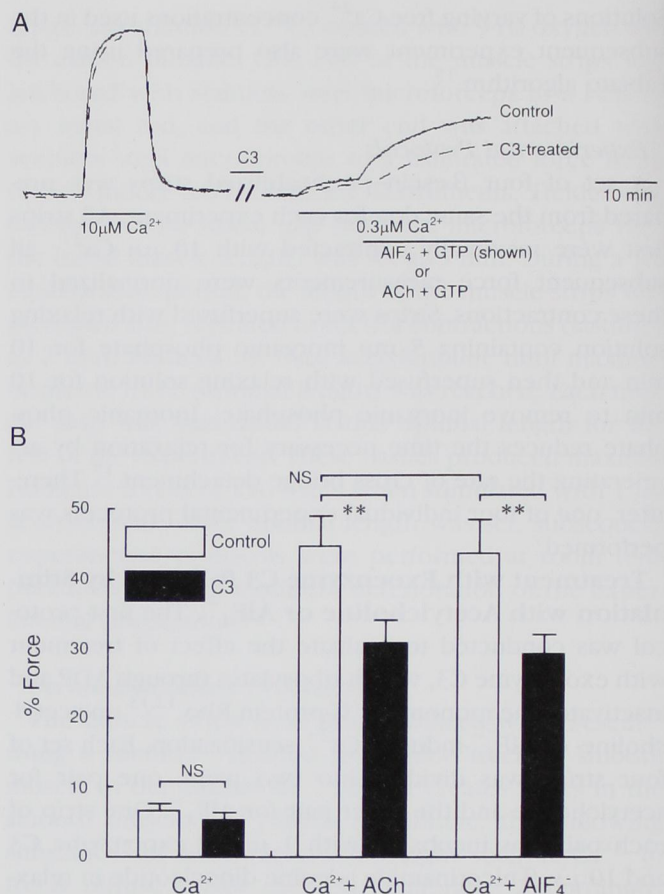

Fig. 1. The effect of treatment with exoenzyme C3 on Ca^{2+} sensitization induced by acetylcholine or aluminum fluoride (AlF$_4^-$). (*A*) Representative recordings of the contractions induced by 0.3 μM Ca^{2+} and by additional AlF$_4^-$ plus guanosine triphosphate (GTP) in the strips without (control) or with the preceding treatment with 1 μg/ml exoenzyme C3 and 10 μM β-nicotinamide adenine dinucleotide for 20 min. Traces are overlapped so the forces induced by 10 μM Ca^{2+} conform with each other. (*B*) Forces induced by 0.3 μM Ca^{2+} alone, Ca^{2+} plus 10 μM acetylcholine and 10 μM GTP, Ca^{2+} plus AlF$_4^-$ (1 mM NaF, 10 μM AlCl$_3$), and 10 μM GTP without (open column; control) or with (filled column) exoenzyme C3 treatment are expressed as percentages of maximal force induced by 10 μM Ca^{2+}, with SD shown by vertical bars (n = 5). Exoenzyme C3 did not affect the contractions induced by 0.3 μM Ca^{2+} alone, whereas it attenuated subsequent increases in force (Ca^{2+} sensitization) induced by either acetylcholine or AlF$_4^-$ (**$P < 0.01$). The control contractions induced by either acetylcholine or AlF$_4^-$ at 0.3 μM Ca^{2+} did not differ, nor did the inhibition produced by exoenzyme C3 on the force produced by either stimulation method.

contractions induced by 0.3 μM Ca^{2+} alone but attenuated subsequent increases in force induced by either acetylcholine or AlF$_4^-$ at 0.3 μM Ca^{2+} ($P < 0.01$; figs. 1A, B). The amount of inhibition produced by exoen-

zyme C3 did not differ between the two methods of stimulation.

Application of Halothane before Stimulation with Acetylcholine or AlF$_4^-$

Figure 2A shows a representative recording from a strip incubated with halothane, stimulated first with 0.3 μM Ca^{2+} and then with 0.3 μM Ca^{2+} and acetylcholine in the presence of halothane. Also shown is a control strip that was not exposed to halothane. The control contractions induced by acetylcholine or AlF$_4^-$ at 0.3 μM Ca^{2+} did not differ (fig. 2B). Halothane (0.80 $\pm$ 0.03 mM) did not affect the contractions induced by 0.3 μM Ca^{2+} alone but attenuated subsequent increases in force induced by either acetylcholine or AlF$_4^-$ ($P < 0.05$ for acetylcholine and $P < 0.01$ for AlF$_4^-$; figs. 2A and 2B). The amount of inhibition produced by halothane did not differ between the two methods of stimulation. Thus, when added before stimulation, halothane had similar effects on acetylcholine- and AlF$_4^-$-induced Ca^{2+} sensitization.

Application of Halothane during Stimulation with Acetylcholine or AlF$_4^-$

Figures 3A and 3B show representative recordings of the application of halothane to contractions induced by acetylcholine or AlF$_4^-$ at 0.3 μM Ca^{2+}, respectively. Stimulation with acetylcholine or AlF$_4^-$ at 0.3 μM Ca^{2+} produced contractions of 37.4 $\pm$ 5.8% and 38.5 $\pm$ 7.4% ($n = 5$) of the contraction induced by 10 μM Ca^{2+}, respectively, which were not significantly different. Halothane (0.65 $\pm$ 0.04 mM) reduced force during stimulation with either acetylcholine or AlF$_4^-$ ($P < 0.01$ for acetylcholine and $P < 0.05$ for AlF$_4^-$; fig. 3C). However, the effect of halothane during acetylcholine stimulation was greater than its effect during AlF$_4^-$ stimulation ($P < 0.01$). Thus, when added to strips stimulated at comparable levels (as measured by isometric force), the effect of halothane was greater on acetylcholine-induced Ca^{2+} sensitization than on AlF$_4^-$-induced Ca^{2+} sensitization.

Application of Halothane during Stimulation with GTP$_\gamma$S or Acetylcholine

Figure 4 shows a representative recording of the application of halothane on the contraction induced by GTP$_\gamma$S at 0.3 μM Ca^{2+}. Stimulation with GTP$_\gamma$S or acetylcholine at 0.3 μM Ca^{2+} produced contractions of 47.0 $\pm$ 5.8% and 47.6 $\pm$ 7.7% ($n = 5$) of the contraction induced by 10 μM Ca^{2+}, respectively, which were not significantly different. Halothane (0.69 $\pm$ 0.03 mM) had
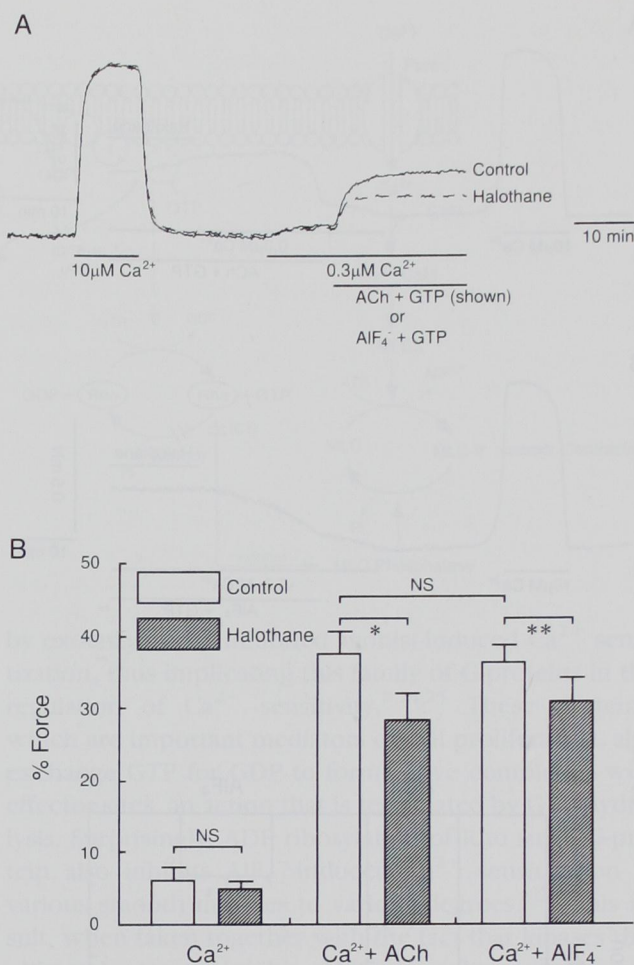
Fig. 2. The effect of halothane applied before stimulation with acetylcholine or aluminum fluoride (AlF$_4^-$). (*A*) Representative recordings from the strips stimulated first with 0.3 μM Ca^{2+} and with additional 10 μM acetylcholine plus 10 μM guanosine triphosphate (GTP) in the absence (control) or presence of halothane. Halothane was applied from 10 min before the stimulation with 0.3 μM Ca^{2+}. Traces are overlapped so the forces induced by 10 μM Ca^{2+} conform with each other. (*B*) Forces induced by 0.3 μM Ca^{2+} alone, Ca^{2+} plus 10 μM acetylcholine and 10 μM GTP, Ca^{2+} plus AlF$_4^-$ (1 mM NaF, 10 μM AlCl$_3$), and 10 μM GTP in the absence (open column; control) or presence (hatched column) of halothane are expressed as percentages of maximal force induced by 10 μM Ca^{2+}, with SD shown by vertical bars ($n = 5$). Halothane did not affect the contractions induced by 0.3 μM Ca^{2+} alone, but it attenuated subsequent increases in force (Ca^{2+} sensitization) induced by either acetylcholine (*$P < 0.05$) or AlF$_4^-$ (**$P < 0.01$). The control contractions induced by acetylcholine or AlF$_4^-$ at 0.3 μM Ca^{2+} did not differ, nor did the inhibition produced by halothane on the force produced by either stimulation method.

no significant effect on GTP$_\gamma$S-induced contractions (6.4 $\pm$ 5.3% relaxation, corrected for time), whereas it relaxed acetylcholine-induced contractions (26.2 $\pm$

A

B

Fig. 4. Representative recording of the application of halothane on the contraction induced by 0.3 μM Ca^{2+}, and 10 μM guanosine 5'-O-(3-thiotriphosphate)–induced Ca^{2+} sensitization.

10.2% relaxation, corrected for time; $P < 0.01$), which is similar to the result of the third protocol.

Discussion

G-Protein Regulation of Ca^{2+} Sensitivity in Airway Smooth Muscle

G-proteins play a pivotal role in the transduction of signals from membrane receptors to intracellular effectors in various cell types. Examples of such mechanisms in smooth muscle include activation of phospholipase C, regulation of adenylate cyclase activity, and regulation of the amount of force developed for a particular [Ca^{2+}]$_i$ (*i.e.*, Ca^{2+} sensitivity). The role of G-proteins in Ca^{2+} sensitization has been firmly established in various permeabilized smooth muscle preparations by the demonstration that the agonist-induced Ca^{2+} sensitization is inhibited by guanosine 5'-O-(β-thiodiphosphate) (GDP$_\beta$S)[20] and is mimicked by GTP or GTP$_\gamma$S.[20,21] Such G-protein–coupled Ca^{2+} sensitization is associated with an increase in rMLC phosphorylation.[16] More recently it was shown that GTP$_\gamma$S inhibits the activity of rMLC phosphatases by activating Rho monomeric G-proteins without affecting the activity of myosin light chain kinase.[18] The subsequent increase in rMLC phosphorylation increases actomyosin adenosine 5'-triphosphatase activity, the cross bridge cycling rate, and force.

G-protein–coupled receptors, such as the muscarinic receptor, are associated with heterotrimeric G-proteins composed of α-, β-, and γ-subunits. The α-subunit contains a binding site for guanine nucleotides and possesses GTPase activity. The β- and γ-subunits are tightly associated and anchor the α-subunit to the cytoplasmic surface of the cell membrane. In its resting state, the G-protein exists as an inactive G$_{\alpha\beta\gamma}$ trimer with GDP occupying the binding site of the α-subunit. The binding
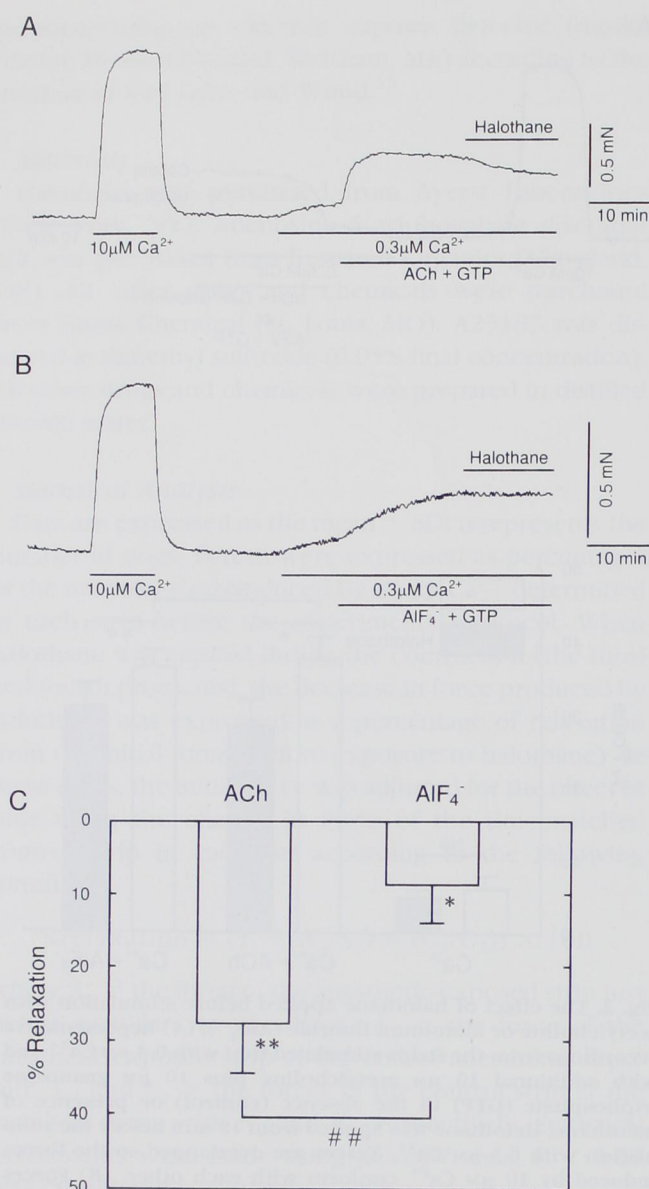
C

Fig. 3. The effect of halothane applied during stimulation with acetylcholine or aluminum fluoride (AlF$_4^-$) at a constant Ca^{2+} concentration. (*A,B*) Representative recordings of the application of halothane on the contraction induced by 10 μM acetylcholine plus 10 μM guanosine triphosphate (GTP; *A*) or AlF$_4^-$ (1 mM NaF, 10 μM AlCl$_3$) plus 10 μM GTP (*B*) at 0.3 μM Ca^{2+}. The degree of precontraction before the addition of halothane was not significantly different for the stimulants. (*C*) Halothane-induced percentage relaxation corrected for time from the initial force induced by acetylcholine or AlF$_4^-$ at 0.3 μM Ca^{2+}, with SD shown by vertical bars (n = 5). Halothane relaxed both contractions (*$P < 0.05$; **$P < 0.01$). However, the extent of relaxation was different for the stimulants (##$P < 0.01$). The effect on acetylcholine was approximately three times greater than the effect on AlF$_4^-$.
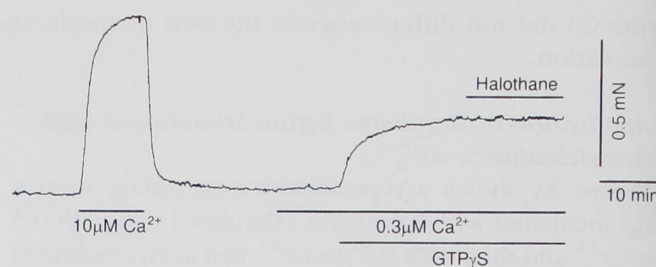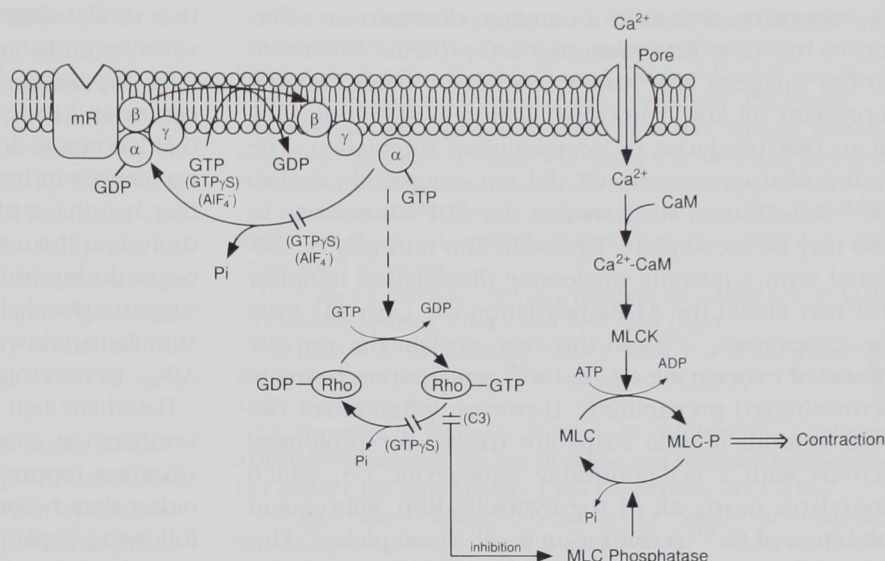
Fig. 5. A model of signal transduction of acetylcholine-induced Ca^{2+} sensitization in permeabilized smooth muscle. mR = muscarinic receptor; α, β, and γ subunits of heterotrimeric G-protein; Rho = monomeric G-protein; CaM = calmodulin; MLCK = myosin light chain kinase; MLC = myosin light chain; MLC-P = phosphorylated MLC; pore = transmembranous pore created by β-escin. The breaks in the lines indicate actions of guanosine 5'-O-(3-thiotriphosphate), aluminum fluoride, and exoenzyme C3.

of an agonist to the receptor produces the release of GDP and the subsequent binding of GTP to the α-subunit. This exchange of guanine nucleotides triggers dissociation of the $\alpha\beta\gamma$-complex from the receptor and separation of the α-subunit from the $\beta\gamma$ dimer. This GTP-bound active G_{α} monomer activates intracellular effectors. The hydrolysis of bound GTP by the intrinsic GTPase activity in the α-subunit permits reassociation of the subunits to a heterotrimer and terminates the activation of the effector.[22]

Fluoroaluminate complexes (mainly as AlF_4^-) have been used as a probe to selectively activate heterotrimeric G-proteins.[11] The structural similarity of AlF_4^- to phosphate (PO_4^{3-}) allows it to bind next to the β phosphate of GDP and mimic the terminal γ phosphate of GTP.[23] This eliminates the requirement for the GDP–GTP exchange to cause conformational change and consequent activation of G-protein. The bound AlF_4^- cannot be hydrolyzed, therefore the G-protein complex is maintained in its active, dissociated state, interrupting its normal cycling through the GDP- and GTP-bound states. $GTP_\gamma S$, a nonhydrolyzable analog of GTP, produces a stable $GTP_\gamma S$–G-protein complex and, thus, maintains both the heterotrimeric and the monomeric G-proteins in their active forms.

The role of heterotrimeric G-proteins in the regulation of Ca^{2+} sensitivity was anticipated, considering the nature of the involved membrane receptors. However, further studies in various types of smooth muscle found that ADP-ribosylation of the Rho monomeric G-protein

by exoenzyme C3 inhibited agonist-induced Ca^{2+} sensitization, thus implicating this family of G-proteins in the regulation of Ca^{2+} sensitivity.[8,24,25] These proteins, which are important mediators of cell proliferation, also exchange GTP for GDP to form active complexes with effector sites, an action that is terminated by GTP hydrolysis. Surprisingly, ADP ribosylation of Rho small G-protein also inhibits AlF_4^--induced Ca^{2+} sensitization in various smooth muscles to various degrees.[8,26] This result, when taken together with the fact that kinases that ultimately act to inhibit rMLC phosphatases are substrates for Rho, has led to the model shown in figure 5. Receptor binding produces dissociation of a heterotrimeric G-protein, which in turn activates a monomeric G-protein that subsequently inhibits rMLC phosphatase and increases rMLC phosphorylation. This scheme is further supported by recent findings in vascular smooth muscle that Ca^{2+} sensitization produced by receptor agonists $GTP_\gamma S$ and AlF_4^- is associated with translocation of RhoA from the cytosol to plasma membrane,[27] and that ADP ribosylation of RhoA inhibits translocation and Ca^{2+} sensitization.[28]

Our results are consistent with this model, which has not been evaluated before in airway smooth muscle. Using developed force as a criterion, we matched heterotrimeric G-protein stimulation provided by membrane receptor stimulation with acetylcholine with that produced by direct stimulation with AlF_4^-. Pretreatment with exoenzyme C3 produced an equal inhibition of acetylcholine- and AlF_4^--induced Ca^{2+} sensitization (fig.

1), suggesting action on a common downstream effector, in this case Rho monomeric G-protein. This result further suggests that the activation of heterotrimeric G-proteins by AlF_4^- produces G-protein activation similar to that produced by acetylcholine stimulation. The finding that exoenzyme C3 did not completely abolish Ca^{2+} sensitization suggests that the ADP ribosylation of Rho may be incomplete. Cytosolic Rho normally is associated with a guanine nucleotide dissociation inhibitor that may shield the ADP-ribosylation site (Asn 41) from the exoenzyme,[27,28] and this may explain the variable effects of exoenzyme C3 on Ca^{2+} sensitization noted in permeabilized preparations. However, when intact vascular smooth muscle strips are treated for prolonged periods with a cell-permeable exoenzyme C3, which ribosylates nearly all of the cytosolic Rho, subsequent inhibition of Ca^{2+} sensitization is still incomplete.[28] This finding suggests that other pathways not involving Rho may also be involved. For example, other monomeric G-proteins, such as ras p21, may be involved in Ca^{2+} sensitization.[29] We cannot distinguish between these possibilities in the current study.

The identity of the G-proteins involved in this cascade in the airways is unknown. Previously we showed in intact canine tracheal smooth muscle that pretreatment with pertussis toxin, which ADP ribosylates heterotrimeric G-proteins in the G_i family, does not affect responses to acetylcholine.[30] In preliminary work in the β-escin–permeabilized preparation, we also found that pertussis toxin does not affect acetylcholine-induced Ca^{2+} sensitization (data not shown), suggesting that the relevant heterotrimeric G-protein coupled to the muscarinic receptor in canine tracheal smooth muscle is a subfamily other than G_i.

Effects of Halothane

The addition of halothane did not affect Ca^{2+} sensitization produced by $GTP_\gamma S$, which directly activates heterotrimeric and monomeric G-proteins. This finding is consistent with our previous work, in which halothane did not affect $GTP_\gamma S$-induced Ca^{2+} sensitization when it was added before stimulation.[10] It shows that halothane does not affect sites downstream of the monomeric G-proteins, such as rMLC phosphatase or actomyosin adenosine 5′-triphosphatase. This conclusion is also supported by halothane's lack of effect on contractions produced by Ca^{2+} alone.[5]

When added before stimulation, halothane attenuated both acetylcholine- and AlF_4^--induced Ca^{2+} sensitization

to a similar degree (fig. 2). Because AlF_4^- directly activates heterotrimeric G-proteins by binding to the α-subunit, bypassing the normal interaction between the receptor and its associated G-protein, this finding implies that halothane does not affect receptor binding or receptor–G-protein interaction. It would also appear unlikely that halothane affects GDP–GTP exchange or GTP hydrolysis at the α-subunit, because these processes do not occur during stimulation with AlF_4^-. Rather, this finding suggests that halothane acts at a site common to both stimulants, downstream from the binding of GTP or AlF_4^- to heterotrimeric G-proteins.

Halothane had less of an effect on AlF_4^--induced Ca^{2+} sensitization compared with acetylcholine-induced sensitization (approximately one third) when added after, rather than before, stimulation (fig. 3). We propose the following explanation for this finding. Recall that AlF_4^- bound to the α-subunit cannot be hydrolyzed, so the G-protein complex is maintained in its active, dissociated state. If halothane primarily inhibits dissociation of the heterotrimeric G-protein subunits, it should have little effect on established AlF_4^--induced Ca^{2+} sensitization, because it was applied after dissociation. Conversely, during stimulation with acetylcholine, the normal cycle of GDP–GTP exchange with subsequent hydrolysis occurs, with an ongoing cycle of subunit dissociation susceptible to inhibition by halothane. Thus, the finding that halothane had a greater effect when added before rather than after exposure to AlF_4^- suggests that halothane stabilizes the heterotrimeric G-protein, inhibiting dissociation of its subunits. The finding that halothane still has a small effect when added after AlF_4^- exposure (*i.e.*, after subunit dissociation) suggests that it may also inhibit the interaction between dissociated subunits, such as the GTP-bound α-subunit, and their effectors, such as monomeric G-proteins. However, based on this interpretation of the results shown in figures 2 and 3, the major action of halothane would be to attenuate dissociation of the heterotrimeric G-proteins in response to receptor activation.

Studies of other tissue types support the concept of anesthetic effects on heterotrimeric G-proteins coupled to muscarinic receptors. In a series of studies using the binding properties of receptor ligands as an index of receptor–G-protein interaction, Anthony *et al.*[31] found that halothane and other volatile anesthetics stabilized the receptor–G-protein complex, interfering with the activation of the G-protein in muscarinic signal transduction. This stabilization did not involve an inhibition of

GTP binding.[32] They proposed that anesthetics inhibited GDP release from the inactive G-protein, inhibited the dissociation of the receptor–G-protein complex, or both. The former possibility is unlikely in our tissue, because AlF_4^--induced Ca^{2+} sensitization (which does not require the release of GDP) was attenuated by halothane applied before stimulation in our study. Halothane also inhibits signaling mediated by muscarinic receptors expressed in *Xenopus* oocytes[33]; similar results have been shown for enflurane.[34] Similar to our findings, these studies found evidence for inhibition only at the level of the receptor–G-protein complex, not at effector sites downstream from these processes.

We conclude that halothane inhibits increases in Ca^{2+} sensitivity produced by muscarinic receptor stimulation of canine tracheal smooth muscle primarily by interfering with activation of heterotrimeric G-proteins, probably by inhibiting the dissociation of G-protein subunits, and, to a lessor extent, by inhibiting interactions between heterotrimeric and small monomeric G-proteins. Because these proteins are ubiquitous second messengers in various cells, these interactions deserve further study as a site of anesthetic action in other types of tissues.

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