

Halothane Attenuation of Calcium Sensitivity in Airway Smooth Muscle

Mechanisms of Action during Muscarinic Receptor Stimulation

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Background: In airway smooth muscle, muscarinic receptor stimulation is thought to increase calcium (Ca^{2+}) sensitivity via a guanosine 5'-triphosphate (GTP)-binding protein/protein kinase C (PKC)-mediated mechanism. This study tested the hypothesis that halothane reduces Ca^{2+} sensitivity during muscarinic receptor stimulation by inhibiting these second messenger pathways.

Methods: A β -escin permeabilized canine tracheal smooth muscle preparation was used in which the cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) is controlled and the GTP-binding protein/PKC pathways remain intact and can be activated. The muscarinic receptor was activated with acetylcholine plus GTP; the GTP-binding proteins were directly activated with a nonhydrolyzable form of GTP, guanosine 5'-O-(3-thiotriphosphate; $\text{GTP}\gamma\text{S}$); and PKC was directly activated with the PKC agonist phorbol 12,13-dibutyrate (PDBu).

Results: Free Ca^{2+} caused a concentration-dependent increase in force. Acetylcholine plus GTP significantly decreased the median effective concentration for free Ca^{2+} from $0.52 \pm 0.06 \mu\text{M}$ to $0.21 \pm 0.02 \mu\text{M}$, demonstrating an increase in Ca^{2+} sensitivity. Halothane ($0.99 \pm 0.04 \text{ mm}$, equivalent to approximately 4 minimum alveolar concentration in dogs) significantly attenuated this increase in Ca^{2+} sensitivity induced by acetylcholine plus GTP, increasing the median effective concentration for free Ca^{2+} from $0.21 \pm 0.02 \mu\text{M}$ to $0.31 \pm 0.03 \mu\text{M}$. However, halothane did not affect the increases in Ca^{2+} sensitivity induced by $\text{GTP}\gamma\text{S}$ or PDBu.

Conclusions: Halothane had no effect on increased Ca^{2+} sensitivity caused by direct activation of GTP-binding proteins with $\text{GTP}\gamma\text{S}$ or PKC with PDBu, suggesting that halothane at-

tenuates acetylcholine-induced Ca^{2+} sensitization via a mechanism independent of these pathways in β -escin-permeabilized canine tracheal smooth muscle. (Key words: β -escin. Calcium sensitivity. Lung, trachea: canine; smooth muscle. Muscle, smooth: airway, trachea. Pharmacology: acetylcholine; guanosine 5'-triphosphate (GTP); guanosine 5'-O-(3-thiotriphosphate) ($\text{GTP}\gamma\text{S}$); guanosine 5'-O-(2-thiodiphosphate) ($\text{GDP}\beta\text{S}$); halothane; phorbol 12,13-dibutyrate (PDBu). Second messenger systems: GTP-binding proteins; protein kinase C.)

VOLATILE anesthetics are potent bronchodilators, relaxing airway smooth muscle *in vitro* in part by a direct effect on the smooth muscle cell.¹⁻⁴ They reduce the cytosolic free calcium concentration ($[\text{Ca}^{2+}]_i$)¹⁻⁴ and the amount of force produced at a constant $[\text{Ca}^{2+}]_i$ (*i.e.*, calcium sensitivity^{2,3}). Although the mechanisms by which volatile anesthetics decrease $[\text{Ca}^{2+}]_i$ have been investigated extensively,¹⁻⁴ few studies have elucidated the intracellular pathways by which these compounds reduce calcium sensitivity in airway smooth muscle,⁵ a potentially important anesthetic mechanism.

Airway smooth muscle contraction produced by muscarinic receptor agonists is mediated by an increase in $[\text{Ca}^{2+}]_i$. Calcium binds calmodulin and subsequently increases myosin light chain kinase activity and phosphorylation of the 20-kDa regulatory myosin light chain.^{6,7} Contractile force, however, does not depend on the increase in $[\text{Ca}^{2+}]_i$ alone. Muscarinic receptor agonists also activate a guanosine 5'-triphosphate (GTP)-dependent second messenger cascade that increases Ca^{2+} sensitivity^{8,9} in permeabilized smooth muscle preparations exposed to calcium. The mechanism of this action is not fully characterized, but there is considerable evidence that GTP-binding proteins¹⁰⁻¹⁸ and protein kinase C (PKC)¹⁹⁻²² play key roles.

The purpose of the current study was to determine the role of GTP-binding proteins and PKC as potential targets for halothane's action on acetylcholine-induced enhancement of Ca^{2+} sensitivity in canine tracheal smooth muscle (CTSM). We used a β -escin-permeabil-

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Received from the Departments of Anesthesiology, Physiology, and Biophysics, Mayo Clinic and Mayo Foundation, Rochester, Minnesota. Submitted for publication October 3, 1996. Accepted for publication February 13, 1997. Supported in part by grants HL-45532 and HL-54757 from the National Institutes of Health. Presented in part at the American Society of Anesthesiologists annual meeting, October 19-23, 1996, New Orleans, Louisiana.

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ized CTSM preparation in which pores are produced in the sarcolemma, permitting control of $[\text{Ca}^{2+}]_i$ by manipulation of extracellular Ca^{2+} concentration, yet GTP-binding protein and PKC second messenger cascades remain intact and can be activated.^{5,23}

Materials and Methods

Tissue Preparation

After we received approval from the Institutional Animal Care and Use Committee, and conforming to the Guiding Principles in the Care and Use of Animals as approved by the Council of the American Physiological Society, we anesthetized 33 mongrel dogs (15–25 kg) of either sex with an intravenous injection of pentobarbital (30 mg/kg) and then killed them by exsanguination. A 15- to 20-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 . Fat, connective tissue, and the epithelium were removed with tissue forceps and scissors under microscopic observation.

Experimental Techniques

Isometric Force Measurements. Muscle strips (width, 0.1–0.2 mm; length, 3–5 mm; wet weight, 0.05–0.1 mg) were mounted in a 0.1-ml cuvette and continuously perfused at 2 ml/min with physiologic salt solution (at 37°C) aerated with 94% oxygen and 6% carbon dioxide. One end of the strips was anchored with stainless steel microforceps to a stationary metal rod and the other end was anchored with stainless steel microforceps to a calibrated force transducer (model KG4; Scientific Instruments, Heidelberg, Germany). During a 3-h equilibration period, the length of the strips was increased after repeated isometric contractions (lasting 2 or 3 min) induced by 1 μM acetylcholine until the optimal length was obtained. Each strip was maintained at this optimal length and cooled to room temperature (25°C) for the rest of the experiment. These tissues produced isometric forces of 0.8–2.5 mN.

Permeabilization Procedure. Muscle strips were permeabilized with β -escin as previously described^{12,18,23} and validated in our laboratory.⁵ β -Escin creates pores in the sarcolemma, thus allowing substances of small molecular weight, such as Ca^{2+} , to diffuse freely across the cell membrane. Accordingly,

$[\text{Ca}^{2+}]_i$ can be manipulated by changing the extracellular Ca^{2+} concentration with ethyleneglycol-bis-(β -aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA)-buffered solutions in the bathing media. Larger cellular proteins necessary for contraction are preserved and the membrane receptor-coupled second messenger systems thought to regulate Ca^{2+} sensitivity, such as GTP-binding proteins and PKC, remain intact and can be activated.⁵ Thus effects of halothane on Ca^{2+} -calmodulin activation of the contractile proteins can be distinguished from those on the membrane receptor-coupled second messenger systems that regulate Ca^{2+} sensitivity.

Muscle strips were permeabilized for 20 min with 100 μM β -escin in relaxing solution. The composition of the relaxing solution was 7.5 mM adenosine 5'-triphosphate, disodium salt; 4 mM EGTA; 20 mM imidazole; 1 mM dithiothreitol; 1 nM free Ca^{2+} ; 10 mM creatinine phosphate; and 0.1 mg/ml creatinine phosphokinase. The pH was buffered to 7.0 at 25°C with potassium hydroxide; the ionic strength was kept constant at 0.20 M by adjusting the concentration of potassium acetate. After the permeabilization procedure, strips were perfused with relaxing solution for 10 min to wash out the excess β -escin. Calcium ionophore A23187 (10 μM) was added to the relaxing solution and all subsequent experimental solutions to deplete the sarcoplasmic reticulum Ca^{2+} stores. Solutions of various free Ca^{2+} concentrations were prepared using the algorithm of Fabiato and Fabiato.²⁴ In all experimental solutions, the final concentration of dimethyl sulfoxide, used as a vehicle to dissolve compounds insoluble in water, did not exceed 0.01%; at this concentration, dimethyl sulfoxide had no effect on the isometric force development (data not shown).

Experimental Protocols

Experiments were conducted to determine the effects of halothane on Ca^{2+} sensitization induced by muscarinic receptor stimulation with 3 μM acetylcholine plus 10 μM GTP, by direct activation of GTP-binding proteins with a nonhydrolyzable analog of GTP, guanosine 5'-O-(3-thiotriphosphate) (GTP γ S) and by direct activation of PKC with the phorbol ester phorbol 12, 13-dibutyrate (PDBu). Previously we found that halothane has no effect on Ca^{2+} sensitivity in the absence of muscarinic receptor stimulation,⁵ indicating that halothane has no effect on Ca^{2+} -calmodulin activation of myosin light chain kinase or the contractile proteins.

Two experimental protocols were performed. In the

first protocol, the effect of agonist-induced Ca^{2+} sensitization in the absence and presence of halothane (0.99 ± 0.04 mm, equivalent to approximately 4 minimum alveolar concentration for dogs expressed as an aqueous concentration in saline, corrected to room temperature²⁵) was determined by generating free Ca^{2+} concentration-response curves (0.001 – 10 μM). These experiments included three tissue sets, one for each agonist. In each tissue set, three permeabilized CTSM strips were prepared and studied concomitantly. One strip of each set was perfused with relaxing solution alone (Ca^{2+} alone, control). The other two strips of each set were perfused with relaxing solution containing either 3 μM acetylcholine (a concentration causing 80% of its maximal effect on isometric force development at a constant $[\text{Ca}^{2+}]_i$ in this preparation⁵) plus 10 μM GTP to activate muscarinic receptors ($n = 6$), 3 μM GTP γS ($n = 6$), or 1 μM PDBu ($n = 6$). One strip was also exposed to halothane. To generate the Ca^{2+} concentration-response curves, muscle strips were allowed to contract for 10 min after each increment of free Ca^{2+} concentration. After these determinations, strips were perfused with relaxing solution for 10 min, including inorganic phosphate (P_i ; 5 mM) to reduce the time required for relaxation by accelerating the rate of cross-bridge detachment²⁶ and then perfused with relaxing solution for 10 min to remove P_i . Isometric force measurements were normalized to those determined in the same tissues in response to 10 μM free Ca^{2+} at the end of the experiment.

The second experimental protocol was performed to determine the effect of halothane on different intensities of GTP-binding protein or PKC activation. Concentration-response curves for GTP γS and PDBu were generated at a constant submaximal $[\text{Ca}^{2+}]_i$ of 0.1 μM and 0.3 μM , respectively. Preliminary studies showed that force developed in response to PDBu only in the presence of ≥ 0.1 μM $[\text{Ca}^{2+}]_i$. Two pairs of two permeabilized CTSM strips were prepared and the contractile response to increasing concentrations of GTP γS (0.01 – 31.6 μM ; $n = 5$) or PDBu (0.001 – 3 μM ; $n = 5$) was measured. The solutions perfusing one strip of each pair also contained 0.97 ± 0.13 mm halothane. Muscle strips were allowed to contract for 10 min after each increment of concentration, the time for stable contractile responses. Isometric force measurements were normalized to those determined in the same tissues in response to 10 μM free Ca^{2+} .

Administration of Halothane. Halothane was delivered to solutions *via* an on-line calibrated vaporizer.

Each solution was equilibrated with halothane for at least 5 min before being introduced to the system. The concentrations of halothane in the solutions at the cuvette were determined by gas chromatography from anaerobically obtained samples using an electron capture detector (model 5880A; Hewlett-Packard, Waltham, MA) according to the method of Van Dyke and Wood.²⁷

Materials. Halothane was purchased from Wyeth-Ayerst Laboratories, Inc. (Philadelphia, PA). A23187 was purchased from Molecular Probes, Inc. (Eugene, OR). Adenosine 5'-triphosphate, disodium salt, was purchased from Research Organics, Inc. (Cleveland, OH). All other drugs and chemicals were purchased from Sigma Chemical Company (St. Louis, MO). Stock solutions and all other drugs and chemicals were prepared in distilled water or dimethyl sulfoxide.

Statistical Analysis. Data are expressed as mean values \pm SD; n represents the number of dogs. All forces were expressed as a percentage of the response to 10 μM free Ca^{2+} in that strip. Concentration-response curves were compared by nonlinear regression described by Meddings *et al.*²⁸ In this method, force (F) at any concentration of drug C was given by the equation:

$$F = F_m C / (EC_{50} + C)$$

where F_m represents the maximal isometric force generated under that condition and EC_{50} represents the concentration that produces one half of this maximal isometric force for that drug. Nonlinear regression analysis was used to fit values of F_m and EC_{50} to data for F and C for each condition studied. This method allowed us to compare curves to determine whether they were significantly different and whether this overall difference could be attributed to differences in F_m , EC_{50} , or both parameters. Probability values of 0.05 or less were considered significant.

Results

Effect of Halothane on Free Ca^{2+} Concentration Response Curves during Muscarinic Receptor Stimulation

Ca^{2+} alone caused a concentration-dependent increase in force. Muscarinic receptor stimulation with acetylcholine plus GTP caused a leftward shift of the free Ca^{2+} concentration response curves (fig. 1), producing a significant additional force at a constant submaximal $[\text{Ca}^{2+}]_i$ as compared to the force induced by free Ca^{2+} alone

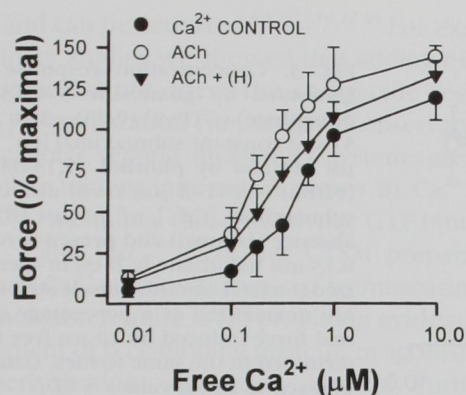
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Fig. 1. Free Ca^{2+} concentration–response curves generated by free Ca^{2+} alone (0.01–10 μM ; Ca^{2+} control), and during muscarinic receptor stimulation with 3 μM acetylcholine (EC_{50}) plus 10 μM GTP (ACh) in the absence and presence of 0.99 \pm 0.04 mM halothane (ACh + H) in β -escin-permeabilized canine tracheal smooth muscle strips. Values are normalized as a percentage of maximal force induced by 10 μM free Ca^{2+} determined in the same tissues. Data are expressed as mean values \pm SD ($n = 6$).

(i.e., increasing Ca^{2+} sensitivity). The concentration of free Ca^{2+} required to produce 50% of maximal force (EC_{50}) was significantly decreased by acetylcholine plus GTP from $0.52 \pm 0.06 \mu\text{M}$ to $0.21 \pm 0.02 \mu\text{M}$. In the presence of acetylcholine and GTP, halothane caused a rightward shift of the free Ca^{2+} concentration–response curve, significantly increasing the EC_{50} for free Ca^{2+} from $0.21 \pm 0.02 \mu\text{M}$ to $0.31 \pm 0.03 \mu\text{M}$.

Effect of Halothane on Ca^{2+} Sensitization Induced by Guanosine 5'-O-(3-thiotriphosphate) or Phorbol 12, 13-dibutyrate

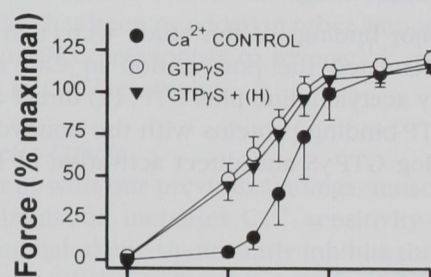
The possible involvement of GTP-binding proteins and PKC in halothane's attenuation of acetylcholine-induced Ca^{2+} sensitization was determined during activation with $\text{GTP}\gamma\text{S}$ and PDBu, respectively. Both $\text{GTP}\gamma\text{S}$ (fig. 2A) and PDBu (fig. 2B) caused a significant leftward shift of the free Ca^{2+} concentration–response curves, reducing the EC_{50} for free Ca^{2+} from $0.61 \pm 0.09 \mu\text{M}$ to $0.16 \pm 0.01 \mu\text{M}$ and from $0.87 \pm 0.09 \mu\text{M}$ to $0.29 \pm 0.04 \mu\text{M}$, respectively. In contrast to its effect on acetylcholine-induced Ca^{2+} sensitization, halothane had no effect on this leftward shift of the curves ($\text{GTP}\gamma\text{S}$: $0.16 \pm 0.01 \mu\text{M}$ to $0.18 \pm 0.02 \mu\text{M}$; PDBu: $0.29 \pm 0.04 \mu\text{M}$ to $0.29 \pm 0.04 \mu\text{M}$, respectively).

$\text{GTP}\gamma\text{S}$ (fig. 3A) and PDBu (fig. 3B) increased force at a constant $[\text{Ca}^{2+}]_i$ of 0.1 μM or 0.3 μM free Ca^{2+} , respectively, in a concentration-dependent manner (i.e., enhanced Ca^{2+} sensitivity). The EC_{50} for $\text{GTP}\gamma\text{S}$ was

$1.17 \pm 0.31 \mu\text{M}$ and was $0.21 \pm 0.02 \mu\text{M}$ for PDBu. Halothane had no effect on this increase in Ca^{2+} sensitivity over the entire range of the agonist concentrations studied (EC_{50} values of $1.46 \pm 0.25 \mu\text{M}$ for $\text{GTP}\gamma\text{S}$ and $0.18 \pm 0.07 \mu\text{M}$ for PDBu).

To confirm the involvement of GTP-binding proteins in acetylcholine-induced Ca^{2+} sensitization, the effect of guanosine 5'-O-(2-thiodiphosphate) ($\text{GDP}\beta\text{S}$), a non-hydrolyzable form of GDP that antagonizes GTP-binding protein activation, was studied ($n = 2$). One mM $\text{GDP}\beta\text{S}$ had no effect on force induced by Ca^{2+} alone. Preincubation with 1 mM $\text{GDP}\beta\text{S}$ in relaxing solution inhibited subsequent force development induced by muscarinic receptor stimulation with 3 μM acetylcholine and 10 μM GTP in the presence of 0.3 μM $[\text{Ca}^{2+}]_i$ by approximately 90%. One mM $\text{GDP}\beta\text{S}$ added after muscarinic receptor stimulation caused an approximately 65% re-

2A



2B

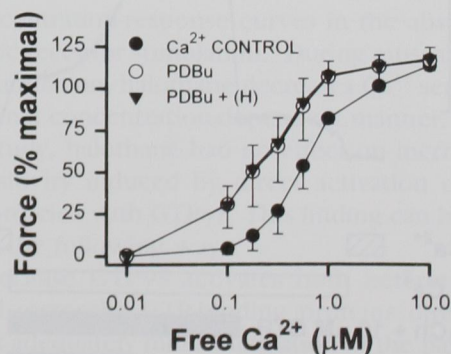


Fig. 2. Free Ca^{2+} concentration–response curves (0.01–10 μM) generated by free Ca^{2+} alone (Ca^{2+} control) and during activation of GTP-binding proteins with 3 μM guanosine 5'-O-(3-thiotriphosphate) ($\text{GTP}\gamma\text{S}$; $n = 6$) (A) and protein kinase C with 1 μM phorbol 12,13-dibutyrate (PDBu; $n = 6$) (B) in the presence and absence of 0.99 \pm 0.04 mM halothane ($\text{GTP}\gamma\text{S} + \text{H}$; PDBu + H, respectively) in β -escin-permeabilized canine tracheal smooth muscle strips. Values are normalized as a percentage of maximal force induced by 10 μM free Ca^{2+} determined in the same tissues. Data are expressed as mean values \pm SD.

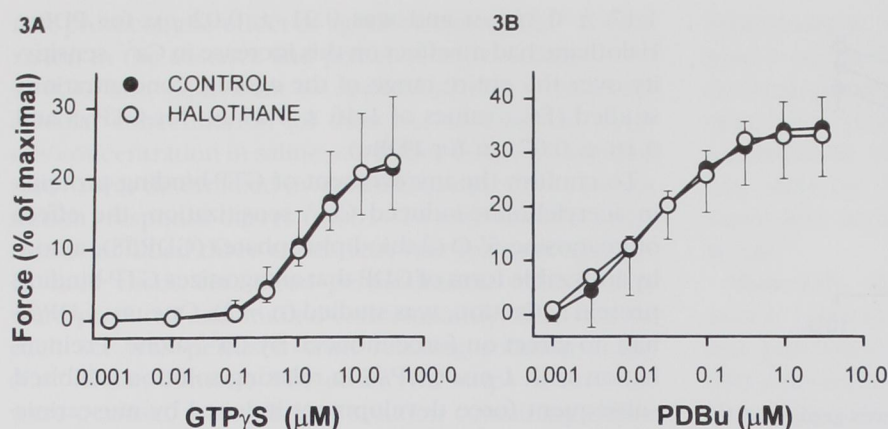


Fig. 3. Concentration-response curves generated by guanosine 5'-O-(3-thiotriphosphate) (GTP γ S) (0.001–31.6 μ M; $n = 5$) at a constant submaximal $[Ca^{2+}]_i$ of 0.1 μ M (A) and by phorbol 12,13-dibutyrate (PDBu) (0.001–3 μ M; $n=5$) at a constant submaximal $[Ca^{2+}]_i$ of 0.3 μ M (B) in the absence (control) and presence of 0.97 ± 0.13 mM halothane in β -escin-permeabilized tracheal smooth muscle strips. Values are normalized as a percentage of maximal force induced by 10 μ M free Ca^{2+} determined in the same tissues. Data are expressed as mean values \pm SD.

laxation of the force induced by 3 μ M acetylcholine and 10 μ M GTP at 0.3 μ M $[Ca^{2+}]_i$ (fig. 4).

Discussion

The major findings of this study were that (1) halothane attenuated the potentiation in Ca^{2+} sensitivity caused by acetylcholine plus GTP; (2) direct activation of the GTP-binding proteins with the nonhydrolyzable GTP analog GTP γ S and direct activation of PKC with

the phorbol ester PDBu increased Ca^{2+} sensitivity in a concentration-dependent manner; and (3) halothane had no effect on the increase in Ca^{2+} sensitivity induced by GTP γ S or PDBu.

Regulation of Ca^{2+} Sensitivity

In many types of smooth muscle, membrane receptor stimulation enhances Ca^{2+} sensitivity.^{2,5,12} In this CTSM preparation, we used acetylcholine plus GTP to submaximally activate m_3 muscarinic receptor subtypes mediating the contractile response.²⁹ Permeabilized smooth muscle preparations have been used as a tool to investigate the mechanisms regulating Ca^{2+} sensitivity.^{8,9,12,30} Compounds to create these preparations include staphylococcus α -toxin and the saponin ester β -escin,^{9,13,18,31} which produce pores in the plasma membrane. The advantage of these preparations is that $[Ca^{2+}]_i$ may be manipulated by changing the composition of the extracellular solution bathing the smooth muscle. In the present study, $[Ca^{2+}]_i$ was clamped by solutions buffered with EGTA, and as a further precaution, all solutions contained the calcium ionophore A23187, which depletes intracellular Ca^{2+} stores. As verified in permeabilized vascular smooth muscle by electron probe X-ray microanalysis,⁹ we previously demonstrated using fura-2 fluorescence measurements in this CTSM preparation that these experimental conditions eliminate intracellular Ca^{2+} gradients and maintain $[Ca^{2+}]_i$ constant during muscarinic receptor stimulation.⁵ Large cellular proteins necessary for contraction, such as calmodulin, myosin light chain kinase, regulatory myosin light chain, actin, and myosin are preserved, and coupling of membrane receptors to second messenger systems that enhance Ca^{2+} sensitivity is re-

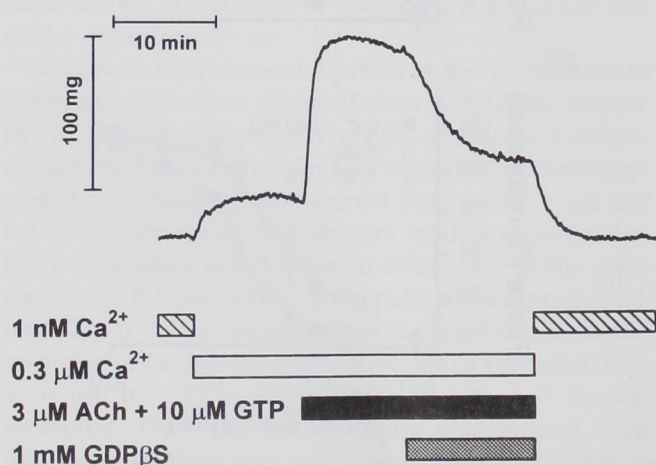


Fig. 4. Representative tracing of the effect of 1 mM guanosine 5'-O-(2-thiodiphosphate) (GDP β S) on acetylcholine-induced Ca^{2+} sensitization in β -escin-permeabilized canine tracheal smooth muscle. Muscarinic receptor stimulation with 3 μ M acetylcholine (EC_{50}) plus 10 μ M GTP at constant $[Ca^{2+}]_i$ (0.3 μ M) caused an increase in isometric force; that is, it enhanced Ca^{2+} sensitivity. The addition of 1 mM GDP β S caused 65% relaxation, expressed as a percentage relative to the force induced by 10 μ M free Ca^{2+} .

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tained and can be activated.^{5,8,9,12,18,22,30} For example, in preliminary studies we showed that adding exogenous calmodulin did not affect the contractile response in our CTSM preparation (unpublished observation).

Intracellular second messenger systems are thought to regulate agonist-induced increases in Ca^{2+} sensitivity,^{9,12,13,23} including those involving GTP-binding proteins¹⁰⁻¹⁷ and PKC.¹⁹⁻²² In our CTSM preparation, increases in Ca^{2+} sensitivity induced by muscarinic receptor stimulation require GTP, providing evidence for the involvement of GTP-binding proteins in agonist-induced Ca^{2+} sensitization (data not shown). Furthermore, $\text{GTP}\gamma\text{S}$, a nonhydrolyzable analog of GTP, mimics^{8,11,12,15,17} and $\text{GDP}\beta\text{S}$, which competitively prevents the binding of guanine nucleotides to GTP-binding proteins, inhibits⁹ agonist-induced Ca^{2+} sensitization in various smooth muscle types. We confirmed these findings in the present study, showing that $\text{GTP}\gamma\text{S}$ produced a concentration-dependent increase in Ca^{2+} sensitivity (fig. 3A). In the permeabilized CTSM preparation used, $\text{GDP}\beta\text{S}$ markedly reversed the acetylcholine-induced contraction at constant submaximal $[\text{Ca}^{2+}]_i$ (fig. 4). Preincubation with $\text{GDP}\beta\text{S}$ also inhibited force development induced by acetylcholine plus GTP. In addition, after extensive permeabilization with Triton X-100, the Ca^{2+} -sensitizing effect of receptor agonists, GTP and $\text{GTP}\gamma\text{S}$ is abolished^{10,30} (unpublished observation, data not shown).

Two different classes of GTP-binding proteins, heterotrimeric and small, monomeric GTP-binding proteins, are involved in receptor-mediated Ca^{2+} sensitization. Both the heterotrimeric^{10,16} and small molecular weight cytosolic GTP-binding proteins, such as rhoA p21^{10,11,32} or ras p21,¹⁷ are activated by $\text{GTP}\gamma\text{S}$. Heterotrimeric GTP-binding proteins induce Ca^{2+} sensitization in permeabilized smooth muscle preparations.^{10,16} Activated ras p21 GTP-binding proteins mimic the Ca^{2+} -sensitizing effect of $\text{GTP}\gamma\text{S}$ and GTP in guinea pig mesenteric microarteries¹⁷ and adenosine diphosphate ribosylation of the rho family proteins completely abolishes $\text{GTP}\gamma\text{S}$ and agonist-induced Ca^{2+} sensitization in permeabilized porcine aortic smooth muscle cells³² and guinea pig vas deferens.¹⁶ The current hypothesis suggests that a cascade of GTP-binding proteins may mediate agonist-induced Ca^{2+} sensitization, with membrane-bound receptors activating heterotrimeric GTP-binding proteins, which in turn activate small cytosolic GTP-binding proteins that affect downstream effector proteins such as protein phosphatases.

It has been suggested that Ca^{2+} sensitizing agonists

acting *via* GTP-binding proteins increase Ca^{2+} sensitivity in permeabilized smooth muscle preparations in part by activating PKC.^{13,20,21,33} After receptor binding, phospholipase C is activated by a GTP-dependent process, producing diacylglycerol, a physiologic activator of PKC, from phosphatidylinositol biphosphate and phosphatidylcholine.³⁴ Once activated, PKC may directly or indirectly inhibit the regulatory myosin light chain phosphatases,³⁵ increasing regulatory myosin light chain phosphorylation and force at a constant $[\text{Ca}^{2+}]_i$.⁸ This hypothesis is supported by the observation that phorbol esters such as PDBu that activate PKC³⁶ increase Ca^{2+} sensitivity in permeabilized smooth muscle preparations.^{16,19,21} We confirmed that PDBu increases Ca^{2+} sensitivity in a concentration-dependent manner. In preliminary studies (data not shown), we found that contractions induced by PDBu developed only in the presence of $[\text{Ca}^{2+}]_i$ of 0.1 μM or greater, suggesting that the majority of PKC isoforms involved in agonist-induced Ca^{2+} sensitization are calcium dependent. Further evidence for the involvement of PKC in agonist-induced Ca^{2+} sensitization has been provided in other smooth muscle types using PKC antagonists to reverse^{31,37} or inhibit³¹ increases in Ca^{2+} sensitivity.

Anesthetic Effects

Consistent with our previous findings, muscarinic receptor stimulation increases Ca^{2+} sensitivity in CTSM (fig. 1), and halothane significantly inhibits this agonist-induced potentiation in force at constant $[\text{Ca}^{2+}]_i$.^{2,5} Previously we showed that halothane does not affect free Ca^{2+} concentration-response curves in the absence of muscarinic receptor stimulation.⁵ During muscarinic receptor stimulation, halothane decreases Ca^{2+} sensitivity in CTSM in a concentration-dependent manner.⁵ In the current study, halothane had no effect on increases in Ca^{2+} sensitivity induced by direct activation of GTP-binding proteins with $\text{GTP}\gamma\text{S}$. This finding can be interpreted in the following ways:

First, because $\text{GTP}\gamma\text{S}$ activates both heterotrimeric and small, cytosolic GTP-binding proteins directly, it might not adequately mimic activation of the particular GTP-binding proteins involved in acetylcholine-induced Ca^{2+} sensitization in airway smooth muscle. For example, $\text{GTP}\gamma\text{S}$ may activate parallel sensitization pathways not stimulated by acetylcholine.

Second, if the final GTP-binding protein of the signal transduction cascade is activated by $\text{GTP}\gamma\text{S}$, any halothane effects on GTP-binding proteins upstream of this target GTP-binding protein may not be detected. For

example, halothane would not be expected to affect the response to GTP γ S if it acted to disrupt the coupling between heterotrimeric and small, cytosolic GTP-binding proteins.

Third, halothane could affect processes involving GTP hydrolysis during muscarinic receptor stimulation. Because we used GTP γ S, the nonhydrolyzable form of GTP, the results of any such effects would not be observed in our experiments. Therefore, we performed additional experiments investigating the effect of halothane on GTP-induced Ca²⁺ sensitization. Halothane (0.89 \pm 0.12 mM) had no effect at any GTP concentration studied (0.01–1 mM; experiments performed at a constant [Ca²⁺]_i of 0.3 μ M; n = 3), which is similar to results obtained using GTP γ S. This finding suggests that halothane's effect on Ca²⁺ sensitivity does not depend on GTP hydrolysis.

Finally, when combined with the findings that acetylcholine-induced Ca²⁺ sensitization can be attenuated by GDP β S and depends on GTP, the lack of halothane's effect on GTP γ S-induced Ca²⁺ sensitization implies that the site of its action may be the coupling between the muscarinic receptor and GTP-binding proteins. Other studies have also suggested that volatile anesthetics may interfere with GTP-binding protein function.^{38–40} Further investigations of this mechanism will require better characterization of GTP-dependent pathways regulating Ca²⁺ sensitivity.

In the present study, halothane did not affect PDBu-induced Ca²⁺ sensitization. Alterations in PKC activity have been suggested to be a mechanism for general anesthetic effects^{3,41–43} in several cell types. Yamakage³ found that halothane inhibits the enzyme translocation from the cytosol to the plasma membrane during muscarinic receptor stimulation. Because the membrane fraction is thought to be the active form of PKC, Yamakage suggested that inhibition of PKC activity might be responsible for the halothane-induced decrease in Ca²⁺ sensitivity. However, because halothane also decreased [Ca²⁺]_i and because many PKC isoforms are Ca²⁺ dependent, halothane's effect on PKC translocation may simply reflect its effect on [Ca²⁺]_i. We conclude that halothane attenuates Ca²⁺ sensitivity in permeabilized CTSM during muscarinic receptor stimulation by acting at a site of the signal transduction prior to any activation of PKC.

In summary, in β -escin-permeabilized CTSM strips, acetylcholine plus GTP, GTP γ S, and PDBu each increased Ca²⁺ sensitivity at constant submaximal [Ca²⁺]_i. Halothane significantly inhibited acetylcholine-induced

Ca²⁺ sensitization, whereas it had no effect on PDBu- or GTP γ S-induced Ca²⁺ sensitization. These results suggest that the site of halothane's action may be the coupling between the muscarinic membrane receptor and GTP-binding proteins, implicating a potentially important anesthetic mechanism in airway smooth muscle.

The authors thank Kathy Street and Robert Lorenz for technical assistance and Janet Beckman and Cathy Nelson for preparing the manuscript.

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