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Dual Effects of Hexanol and Halothane on the Regulation of Calcium Sensitivity in Airway Smooth Muscle

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Background: **Contraction of airway smooth muscle is regulated by receptor-coupled mechanisms that control the force developed for a given cytosolic calcium concentration (*i.e.*, calcium sensitivity). Halothane antagonizes acetylcholine-induced increases in calcium sensitivity by inhibiting GTP-binding (G)–protein pathways. The authors tested the hypothesis that hexanol, like halothane, inhibits agonist-induced increases in calcium sensitivity in airway smooth muscle by inhibiting G-protein pathways.**

Methods: **Calcium sensitivity was assessed using α-toxin–permeabilized canine tracheal smooth muscle. In selected experiments, regulatory myosin light chain phosphorylation was also determined by Western blotting in the presence and absence of 10 mM hexanol and/or 100 μM acetylcholine.**

Results: **Hexanol (10 mM) and halothane (0.76 mM) attenuated acetylcholine-induced calcium sensitization by decreasing regulatory myosin light chain phosphorylation during receptor stimulation. Hexanol also inhibited increases in calcium sensitivity due to direct stimulation of heterotrimeric G-proteins with tetrafluoroaluminate but not with 3 μM GTPγS, consistent with prior results obtained with halothane. In contrast, in the absence of receptor stimulation, both compounds produced a small increase in calcium sensitivity by a G-protein–mediated increase in regulatory myosin light chain phosphorylation that was not affected by pertussis toxin treatment.**

Conclusions: **The authors noted dual effects of hexanol and halothane. In the presence of muscarinic receptor stimulation, hexanol, like halothane, decreases calcium sensitivity by interfering with heterotrimeric G-protein function. However, in the absence of muscarinic receptor stimulation, hexanol and halothane slightly increase calcium sensitivity by a G-protein–mediated process not sensitive to pertussis toxin. Hexanol may represent a useful experimental tool to study the effect of anesthetics on heterotrimeric G-protein function.**

VOLATILE anesthetics directly relax airway smooth muscle (ASM) by decreasing cytosolic calcium concentration ($[Ca^{2+}]_i$)[1–4] and by decreasing the amount of force produced for a given $[Ca^{2+}]_i$ (*i.e.*, calcium sensitivity) during membrane receptor stimulation.[2,5–10] This latter effect is especially important during maximal stimulation, such as is present during severe bronchospasm. We have shown that volatile anesthetics inhibit receptor-linked pathways that increase calcium sensitivity in response to the physiologic agonist acetylcholine.[3,5–10] Studies of both airway and other types of smooth muscle have shown that receptor stimulation activates specific guanine nucleotide–binding proteins (G-proteins). We and others have shown that two families of G-proteins, heterotrimeric and small monomeric G-proteins, are involved in the regulation of calcium sensitivity.[10,11] Members of these two families form a cascade, 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, such as Rho. This event increases the phosphorylation of the regulatory myosin light chain (rMLC) by inhibiting the activity of a smooth muscle protein phosphatase. Increases in rMLC phosphorylation increase crossbridge cycling and produce force. In prior work, we found that halothane acts on this pathway specifically by inhibiting the function of heterotrimeric G-proteins coupled to muscarinic receptors, probably by inhibiting subunit dissociation.[10,12]

Recently, we have shown primary alcohols such as ethanol and hexanol also inhibit agonist-mediated increases in calcium sensitivity.[13,14] Other authors have noted parallels between the actions of alcohols (which themselves have anesthetic properties) and those of volatile anesthetics[15,16] and have found that alcohols can affect G-protein function.[17–19] If the mechanism of action of volatile anesthetics and the alcohols on G-protein–coupled responses are similar, the alcohols would represent useful experimental tools to further probe mechanism, not only to explore structure–activity relations but because they are more convenient to work with in aqueous solution. Our prior experiments with hexanol documented that it inhibits agonist-induced calcium sensitivity in intact airway smooth muscle but did not provide further mechanistic information.[14]

The purpose of the current study was to determine the mechanism by which hexanol, a representative primary alcohol, inhibits agonist-induced increases in calcium sensitivity in airway smooth muscle. Using an α-toxin–permeabilized canine airway smooth muscle preparation, we tested the hypothesis that, like halothane, hexanol inhibits the function of heterotrimeric G-proteins coupled to muscarinic receptors. To allow comparison of hexanol's effect with halothane, in selected experiments, we also characterized the effects of halothane in the α-toxin–permeabilized preparation, which have not been previously described. We then further explored the role of pertussis-sensitive G-proteins in the actions of both hexanol and halothane.

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Materials and Methods

Tissue Preparation

After approval by the Institutional Animal Care and Use Committee (Mayo Clinic, Rochester, Minnesota), mongrel dogs (weight, 20–25 kg) of either sex were anesthetized with 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 of the following composition: 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 removed under microscopic observation to make muscle strips of 0.1–0.2 mm width, approximately 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% O_2 and 6% CO_2. 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 length of the muscle strips was increased after repeated isometric contractions (of 1- to 2-min duration) induced by 1 μM acetylcholine until isometric force was maximal (optimal length). Each muscle strip was maintained at this optimal length for the remainder of the experiment. These tissues produced maximal isometric forces of approximately 2 mN when stimulated with 1 μM acetylcholine.

Permeabilization Procedure

The muscle strips were permeabilized with α-toxin by a method validated for canine tracheal smooth muscle in our laboratory.[20] To summarize, muscle strips were treated for 20 min with 2,500 U/ml α-toxin in the relaxing solution. The relaxing solution was prepared using the algorithm of Fabiato and Fabiato[21]: 7.5 mM Mg_2ATP, 4 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 20 mM imidazole, 1 mM dithiothreitol, 1 mM free Mg^{2+}, 1 nM free calcium, 10 mM creatine phosphate, and 0.1 mg/ml creatine phosphokinase. 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. The calcium ionophore A23187 (10 μM) was added to the relaxing solution and all subsequent experimental solutions to deplete the sarcoplasmic reticulum calcium stores. Solutions of varying free calcium concentrations were also prepared using the Fabiato algorithm. After the permeabilization procedure, reproducible maximal responses to 10 μM calcium were established as previously described.[20] During experimental protocols, whenever acetylcholine was applied to stimulate muscarinic receptors, 1 μM GTP was also included to assure adequate functioning of G-proteins.

Regulatory Myosin Light Chain Phosphorylation Measurements

Samples for rMLC phosphorylation measurements were separately prepared according to the same procedures as force measurements but incubated in wells at approximately optimal length instead of being superfused. Preliminary experiments revealed that muscle length does not affect rMLC phosphorylation under these conditions (unpublished observations conducted on August 4, 9, 17, and 24, 1999 by Hayashi Yoshimura, M.D., Research Fellow, Department of Anesthesiology Mayo Clinic and Foundation, Rochester, Minnesota). The data included measurements of regulatory myosin light chain phosphorylation at various muscle lengths both at rest (free $[Ca^{2+}]$ = 1 nM) and during maximal stimulation (free $[Ca^{2+}]$ = 10 μm). After experimental protocols, muscle strips were flash-frozen with dry ice–cooled acetone containing 10% (wt/vol) trichloroacetic acid and 10 mM dithiothreitol. Strips were then allowed to warm to room temperature in the same solution. After washing out trichloroacetic acid with acetone–dithiothreitol, strips were then allowed to dry. The dry weight of the strips was 0.07–0.13 mg. rMLC was extracted as described by Gunst *et al.*,[22] and phosphorylation was determined by glycerol-urea gel electrophoresis followed by Western blotting as previously described.[10] Unphosphorylated and phosphorylated bands of rMLC were visualized by phosphorimage analysis (PhosphorImager; Molecular Dynamics, Sunnyvale, CA), and fractional phosphorylation was calculated as the density ratio of the sum of monophosphorylated and diphosphorylated rMLC to total rMLC using ImageQuaNT software (Molecular Dynamics).

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 samples obtained at the end of the protocol using an electron capture detector (model 5880A; Hewlett-Packard, Waltham, MA) according to the method of Van Dyke and Wood.[23] The concentration of halothane achieved in solution in these experiments was 0.76 ± 0.13 mM (mean ± SD).

Experimental Protocols

Effects of Hexanol and Halothane on Calcium Sensitization. Two pairs of permeabilized strips were stimulated with varying free $[Ca^{2+}]$ (1 nM to 100 μM). In one pair, 100 μM acetylcholine was added 5 min before

the first increment in [Ca^{2+}]. In one strip of each pair, 10 mM hexanol was also added to the solutions. This concentration was chosen based on prior results showing that it produces maximal relaxation in intact canine trachea.[14] Because we have not previously examined the effects of halothane in a permeabilized preparations under these specific conditions (α-toxin permeabilization at 37°C), the experiment was repeated in another set of strips with halothane added to one strip of each pair before calcium stimulation to permit qualitative comparison of the effects of these two drugs.

Effects of Hexanol on Regulatory Myosin Light Chain Phosphorylation. Nine strips were permeabilized. One strip was frozen at baseline (1 nM calcium). The remaining pairs of strips were incubated with 0, 1, 3, or 10 mM hexanol for 10 min before increasing the [Ca^{2+}] to 0.18 μM (in the continued presence of the given concentration of hexanol). One strip of each pair also received 100 μM acetylcholine when the [Ca^{2+}] was increased. After 10 min, the strips were frozen.

Effects of Hexanol on Regulatory Myosin Light Chain Phosphorylation. To probe the site of hexanol's action, we utilized the pharmacological probes AlF_4^-, which directly activates heterotrimeric G-proteins without directly activating monomeric G-proteins, and GTPγS, which directly activates both heterotrimeric and monomeric G-proteins.[10] Two pairs of permeabilized strips were stimulated with varying free [Ca^{2+}] (1 nM to 100 μM). In one pair, AlF_4^- (2 mM NaF + 20 μM $AlCl_3$) was added 5 min before the first increment in [Ca^{2+}]. In one strip of each pair, 10 mM hexanol was also added to the solutions. The experiment was repeated in another set of strips with 3 μM GTPγS.

Involvement of G-proteins and Muscarinic Receptors in Hexanol-induced Changes in Calcium Sensitivity. To determine if effects of hexanol on calcium sensitivity were mediated by G-proteins, a set of three strips was permeabilized. After treatment with 0.18 μM calcium for 10 min, 100 μM acetylcholine was added to one strip, and 10 mM hexanol (without acetylcholine) was added to another. After a further 20 min, 1 mM guanosine 5'-O-(2-thiodiphosphate) (GDPβS), which inhibits G-protein function, was added to all three strips.

To determine if hexanol acts as a muscarinic agonist, permeabilized strips were first contracted with 0.18 μM calcium, and then 10 mM hexanol was added. Ten minutes after the addition of hexanol, 10 μM atropine was added to the solutions.

Effect of Pertussis Toxin on Responses to Hexanol and Halothane. These experiments determined if the effects of hexanol and halothane were mediated by pertussis-sensitive G-proteins. Two pairs of strips were prepared and incubated for 21 h at 31°C in 94% O_2–6% CO_2 in physiologic salt solution containing 0.1% fetal bovine serum, and 100 U/ml streptomycin and penicillin. Pertussis toxin (20 μg/ml) was added to the solutions, bathing one strip of each pair. After incubation, the strips were mounted, placed at optimal length, and permeabilized. After exposure to 0.3 μM calcium for 10 min, 100 μM acetylcholine was added to one pair of strips. After a further 15 min, 10 mM hexanol was added to all strips. The experiment was repeated in other sets of strips, with halothane, instead of hexanol, added to all strips.

Materials

Halothane was purchased from Ayerst Laboratories Inc. (New York, NY). Adenosine 5'-triphosphate (ATP) disodium salt was purchased from Research Organics Inc. (Cleveland, OH). All other drugs and chemicals were purchased from Sigma Chemical Co. (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 mean ± SD; n represents the number of dogs. Forces were expressed as percentage of the maximal force induced by 10 μM calcium determined in each individual strip immediately prior to the experimental protocol. Comparisons between two groups were made with paired *t* tests. Changes in rMLC phosphorylation with multiple doses of hexanol were assessed by repeated-measures analysis of variance within each condition and compared with control values using the Dunnett test. Parameters for concentration–response curves were determined using nonlinear regression fit to a three-parameter Hill equation (Sigma Stat; Jandel Scientific, San Rafael, CA), and parameter coefficients were compared using paired *t* tests. $P < 0.05$ was considered significant.

Results

Effects of Hexanol and Halothane on Calcium Sensitization

Increasing [Ca^{2+}] in the solutions bathing the permeabilized muscle produced a progressive increase in force (fig. 1). Addition of 100 μM acetylcholine increased the force developed at a given [Ca^{2+}], causing a leftward shift of the force–[Ca^{2+}] relation (*i.e.*, a decrease in EC_{50}) without changing the maximum force (fig. 1 and table 1). Both hexanol and halothane reduced the force developed at a given [Ca^{2+}] during acetylcholine exposure, causing a rightward shift in the force–[Ca^{2+}] relation (*i.e.*, an increase in EC_{50}) without changing maximal force (fig. 1 and table 1). When added in the absence of acetylcholine, both hexanol and halothane caused a significant leftward shift in the force–[Ca^{2+}] relation (fig. 2 and table 1). Thus, in the absence of muscarinic stimulation, these drugs increased calcium sensitivity, whereas

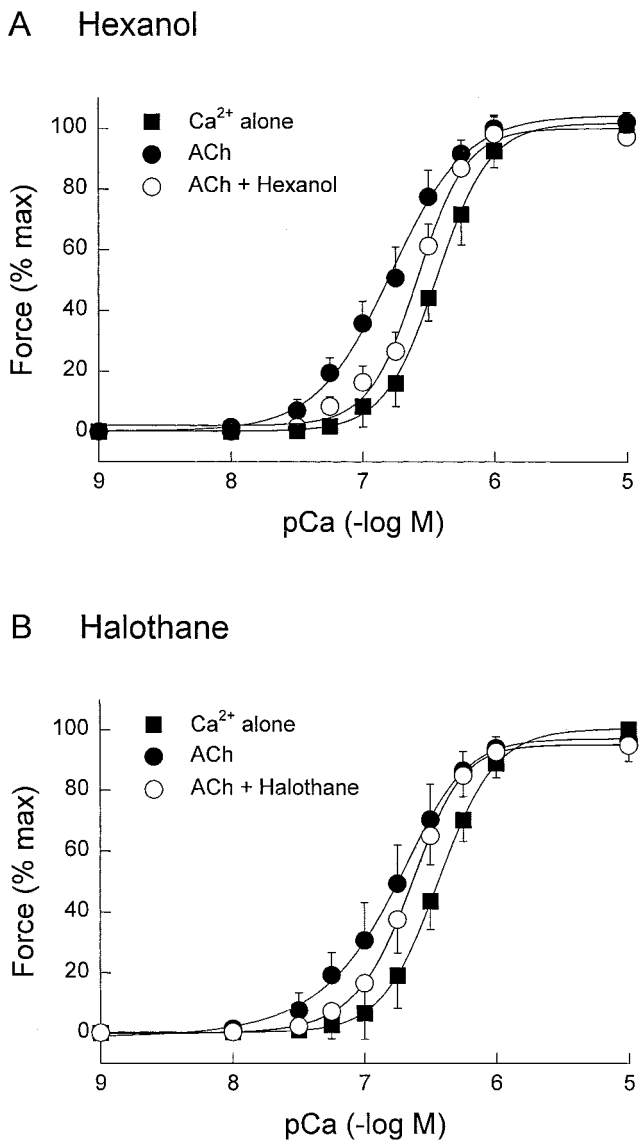

Fig. 1. Relation between force and [Ca^{2+}] in solutions bathing α-toxin–permeabilized canine tracheal smooth muscle, in absence (calcium alone) or presence of 100 μM acetylcholine (ACh). One set of strips stimulated with acetylcholine was also exposed to 10 mM hexanol (*A*) or 0.76 mM halothane (*B*). Acetylcholine increased calcium sensitivity (*i.e.*, produced a leftward shift in the concentration–response curve), an increase that was attenuated by both hexanol and halothane. Values are mean ± SD, n = 6 for each experiment. Force is expressed as a percentage of the response to a maximal [Ca^{2+}] determined in each strip before each experiment.

they attenuated the increase in calcium sensitivity produced by muscarinic stimulation.

Effects of Hexanol on Regulatory Myosin Light Chain Phosphorylation

Increasing the [Ca^{2+}] in solutions bathing the strips from 1 nM to 0.18 μM significantly increased rMLC phosphorylation (from 8 ± 3 to 11 ± 1%). In the absence of hexanol, 100 μM acetylcholine produced a further significant increase (to 27 ± 7%). In the presence of 100 μM acetylcholine, hexanol produced a concentration-dependent decrease in rMLC phosphorylation, which was significant beginning at the lowest hexanol concentration studied (1 mM; fig. 3). In the absence of acetylcholine, hexanol produced a concentration-dependent increase in rMLC phosphorylation (fig. 3). At 3 and 10 mM hexanol, the rMLC phosphorylation was not significantly different in the presence or absence of acetylcholine. Thus, in the absence of muscarinic stimulation, hexanol increases rMLC phosphorylation, whereas it attenuates increases in rMLC phosphorylation produced by muscarinic stimulation.

Effects of Hexanol on AlF_4^- and GTPγS-induced Calcium Sensitization

Addition of either AlF_4^-, which stimulates heterotrimeric G-proteins, or GTPγS, which stimulates all G-proteins, produced an increase in calcium sensitivity, significantly decreasing EC_{50} of the force–[Ca^{2+}] relation when compared with calcium-alone conditions, without changing F_{MAX} (fig. 4 and table 1). Hexanol (10 μM) attenuated the increase in calcium sensitivity produced by AlF_4^- (as indicated by a significant increase in EC_{50} during AlF_4^- stimulation; table 1), but had no effect on calcium sensitization produced by GTPγS (fig. 4 and table 1). This finding suggests that the effect of hexanol occurs at the level of the heterotrimeric G-protein, not at more distal sites in the transduction pathway.

Involvement of G-proteins or Muscarinic Receptors in Hexanol-induced Changes in Calcium Sensitivity

Inhibition of G-protein function by GDPβS produced a 93 ± 3% inhibition of the increase in calcium sensitivity produced by 100 μM acetylcholine in strips exposed to 0.18 μM calcium (n = 5; fig. 5A). This finding confirms that this concentration of GDPβS almost completely inhibits the G-proteins responsible for agonist-induced increases in calcium sensitivity. GDPβS also completely reversed the increase in force produced by the addition of 10 mM hexanol to strips exposed to 0.18 μM calcium (a 103 ± 23% inhibition; n = 5; fig. 5B). Thus, the increase in calcium sensitivity produced by hexanol in the absence of muscarinic stimulation is mediated by G-proteins.

Atropine (10 μM) did not affect calcium sensitivity in strips activated with 0.18 μM calcium and 10 mM hexanol (n = 3; fig. 5C), demonstrating that hexanol does not act as a muscarinic receptor agonist.

Effect of Pertussis Toxin on Responses to Hexanol and Halothane

Treatment with pertussis toxin for 21 h did not significantly change the force developed in response to 0.32 μM calcium alone compared with control strips also incubated for 21 h without pertussis toxin (25 ± 12 and 21 ± 9% maximal, respectively; P = 0.15; n = 11). However, per-

Table 1. Coefficients for Nonlinear Regression of Concentration–Response Relations

	Control		Hexanol		Halothane	
	EC_{50}	FMAX	EC_{50}	FMAX	EC_{50}	FMAX
Calcium alone	0.37 ± 0.06	102 ± 2	0.28 ± 0.04*	101 ± 5	—	—
100 μM acetylcholine	0.15 ± 0.05†	103 ± 3	0.26 ± 0.04*	98 ± 7	—	—
Calcium alone	0.37 ± 0.09	101 ± 4	—	—	0.28 ± 0.08*	100 ± 3
100 μM acetylcholine	0.17 ± 0.06†	98 ± 5	—	—	0.22 ± 0.05*†	96 ± 5
Calcium alone	0.32 ± 0.06	102 ± 3	0.20 ± 0.05*	96 ± 4*	—	—
AlF_4^-	0.12 ± 0.04†	102 ± 5	0.15 ± 0.03*†	95 ± 2*	—	—
Calcium alone	0.38 ± 0.08	99 ± 10	0.24 ± 0.08*	101 ± 4	—	—
GTPγS	0.10 ± 0.03†	102 ± 4	0.08 ± 0.05†	103 ± 6	—	—

Values are mean ± SD.

* Significant difference from control for each condition, paired *t* test. † Significant difference from calcium alone for the corresponding condition, paired *t* test.

AlF_4^- = aluminum fluoride; GTPγS = guanosine 5′-O-(3-thiotriphosphate).

tussis toxin significantly attenuated the additional force produced by 100 μM acetylcholine (from 39 ± 10 to 25 ± 7% maximal; $P < 0.001$; n = 11), indicating that pertussis-sensitive G-proteins at least partially mediate increases in calcium sensitivity produced by muscarinic stimulation. Both hexanol (10 mM) and halothane (0.76 mM) significantly increased calcium sensitivity in the absence of muscarinic receptor stimulation (fig. 6). Treatment with pertussis toxin had no effect on these increases in calcium sensitivity. Both hexanol and halothane attenuated increases in calcium sensitivity produced by 100 μM acetylcholine (fig. 6). Treatment with pertussis toxin abolished this effect of hexanol and halothane, such that neither compound significantly affected increases in calcium sensitivity produced by 100 μM acetylcholine.

Discussion

We noted dual effects of hexanol and halothane. In the absence of receptor stimulation, hexanol and halothane increased calcium sensitivity by a G-protein–mediated process not sensitive to pertussis toxin. However, in the presence of muscarinic receptor stimulation, the net effect of both hexanol and halothane was to decrease calcium sensitivity.

Decreased Calcium Sensitivity in the Presence of Muscarinic Stimulation

In prior work, we noted that halothane decreases the force developed for a given intracellular $[Ca^{2+}]$ (*i.e.*, the calcium sensitivity) in ASM during maximal muscarinic stimulation, both in intact and β-escin–permeabilized ASM.[2,5,6] The mechanism of this effect is inhibition of the heterotrimeric G-proteins that couple the muscarinic receptor to downstream effectors *via* small monomeric G-proteins.[10,24] By disrupting this receptor–G-protein signaling pathway, inhibition of smooth muscle protein phosphatase produced by muscarinic stimulation is partially released, such that rMLC phosphorylation, and consequently force, are decreased.[7,12] The current findings confirm the fundamental observation that halothane affects agonist-induced increases in calcium sensitivity, using a different permeabilization procedure, *Staphylococcal* α-toxin, which we have recently validated for canine airways.[20] Compared with β-escin, α-toxin produces a more stable preparation capable of reproducible contractions for several hours, suggesting that it better maintains intracellular homeostasis.[25,26] This stability also permits studies to be performed at physiologic temperatures (37°C) without the deterioration observed with other permeabilization procedures.

We found that hexanol had similar effects as halothane on calcium sensitivity in the presence of muscarinic stimulation, producing a concentration-dependent decrease in rMLC phosphorylation similar to that noted in prior studies of halothane.[7,12] This is also consistent with the effects of hexanol on the relation between force and $[Ca^{2+}]_i$ noted previously in intact ASM maximally stimulated with acetylcholine.[14] Halothane and other volatile anesthetics affect calcium sensitivity within the range of concentrations that produce clinical anesthesia.[7,9] The EC_{50} for hexanol to produce anesthesia in experimental animals (tadpoles and rats), expressed as a concentration in aqueous solution, is approximately 0.76 mM.[16] We noted significant effects on calcium sensitivity, assessed *via* changes in rMLC phosphorylation, at concentrations of hexanol as low as 1 mM (fig. 3), so that we consider that hexanol also affects calcium sensitivity at concentrations which are relevant to anesthesia.

Additional experiments using GTPγS and AlF_4^- to probe mechanism again produced findings similar to those noted in prior work with halothane.[10] Based on the rMLC phosphorylation results (fig. 3) and prior results in intact ASM,[14] we used hexanol at 10 mM in these mechanistic studies to maximize functional effects on EC_{50} and rMLC phosphorylation during muscarinic stimulation, recognizing that this is a high concentration relative to that required to produce anesthesia. Hexanol significantly attenuated AlF_4^--induced calcium sensitiza-

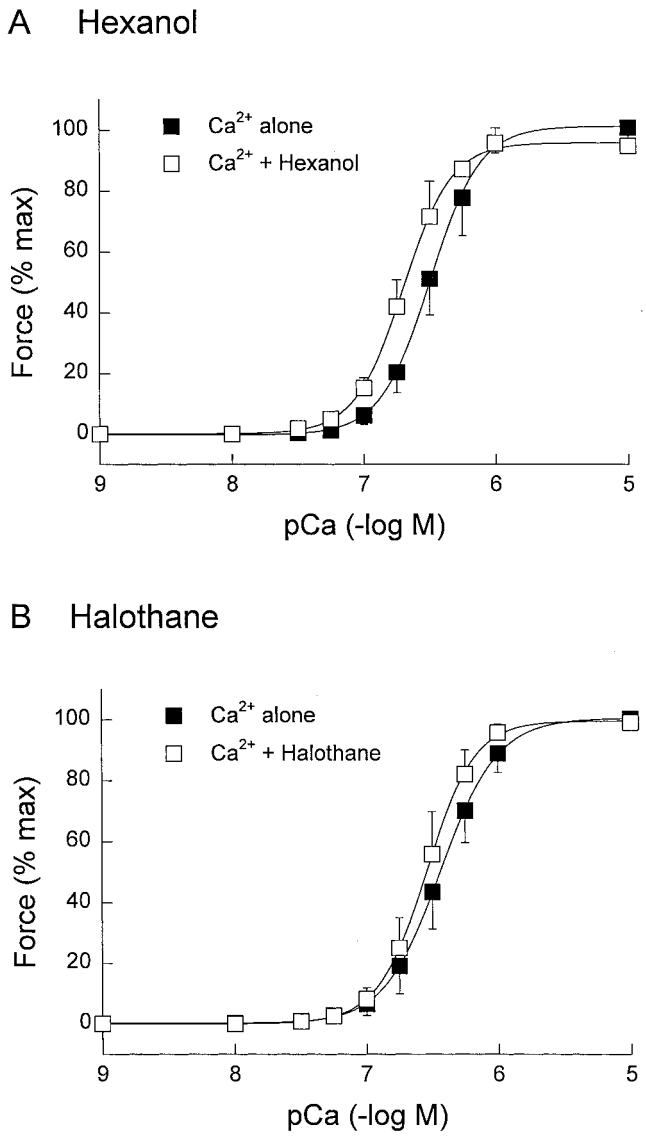

Fig. 2. Relation between force and [Ca^{2+}] in solutions bathing α-toxin–permeabilized canine tracheal smooth muscle in the absence of receptor stimulation. One set of strips was also exposed to 10 mM hexanol (*A*) or 0.76 mM halothane (*B*). In the absence of receptor stimulation, both hexanol and halothane produced a small increase in calcium sensitivity (*i.e.*, a leftward shift in the concentration–response curve). Values are mean ± SD, n = 6 for each experiment. Force is expressed as a percentage of the response to a maximal [Ca^{2+}] determined in each strip before each experiment.

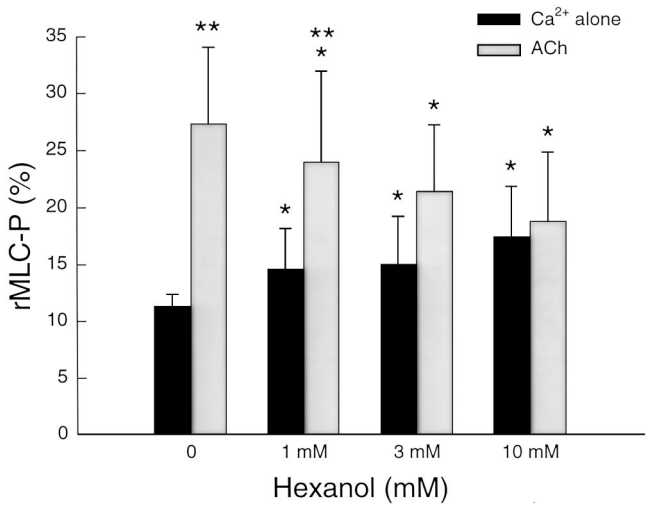

Fig. 3. Phosphorylated regulatory myosin light chain (rMLC-P; expressed as a percent of total rMLC) with and without exposure to increasing concentrations of hexanol, in the absence (calcium alone) or presence of 100 μM acetylcholine (ACh). Hexanol produced a dose-dependent attenuation of increases in rMLC-P caused by acetylcholine but produced a dose-dependent increase in rMLC-P in the absence of receptor stimulation. Values are mean ± SD, n = 6 for each condition. *Significant difference from 0 hexanol, repeated-measures analysis of variance with Dunnett test. **Significant difference from calcium-alone values within each hexanol concentration, paired *t* test with Bonferroni correction for four comparisons.

tion. AlF_4^- directly activates heterotrimeric G-proteins by binding to the α subunit next to the ß phosphate of GDP and mimicking the γ phosphate of GTP.[27] This finding implies that hexanol acts at sites at or distal to the heterotrimeric G-protein. Hexanol did not affect calcium sensitization produced by GTPγS, which directly activates both heterotrimeric and monomeric G-proteins. This demonstrates that hexanol does not affect sites downstream of the monomeric G-proteins, such as smooth muscle protein phosphatase or actomyosin ATPase, and localizes its effect to the heterotrimeric G-protein, as noted for halothane.[10] Our results do not, however, exclude additional effects of hexanol on the muscarinic receptor itself, or its interaction with G-proteins, actions which have been implicated as a potential mechanisms of anesthetic action in other experimental systems.[28–31]

Heterotrimeric G-proteins consist of an α subunit, which confers specificity of function and distinguishes between families of G-proteins, and two other subunits (ß and γ). Of the four major families of G-proteins, the function of two that are coupled to the muscarinic receptor have been examined specifically in ASM.[32,33] Proteins of the $G\alpha_i$ family, which can be inhibited by pertussis toxin catalyzed ADP-ribosylation of a cysteine residue near the C terminus, couple the M_2 muscarinic receptor to adenylyl cyclase and mediate inhibition of this enzyme. $G\alpha_q$ proteins couple the M_3 muscarinic receptor to phospholipase C and thus mediate increases in intracellular [Ca^{2+}] produced by muscarinic stimulation of ASM. Pertussis-sensitive G-proteins contribute to increases in calcium sensitivity in porcine ASM,[34] a finding we extend to canine ASM by showing that pertussis toxin attenuates acetylcholine-induced increases in calcium sensitivity. Consistent with findings in swine,[35,36] this attenuation is only partial (approximately 30%), implying either that the ADP-ribosylation of the G-protein produced by pertussis toxin is incomplete or that G-proteins other that those of the $G\alpha_i$ family are also involved in the regulation of calcium sensitivity. Work by

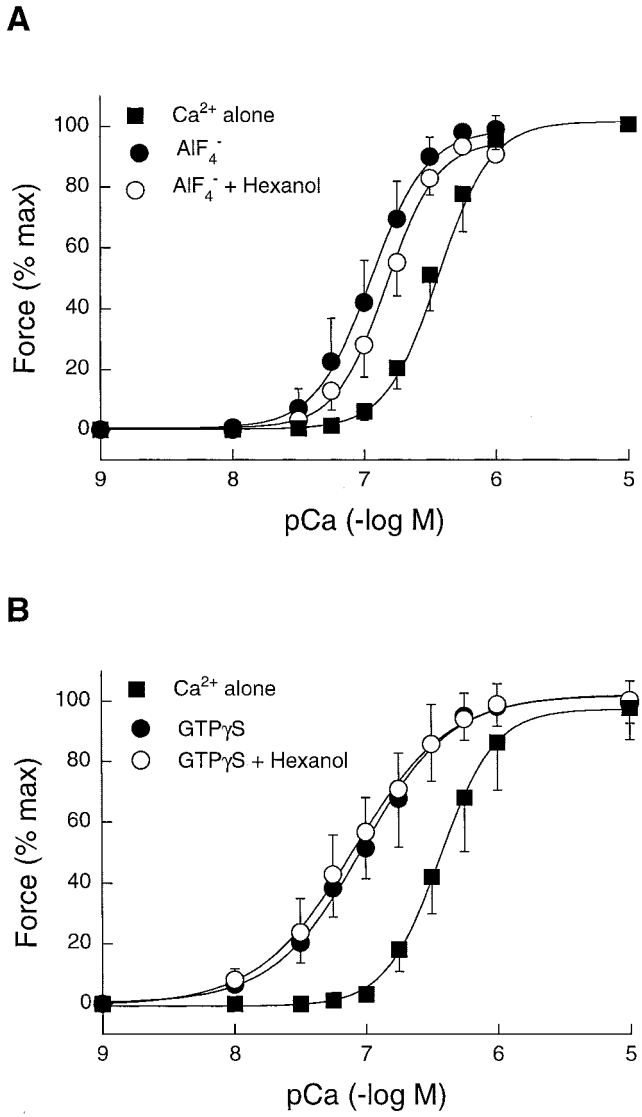

Fig. 4. Relation between force and [Ca^{2+}] in solutions bathing α-toxin–permeabilized canine tracheal smooth muscle, in absence (calcium alone) or presence of AlF_4^- (*A*) or 3 μM GTPγS (*B*). One set of strips stimulated with AlF_4^- or GTPγS was also exposed to 10 mM hexanol. Both AlF_4^- and GTPγS increased calcium sensitivity (*i.e.*, produced a leftward shift in the concentration–response curve). Hexanol attenuated the increase in calcium sensitivity caused by AlF_4^- but not GTPγS. Values are mean ± SD, n = 6 for each experiment. Force is expressed as a percentage of the response to a maximal [Ca^{2+}] determined in each strip before each experiment.

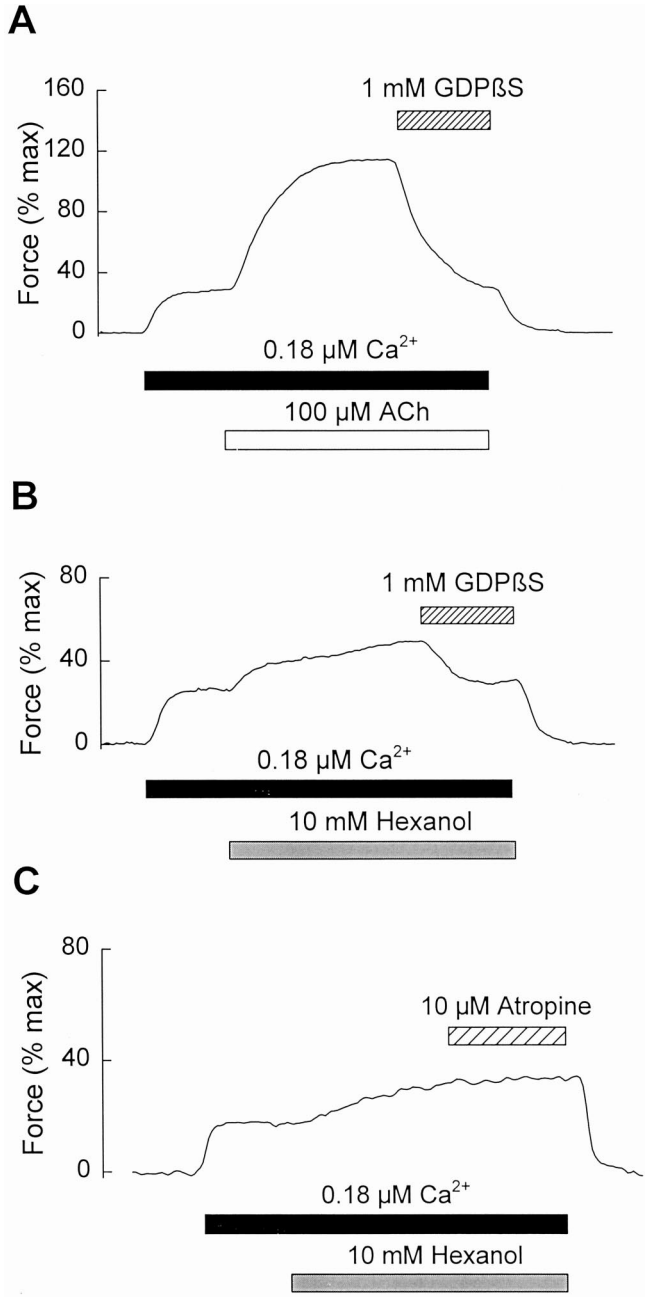

Fig. 5. Representative experiments in permeabilized strips exploring the role of G-proteins and muscarinic receptor stimulation in the action of hexanol to increase calcium sensitivity. Calcium stimulation increased force, an increase that was further augmented by 100 μM acetylcholine (ACh; *A*) or 10 mM hexanol (*B*, *C*). The effects of acetylcholine and hexanol were abolished by G-protein inhibition provided by 1 mM GDPβS (*A*, *B*). Atropine (10 μM) did not affect the increase in force produced by hexanol (*C*; representative of three experiments).

Croxton *et al.*[36] supports the latter possibility, implying that both $G\alpha_q$ and $G\alpha_i$ contribute to calcium sensitization produced by muscarinic stimulation in porcine ASM. Confirmation of this interpretation will require better delineation of the second-messenger pathways regulating calcium sensitivity in ASM. There is precedent for considerable complexity in the regulation of processes mediated by muscarinic receptors in ASM, such as stress fiber formation in cultured human ASM, with multiple G-proteins coupling heterogeneously to multiple receptors.[35,37] The current findings might appear to be inconsistent with our prior work noting that pertussis toxin treatment did not affect the ability of halothane to relax intact canine ASM.[38] However, this prior study was performed under conditions of submaximal muscarinic stimulation, conditions under which increases in calcium sensitivity do not play an important role in maintaining contraction.[2]

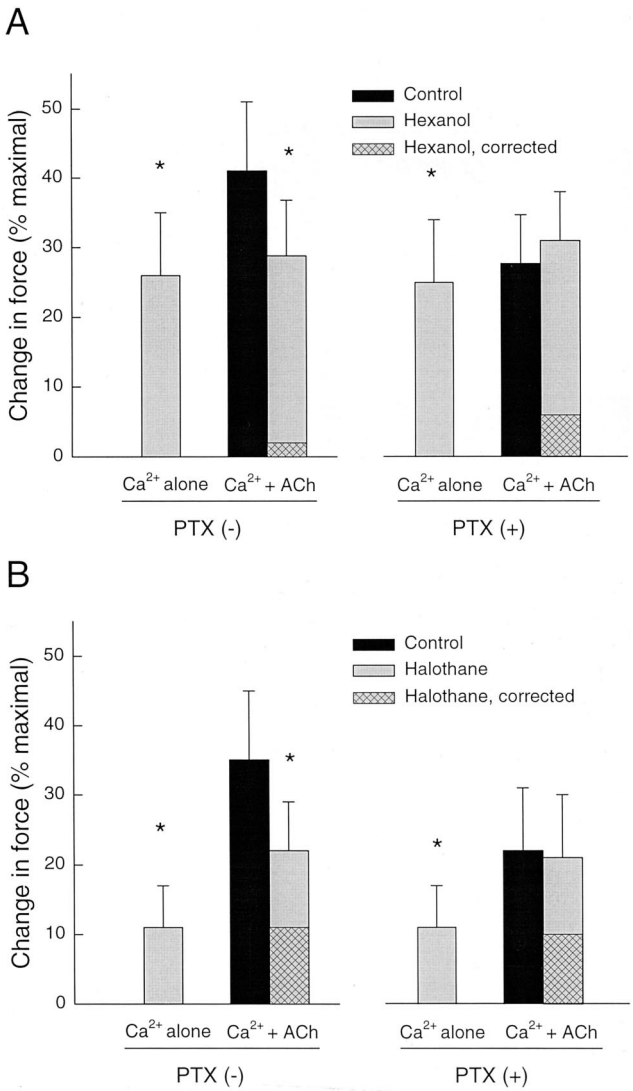

Fig. 6. Changes in force produced by 10 mM hexanol (*A*; n = 6) or 0.76 mM halothane (*B*; n = 5) in permeabilized strips stimulated with 0.32 μM calcium, with (calcium + acetylcholine) or without (calcium alone) 100 μM acetylcholine (ACh). Strips were incubated with (PTX(+)) or without (PTX(−)) 20 μM pertussis toxin for 21 h in physiologic salt solution before permeabilization. Values are mean ± SD and are expressed as the change in force (as a percentage of maximal) from values measured during exposure to 0.32 μM calcium alone in the absence of anesthetics. In the absence of receptor stimulation provided by acetylcholine, both hexanol and halothane increased calcium sensitivity, an increase that was not affected by pertussis toxin. In the presence of receptor stimulation, pertussis toxin abolished the effect of halothane and hexanol on calcium sensitivity. "Corrected" values were calculated for each of the four conditions by subtracting the amount of sensitization produced in the absence of receptor stimulation (Ca^{2+} alone) from the amount of sensitization produced in its presence (Ca^{2+} + ACh). This procedures assumes that the effects of hexanol and halothane in the absence and presence of receptor stimulation are additive (see Discussion for further details). *Significant difference from the corresponding control condition, paired *t* test, *P* < 0.05.

Pertussis toxin abolished the effects of hexanol and halothane on acetylcholine-induced calcium sensitivity. At first glance, this finding may suggest that the anesthetics have a specific effect on $G\alpha_i$ activity. However, interpretation of this finding is complicated by two factors: (1) the probable offsetting effects of these compounds on calcium sensitivity in the absence of muscarinic stimulation, and (2) uncertainty regarding the mechanism responsible for the incomplete attenuation of acetylcholine-induced sensitization by pertussis toxin. As an example of the former, in the case of hexanol, after pertussis toxin treatment, the increase in calcium sensitivity produced by acetylcholine was similar to that produced by the application of hexanol alone (fig. 6). It is thus difficult to evaluate any possible role of hexanol on acetylcholine-induced increases in calcium sensitivity under these conditions. However, this may be estimated if it is assumed that the effects of hexanol and halothane in the absence and presence of receptor stimulation are additive. Because both actions appear to converge on rMLC phosphorylation as an effector, this may be a reasonable assumption. We can then subtract the amount of sensitization produced in the absence of receptor stimulation ("calcium alone" in fig. 6) from the amount of sensitization produced in its presence to estimate the effect of the anesthetic on receptor-mediated sensitization ("Corrected" in fig. 6). Subject to this assumption, it is evident that even following pertussis toxin treatment, both anesthetics still attenuated acetylcholine-induced calcium sensitivity. This finding could reflect either an effect of the anesthetics on a G-protein other than $G\alpha_i$ that may mediate calcium sensitivity (if pertussis toxin completely abolished $G\alpha_i$ activity), an effect on $G\alpha_i$ activity (if the effect of pertussis toxin on $G\alpha_i$ activity was incomplete), or a combination of both factors. Distinguishing between these possibilities will require better delineation of the second-messenger pathways regulating calcium sensitivity in ASM.

Increased Calcium Sensitivity in the Absence of Muscarinic Stimulation

Both hexanol and halothane increased force under conditions of constant extracellular [Ca^{2+}] in α-toxin–permeabilized canine ASM at 37°C in the absence of receptor stimulation. The change in EC_{50} measured over a full range of [Ca^{2+}] was comparable between the two agents at the doses studied (fig. 2 and table 1). At the relatively low [Ca^{2+}] used for the pertussis toxin studies (0.32 μM), the effect of 10 mM hexanol was greater than the effect of 0.75 mM halothane (fig. 6). These comparisons should be made with caution, however, because relative to minimum alveolar concentration (MAC), the concentration of hexanol studied was greater than that of halothane. Using GDPβS as a probe, we found that the increases in calcium sensitivity produced by hexanol were mediated by G-proteins. We confirmed this finding in further experiments using ASM permeabilized with Triton-X, a more stringent permeabilization procedure that eliminates G-protein mediated responses.[39] Under these conditions, hexanol in fact produced a small relax-

ation when added to strips activated with calcium (data not shown). The lack of effect of atropine on calcium sensitization produced by hexanol excludes the possibility that hexanol acts as a muscarinic agonist.

The fact that hexanol- and halothane-induced sensitization is not inhibited by pertussis toxin should be interpreted cautiously. ADP ribosylation by pertussis toxin blocks the interaction of the muscarinic receptor with toxin-sensitive proteins such as G_i without affecting the ability of the G-protein to bind GTP.[40] It is not clear whether ADP ribosylation affects the ability of activated subunits to interact with effectors such as Rho. Because the muscarinic receptor was not activated during anesthetic exposure (as confirmed by the experiment with atropine), the lack of effect of pertussis toxin does not necessarily provide information regarding the identity of the G-protein–mediated process stimulated by the anesthetics. Further, it is possible that anesthetics stimulated small G-proteins downstream of heterotrimeric proteins, such as Rho.

We have not observed significant halothane-induced increases in calcium sensitivity in prior studies using a β-escin–permeabilized ASM preparation studied at 25°C,[5,6,10] although there was a tendency toward such an increase observed at lower $[Ca^{2+}]$ in some studies, and the magnitude of the effect in the current study is small. It is possible that during β-escin permeabilization, which produces larger pores in the plasma membranes and is less stable over time, cellular components necessary for this effect are lost. It is also possible that differences in temperature play a role. Toda and Su[41] also found that a volatile anesthetic (isoflurane) increased calcium sensitivity in permeabilized rabbit femoral artery. However, this would appear to be a different phenomenon compared with that observed in the current study, because (1) they used saponin-permeabilized arteries, a procedure which, similar to Triton-X treatment, eliminates G-protein–coupled responses, and (2) their increases in sensitivity were not associated with increases in rMLC phosphorylation. Other authors have found that volatile anesthetics either do not affect or cause a small inhibition of calcium-mediated contractions in vascular smooth muscle.[42] A prior study of halothane's effect on the force–$[Ca^{2+}]_i$ relation in intact ASM did not detect evidence for such increases in calcium sensitivity,[2] suggesting that it has little functional significance if present *in vivo*.

In a prior study, we also did not observe an effect of hexanol (2 mM) on the relation between force and $[Ca^{2+}]_i$ in intact ASM in the absence of muscarinic stimulation.[14] According to measurements of rMLC in the current study (fig. 3), this concentration of hexanol should have caused a small increase in calcium sensitivity. It is possible that it was not possible to detect a small increase in calcium sensitivity using the protocol employed in intact ASM or that the $[Ca^{2+}]_i$ under conditions of the prior study was insufficient to support contraction. In any event, like the effect of halothane, it appears that this small increase in calcium sensitivity noted in this permeabilized preparation has little functional significance in intact ASM.

Apart from any functional significance that these increases in calcium sensitivity may have in intact smooth muscle, our results represent the first demonstration to our knowledge that a volatile anesthetic can directly stimulate a G-protein–mediated process. However, anesthetics clearly inhibit the ability of G-proteins to mediate agonist-induced increases in calcium sensitivity. We can only speculate as to why this dual effect occurs. One possibility is that anesthetics activate monomeric G-proteins but inhibit receptor activation of heterotrimeric G-proteins. Alternately, as discussed above, several heterotrimeric G-protein subunit families can mediate calcium sensitivity,[36] and it is possible that anesthetics stimulate some families and inhibit others. There is precedent for differential effects of anesthetics on subunit families. Pentyala *et al.*[43] found that anesthetic effects on the binding of guanine nucleotides to purified α subunits depended on subunit type. Volatile anesthetics inhibited the exchange of GDP or GTPγS on the subunits, an action that would inhibit their function. Of the subunits examined, $G\alpha_{i\text{-}2}$ was most sensitive, and one subunit ($G\alpha_o$, a member of the $G\alpha_i$ family) was completely insensitive; $G\alpha_q$ was not examined. Demonstration of differential effects of anesthetics on G-protein function *in situ* requires further study.

Effects of Alcohols on Calcium Sensitivity: Other Studies

There are limited prior data on the effects of other alcohols on calcium sensitivity in smooth muscle. We have found that ethanol also has dual effects in canine ASM, attenuating increases in rMLC phosphorylation produced by muscarinic stimulation while producing a small increase in calcium sensitivity in the absence of receptor stimulation.[13] In the case of ethanol, the net functional effect in terms of force is an increase in calcium sensitivity under all conditions as the latter effect outweighs the former. The effect of ethanol to increase calcium sensitivity in the absence of receptor stimulation in canine ASM is fundamentally different from that of hexanol as it is not associated with the activation of G-proteins and is not accompanied by increases in rMLC phosphorylation.[13] However, in vascular smooth muscle, ethanol-induced increases in calcium sensitivity have been observed that are, like our hexanol-induced increases in calcium sensitivity in the absence of receptor stimulation, dependent on G-proteins.[44]

Summary

In the presence of muscarinic receptor stimulation, hexanol, like halothane, decreases calcium sensitivity by interfering with the function of heterotrimeric G-proteins. However, in the absence of muscarinic receptor stimulation, hexanol and halothane increase calcium sensitivity by a G-protein–mediated process not sensitive to pertussis toxin. Because the actions of hexanol thus are similar to those of halothane, it may represent a useful experimental tool to study the effect of anesthetics on receptor–G-protein function in ASM.

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