

Anesthetics Inhibit Acetylcholine-promoted Guanine Nucleotide Exchange of Heterotrimeric G Proteins of Airway Smooth Muscle

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Background: Anesthetics inhibit airway smooth muscle contraction in part by a direct effect on the smooth muscle cell. This study tested the hypothesis that the anesthetics halothane and hexanol, which both relax airway smooth muscle *in vitro*, inhibit acetylcholine-promoted nucleotide exchange at the α subunit of the $G_{q/11}$ heterotrimeric G protein ($G\alpha_{q/11}$; *i.e.*, they inhibit muscarinic receptor- $G\alpha_{q/11}$ coupling).

Methods: The effect of halothane (0.38 ± 0.02 mM) and hexanol (10 mM) on basal and acetylcholine-stimulated $G\alpha_{q/11}$ guanosine nucleotide exchange was determined in membranes prepared from porcine tracheal smooth muscle. The nonhydrolyzable, radioactive form of guanosine-5'-triphosphate, [3S]GTP γ S, was used as the reporter for $G\alpha_{q/11}$ subunit dissociation from the membrane to soluble fraction, which was immunoprecipitated with rabbit polyclonal anti- $G\alpha_{q/11}$ antiserum.

Results: Acetylcholine caused a significant time- and concentration-dependent increase in the magnitude of $G\alpha_{q/11}$ nucleotide exchange compared with basal values (*i.e.*, without acetylcholine), reaching a maximal difference at 100 μ M (35.9 ± 2.9 vs. 9.8 ± 1.2 fmol/mg protein, respectively). Whereas neither anesthetic had an effect on basal $G\alpha_{q/11}$ nucleotide exchange, both halothane and hexanol significantly inhibited the increase in $G\alpha_{q/11}$ nucleotide exchange produced by 30 μ M acetylcholine (by 59% and 68%, respectively).

Conclusions: Halothane and hexanol interact with the receptor-heterotrimeric G-protein complex in a manner that prevents acetylcholine-promoted exchange of guanosine-5'-triphosphate for guanosine-5'-diphosphate at $G\alpha_{q/11}$. These data are consistent with the ability of anesthetics to interfere with cellular processes mediated by heterotrimeric G proteins in many cells, including effects on muscarinic receptor-G-protein regulation of airway smooth muscle contraction.

ANESTHETICS interfere with numerous cellular processes, including some that are regulated by heterotrimeric guanosine-5'-triphosphate (GTP)-binding proteins (G proteins),^{1–5} such as airway smooth muscle (ASM) contraction.^{6–9} Although the mechanism by which anesthetics interfere with heterotrimeric G-protein-mediated cellular processes is not fully known, a preponderance of evidence suggests inhibition of signaling proteins activated by the α ($G\alpha$) subunit or $\beta\gamma$ ($G\beta\gamma$) dimer of the G protein, such as phospholipase C,¹⁰ protein kinase C,¹¹ and ion channels.¹² Evidence also suggests direct

anesthetic effects on the heterotrimeric G-protein-receptor complex (GPCR),^{4,5,13–15} including direct inhibition of nucleotide exchange at the nucleotide binding (catalytic) site of the $G\alpha$ subunit,¹⁶ although this finding is controversial.¹⁷ Anesthetics have also been shown to bind to membrane-delimited receptors,^{18,19} implying a salient inhibitory effect on the ability of receptor agonists to promote nucleotide exchange at the $G\alpha$ subunit (*i.e.*, receptor-heterotrimeric G-protein coupling), and not on the intrinsic activity of the G protein.¹⁶

Our previous work suggests that some anesthetics relax ASM in part by inhibiting muscarinic receptor-heterotrimeric G-protein-mediated regulation of actomyosin cross-bridge formation and contraction.^{6–9} We proposed that this effect could be due to a direct action on muscarinic receptor-heterotrimeric G-protein coupling.⁶ The purpose of the current study was to test the hypothesis that anesthetics inhibit muscarinic receptor-induced enhancement of guanosine nucleotide exchange at the $G\alpha$ subunit of heterotrimeric G proteins (*i.e.*, inhibit muscarinic receptor-heterotrimeric G-protein coupling). To test this hypothesis, we prepared crude membranes from porcine tracheal smooth muscle in which coupling between the muscarinic receptor and the heterotrimeric G proteins is preserved and nucleotide exchange at the $G\alpha$ subunit could be promoted by exogenous acetylcholine. We specifically examined whether the anesthetics halothane and hexanol, which both relax ASM,^{7,9} inhibit acetylcholine-promoted nucleotide exchange at the $G\alpha$ subunit of the $G_{q/11}$ isoform ($G\alpha_{q/11}$), because $G\alpha_{q/11}$ is present in ASM and plays a crucial role in the regulation of smooth muscle contraction.^{20,21}

Materials and Methods

Tissue Preparation

After the study was approved by the Institutional Animal Care and Use Committee (Mayo Foundation, Rochester, Minnesota), porcine tracheas were obtained from an abattoir or by euthanasia of research animals. In our experience, we have found no physiologic difference in tracheal smooth muscle obtained from these two sources (unpublished observations). For tissue obtained from research animals, euthanasia was accomplished by intramuscular injection of telazol (Ft. Dodge Animal Health, Inc., Ft. Dodge, IA) (10 ml/kg) and xylazine (6 mg/kg) and intravenous injection of 400–600 mg phenobarbital sodium, followed by exsanguination by bilat-

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eral transection of the carotid arteries. For studies using tissue obtained from both sources, the extrathoracic tracheas were excised and immersed in chilled physiologic salt solution with a composition 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 Mg_2SO_4 . After removal of fat, connective tissue, and epithelium, tracheal smooth muscle was cut into strips (1.5 cm long \times 0.25 cm wide), frozen in liquid nitrogen and stored at -70°C until its was used to prepare crude membranes for investigation.

Crude Membrane Preparation

A crude membrane fraction of ASM homogenate was prepared according to a modification of previously described methods.^{22,23} Approximately 350 mg frozen tissue, the amount obtained from a single animal, was ground to a fine powder in liquid nitrogen using a mortar and pestle. The dry powder was suspended for 15 min in ice-cold lysis buffer composed of 20 mM HEPES (pH 8.0), 1 mM EDTA, 0.1 mM phenylmethanesulfonyl fluoride, 10 $\mu\text{g}/\text{ml}$ leupeptin, and 2 $\mu\text{g}/\text{ml}$ aprotinin and then gently homogenized on ice with a Dounce tissue grinder (approximately 10–12 strokes). The homogenate was filtered through a 250- μm nylon filter (Small Parts, Inc., Miami Lakes, FL) and centrifuged at 87,000g for 30 min (4°C). The pellet was washed with lysis buffer and then resuspended by gentle vortex in assay buffer composed of 50 mM Tris-HCl (pH 7.4), 2 mM EDTA, 100 mM NaCl, 4.8 mM MgCl_2 , and 1 μM guanosine-5'-diphosphate (GDP), creating a crude membrane emulsion that was again filtered as described above. A portion of the crude membrane emulsion was solubilized in 6 ml NaOH, 0.1 N, and heated (30 min) for protein concentration determination by Lowry assay.²⁴ The homogenate was then diluted with assay buffer to a protein concentration of 2.5 mg/ml.

Nucleotide Exchange Measurement

This assay, graphically depicted in figure 1, is based on the fundamental principle that the exchange of GTP for GDP at the $G\alpha$ subunit of heterotrimeric G proteins causes the dissociation of this subunit from the membrane-delimited receptor and the $G\beta\gamma$ dimer into solution. In a system with preserved receptor-heterotrimeric G-protein coupling, the extent of this dissociation is promoted by the binding of an agonist to the receptor in the presence of GTP. In the current study, exogenous acetylcholine was used to stimulate muscarinic receptors, and a nonhydrolyzable, radioactive form of GTP, [^{35}S]GTP γS , was used as the reporter for the $G\alpha$ subunit dissociation. The native, soluble $G\alpha_{q/11}$ isotype was then immunoprecipitated from solution using rabbit polyclonal antiserum generated against native, recombinant rat brain $G\alpha_q$.

To perform the assay, $G\alpha_{q/11}$ nucleotide exchange was initiated by the addition of 29 nM (final concentration)

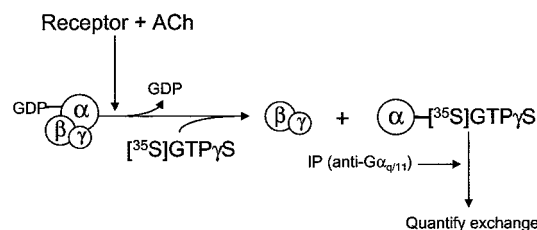


Fig. 1. Schematic diagram depicting the experimental technique for measuring receptor agonist-promoted nucleotide exchange at the α subunit of the $G_{q/11}$ heterotrimeric guanosine-5'-triphosphate (GTP)-binding protein ($G\alpha_{q/11}$). With this technique, the extent of exchange of the nonhydrolyzable, radioactive form of GTP, [^{35}S]GTP γS , for guanosine-5'-diphosphate (GDP) is determined in the absence or presence of receptor agonist. The binding of [^{35}S]GTP γS to $G\alpha_{q/11}$ causes the dissociation of $G\alpha_{q/11}$ from the membrane-associated receptor and the $\beta\gamma$ dimer into solution, where it can be immunoprecipitated with anti- $G\alpha_{q/11}$ antibody. The extent of $G\alpha_{q/11}$ dissociation is then quantified by measuring the amount of radioactivity (see text for detailed description of this technique). ACh = acetylcholine.

[^{35}S]GTP γS (specific activity 1.25 $\mu\text{Ci}/\text{pmol}$) to the crude membrane emulsion (containing 125 μg protein) at 30°C . Reactions were terminated with 600 μl ice-cold immunoprecipitation buffer of the following composition: 50 mM Tris-HCl (pH 7.5), 20 mM MgCl_2 , 150 mM NaCl, 2 $\mu\text{g}/\text{ml}$ aprotinin, 0.5% (volume/volume) IGEPAL CA-630, 100 μM GDP, and 100 μM GTP. All the reaction tubes were then briefly vortex mixed and gently rotated for 30 min (4°C). Finally, the samples were centrifuged at 12,500g for 15 min (4°C), and the supernatant containing soluble, native proteins was used for immunoprecipitation of [^{35}S]GTP γS -bound $G\alpha_{q/11}$ (fig. 1).

For immunoprecipitation, rabbit anti- $G\alpha_{q/11}$ antiserum was bound to protein A-agarose beads by incubating the beads in immunoprecipitation buffer (4°C) containing 1:200 (volume/volume) anti- $G\alpha_{q/11}$ antiserum for at least 2 h before the assay (antibody-coated beads). Unbound antibody was removed by washing the beads twice with immunoprecipitation buffer; the antibody-coated beads were stored at 4°C until used for experiments. The supernatant of the assay was first precleared with normal rabbit serum (1:100 dilution) and 30 μl antibody-free protein A-agarose beads for 60 min (4°C) to reduce subsequent nonspecific protein binding to immune complexes or the agarose matrix. The beads were then pelleted by centrifugation at 3,260g (10 min at 4°C), and the precleared samples were transferred into fresh tubes and incubated for 2 h (4°C) with 40 μl antibody-coated beads. The beads were then washed four times by repeated pelleting and centrifugation at 3,260g (10 min at 4°C) followed by resuspension in immunoprecipitation buffer (1 ml). Finally, the washed beads were placed in 4 ml Ultimate Gold scintillation cocktail (Packard Bioscience, Meriden, CT), and radioactivity was quantified using a Beckman model LS6000IC liquid scintillation counter (Beckman, Palo Alto, CA). Background radioactivity measurements were determined by performing

tandem experiments without protein and were less than 10% of the radioactivity of the basal $G\alpha_{q/11}$ nucleotide exchange measurements. The amount of radioactivity above background measurements was taken to indicate the amount of $G\alpha_{q/11}$ dissociated from the membrane into the soluble fraction due to the exchange of [^{35}S]GTP γ S for GDP. Values were normalized to the amount of protein present in the membrane emulsion before preclearance.

Preparation of Anesthetic Solutions

Stock solutions of assay buffer with saturating concentrations of halothane were prepared by mixing halothane in the assay buffer by stirring overnight in a ground-glass flask.¹⁶ These stocks were diluted with fresh assay buffer to achieve the desired concentration of halothane. Assay tubes were capped with Teflon-coated rubber stoppers (Alltech Associates, Inc., Deerfield, IL) immediately after the addition of halothane-containing solutions. Actual halothane concentrations in solution under assay conditions were measured by gas chromatography according to the method of Van Dyke and Wood.²⁵ Hexanol was added as appropriate directly to the assay buffer. We have verified in previous work using gas chromatography that this procedure provides concentrations of hexanol in aqueous solution expected on the basis of its density and molecular weight.⁷

Experimental Protocols

Effect of Exogenous Acetylcholine on $G\alpha_{q/11}$ Nucleotide Exchange. To determine the time course for the extent of exchange of [^{35}S]GTP γ S for GDP at $G\alpha_{q/11}$ (defined as $G\alpha_{q/11}$ [^{35}S]GTP γ S/GDP exchange), crude membrane samples were incubated without (basal exchange) or with (acetylcholine-promoted exchange) 100 μM acetylcholine. The reactions were terminated at 1, 3, 5, 10, and 20 min after initiating the assay reactions with [^{35}S]GTP γ S. To determine the effect of acetylcholine concentration on $G\alpha_{q/11}$ [^{35}S]GTP γ S/GDP exchange, measurements were obtained in separate experiments using crude membrane samples incubated without (basal exchange) or with 10, 30, 60, and 100 μM acetylcholine, and the reactions were terminated at 10 min after initiating the assay reactions.

Effect of Anesthetics on Acetylcholine-promoted $G\alpha_{q/11}$ Nucleotide Exchange. Assays were performed in the presence or absence of either halothane or 10 mM hexanol. The effects of these agents on $G\alpha_{q/11}$ [^{35}S]GTP γ S/GDP exchange were determined in separate experiments using samples incubated without (to assess effects on basal nucleotide exchange) or with 30 or 100 μM acetylcholine (to assess anesthetic effects on acetylcholine-promoted nucleotide exchange) present in the assay buffer. All reactions were terminated at 10 min after initiating the assay reactions. Hexanol, 10 mM, produces maximal functional effects on ASM and was chosen so that the current results

could be compared to the results of our previous work.⁹ The aqueous halothane concentration was 0.38 ± 0.02 mM, which did not vary significantly over the duration of an experiment (preliminary data not shown) and is a concentration within the range previously shown to inhibit ASM contraction.^{12,26,27} Each condition was assayed in triplicate.

Materials

[^{35}S]GTP γ S (specific activity 1 $\mu\text{Ci}/\mu\text{l}$) was purchased from Amersham Biosciences (Piscataway, NJ). Rabbit polyclonal antiserum generated against recombinant rat brain $G\alpha_q$ protein and rabbit normal serum were purchased from Calbiochem (EMD Biosciences, Inc. Affiliate, San Diego, CA). Protein A-agarose beads were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The Lowry protein assay kits were purchased from Bio-Rad (Hercules, CA). All other chemicals were purchased from Sigma Chemical Company (St. Louis, MO).

Statistical Analysis. Data are reported as mean \pm SD. For time-course studies, a two-way repeated-measures analysis of variance was used to test the effect of time and acetylcholine on $G\alpha_{q/11}$ nucleotide exchange. For the determination of the effects of acetylcholine, halothane, and hexanol on $G\alpha_{q/11}$ nucleotide exchange, one- or two-way repeated-measures analysis of variance was used as appropriate. *Post hoc* testing was performed using the Student-Newman-Keuls test. A value of $P < 0.05$ indicated statistical significance.

Results

Effect of Exogenous Acetylcholine on $G\alpha_{q/11}$ Nucleotide Exchange

In the absence of acetylcholine (basal nucleotide exchange), there was a time-dependent increase in $G\alpha_{q/11}$ [^{35}S]GTP γ S/GDP exchange (fig. 2). At all time points, there was significantly more $G\alpha_{q/11}$ [^{35}S]GTP γ S/GDP exchange in samples treated with 100 μM acetylcholine than in those without acetylcholine, with a maximal difference reached at 20 min (35.9 ± 2.9 vs. 9.8 ± 1.2 fmol/mg protein, respectively). The increase in $G\alpha_{q/11}$ [^{35}S]GTP γ S/GDP exchange was concentration dependent compared with basal values (fig. 3).

Effect of Anesthetics on Acetylcholine-promoted $G\alpha_{q/11}$ Nucleotide Exchange

Halothane significantly inhibited the increase in $G\alpha_{q/11}$ [^{35}S]GTP γ S/GDP exchange (assayed at 10 min) induced by 30 and 100 μM acetylcholine (14.2 ± 1.7 vs. 22.0 ± 2.4 and 22.1 ± 2.5 vs. 30.8 ± 4.2 fmol/mg protein, respectively) (fig. 4), *i.e.*, by 59% and 39%, respectively, when compared to basal values (fig. 4). Likewise, hexanol significantly inhibited the increase in $G\alpha_{q/11}$ [^{35}S]GTP γ S/GDP exchange induced by 30 μM acetylcho-

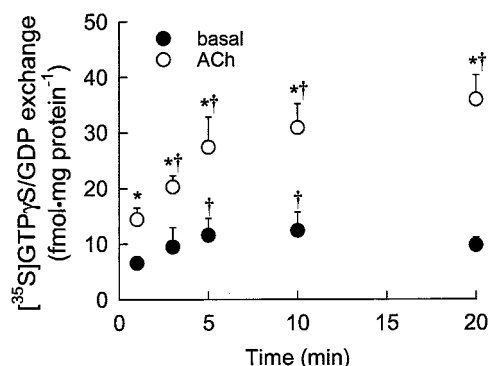


Fig. 2. Time-dependent change in the extent of exchange of the nonhydrolyzable, radioactive form of guanosine-5'-triphosphate (GTP), [³⁵S]GTPγS, for guanosine-5'-diphosphate (GDP) ([³⁵S]GTPγS/GDP exchange) at the α subunit of the G_{q/11} heterotrimeric GTP-binding protein (Gα_{q/11}). Gα_{q/11} [³⁵S]GTPγS/GDP exchange was measured in the absence (basal exchange) or presence (acetylcholine [ACh]-promoted exchange, 100 μM) of exogenous acetylcholine. Gα_{q/11} [³⁵S]GTPγS/GDP exchange values are presented as mean ± SD (n = 9). * Significant difference from the initial (1 min) value; † significant difference from basal values.

line from 27.3 ± 2.1 fmol/mg protein to 19.8 ± 1.9 fmol/mg protein, a 68% inhibition when compared to basal values (fig. 5). A similar tendency was observed during stimulation with 100 μM acetylcholine but did not reach statistical significance. Neither halothane nor hexanol had an effect on basal Gα_{q/11} [³⁵S]GTPγS/GDP exchange (*i.e.*, in the absence of acetylcholine) (figs. 4 and 5).

Discussion

The major finding of this study is that both halothane and hexanol, anesthetics that inhibit ASM contraction,^{7,9,12,28} inhibit acetylcholine-promoted Gα_{q/11} [³⁵S]GTPγS/GDP exchange without affecting basal, intrinsic nucleotide exchange in the absence of receptor

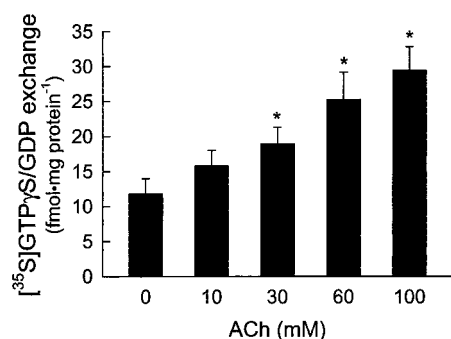


Fig. 3. Concentration-dependent effect of exogenous acetylcholine (ACh; 10, 30, 60, and 100 μM) on the extent of exchange of the nonhydrolyzable, radioactive form of guanosine-5'-triphosphate (GTP), [³⁵S]GTPγS, for guanosine-5'-diphosphate (GDP) ([³⁵S]GTPγS/GDP exchange) at the α subunit of the G_{q/11} heterotrimeric GTP-binding protein (Gα_{q/11}). Gα_{q/11} [³⁵S]GTPγS/GDP exchange values are presented as mean ± SD (n = 3). * Significant difference from the basal value.

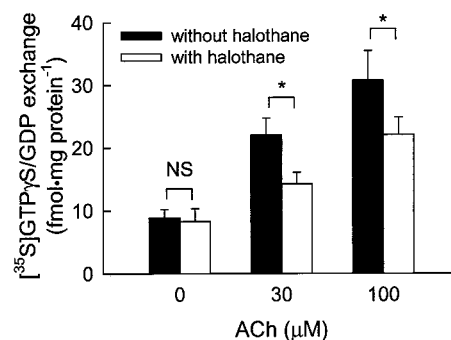


Fig. 4. Effect of halothane (0.38 ± 0.02 mM) on the extent of exchange of the nonhydrolyzable, radioactive form of guanosine-5'-triphosphate (GTP), [³⁵S]GTPγS, for guanosine-5'-diphosphate (GDP) ([³⁵S]GTPγS/GDP exchange) at the α subunit of the G_{q/11} heterotrimeric guanosine-5'-triphosphate (GTP)-binding protein (Gα_{q/11}). Gα_{q/11} [³⁵S]GTPγS/GDP exchange was measured in the absence (basal exchange) or presence (acetylcholine [ACh]-promoted exchange) of 30 or 100 μM acetylcholine. Gα_{q/11} [³⁵S]GTPγS/GDP exchange values are presented as mean ± SD (n = 5). * Significant difference between the bracketed columns. NS = not significant.

stimulation. These data demonstrate in tissue and through direct biochemical assessment that some anesthetics inhibit receptor-heterotrimeric G-protein coupling. This mechanism could be responsible at least in part for the observed effects of anesthetics on numerous receptor-heterotrimeric G-protein-mediated cellular processes, including ASM relaxation.

Muscarinic receptors activate one or more subfamilies of heterotrimeric G proteins, which are comprised of α, β, and γ subunits. The Gα subunit contains a binding site for GTP and GDP and possesses GTPase (catalytic) activity. The Gβγ subunits are tightly associated and anchor the Gα subunit to the cytoplasmic surface of the cell membrane. In its resting state, the G protein exists as an inactive Gαβγ trimer, with GDP occupying the nucleotide binding site of the Gα subunit. The binding of an

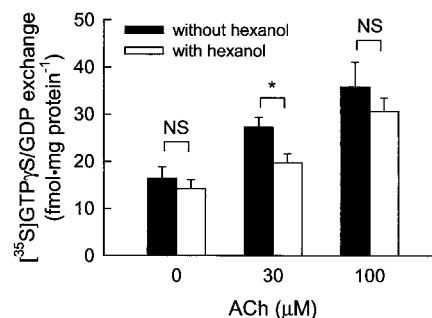


Fig. 5. Effect of hexanol (10 mM) on the extent of exchange of the nonhydrolyzable, radioactive form of guanosine-5'-triphosphate (GTP), [³⁵S]GTPγS, for guanosine-5'-diphosphate (GDP) ([³⁵S]GTPγS/GDP exchange) at the α subunit of the G_{q/11} heterotrimeric GTP-binding protein (Gα_{q/11}). Gα_{q/11} [³⁵S]GTPγS/GDP exchange was measured in the absence (basal exchange) or presence (acetylcholine [ACh]-promoted exchange) of 30 or 100 μM acetylcholine. Gα_{q/11} [³⁵S]GTPγS/GDP values are presented as mean ± SD (n = 5). * Significant difference between the bracketed columns. NS = not significant.

agonist to the receptor promotes GDP release and subsequent GTP (or [^{35}S]GTP γS) binding to the α subunit (*i.e.*, nucleotide exchange). This exchange of nucleotides triggers dissociation of the $G\alpha\beta\gamma$ complex from the receptor and separation of the $G\alpha$ subunit from the $G\beta\gamma$ dimer. The hydrolysis of bound GTP by the intrinsic GTPase activity of the $G\alpha$ subunit permits reassociation of the subunits into a heterotrimer and terminates the activation of the effector. In ASM, this process increases both intracellular calcium concentration and the sensitivity of the contractile machinery to a given concentration of intracellular calcium (*i.e.*, calcium sensitivity), both of which induce smooth muscle contraction.²⁹

The heterotrimeric G proteins are classified according to the identity of the $G\alpha$ subunit into four major subfamilies,³⁰ and within each subfamily, there are several isoforms. For example, the $G\alpha_q$ subfamily consists of at least five distinct proteins, including $G\alpha_q$ itself, $G\alpha_{q11}$, $G\alpha_{q14}$, and others. The role of different heterotrimer G-protein subfamilies in mediating ASM contraction is not completely delineated, but $G\alpha_q$ subfamily proteins, such as G_q and G_{11} , transduce receptor-mediated increases in both intracellular calcium concentration and calcium sensitivity in ASM.²⁰

There is considerable evidence that some anesthetics, including halothane and hexanol, can inhibit cellular processes activated by heterotrimeric G-protein-coupled receptors in ASM, producing the functional effect of ASM relaxation.^{8,9,12,26,27,31-34} In a series of experiments in differentiated ASM tissue using pharmacologic probes, we suggested that this effect could be localized, at least in part, to the GPRC.^{9,27} However, these measurements of cellular function (*e.g.*, intracellular calcium concentration and isometric force) did not permit direct assessment of anesthetic effects on the GPRC, because effects on more distal elements of the signaling pathways could not be excluded (*e.g.*, effects on calcium channels). A similar limitation applies to previous work inferring anesthetic effects on the GPRC from measurements of downstream effector function in other intact tissues or expression systems, such as *Xenopus* oocytes.^{5,13-15,35} Finally, studies of ligand binding to muscarinic receptors derived from various tissues provide indirect evidence of anesthetic effects on the GPRC, because receptor-heterotrimeric G-protein interactions determine ligand-receptor affinity.^{1,2} However, this technique is indirect, which can lead to misinterpretation and erroneous conclusions.

Assessment of nucleotide exchange at the $G\alpha$ subunit in cellular membrane preparations from specific tissues provides a direct measure of receptor-heterotrimeric G-protein coupling. Using the technique described in the current study, the exchange of GDP for GTP at a particular $G\alpha$ protein can be measured, with subfamily specificity determined by the epitope to which the antibody is raised for the immunoprecipitation step. Most

previous studies using these techniques have been conducted using experimental systems in which receptors and heterotrimeric G proteins are overexpressed in mammalian or insect cells^{22,23,36}; one report using freshly dissociated gut smooth muscle cells has been published.³⁷ The current study is the first to demonstrate the use of this technique to measure agonist-promoted nucleotide exchange using crude membrane prepared from smooth muscle tissue.

In addition to the specificity of the antibody used for immunoprecipitation, the ability of the experimental techniques used in the current study to detect agonist-promoted heterotrimeric G-protein nucleotide exchange in a crude membrane preparation is limited by several factors. These include the magnitude of the intrinsic, basal nucleotide exchange, the amount of the endogenous $G\alpha$ subunit isotype of interest expressed in the tissue, and the extent to which the $G\alpha$ subunit isotype of interest is coupled to and dissociates from the membrane receptor and $G\beta\gamma$ dimer with agonist binding. In the case of $G\alpha_{q11}$, the antibody used is highly specific for both the $G\alpha_q$ and $G\alpha_{11}$ subunits,³⁶ a finding confirmed by our preliminary data showing no cross-reactivity with $G\alpha_i$ or $G\alpha_s$ subfamily proteins (data not shown). The kinetics of basal, intrinsic nucleotide exchange for the $G\alpha_{q11}$ using either recombinant, pure protein^{38,39} or crude membrane prepared from mammalian cells in which the receptor and the heterotrimer G-protein subunits have been enriched³⁶ is low. Although this was also true in the current study, the basal $G\alpha_{q11}$ nucleotide exchange was still sufficient to conduct a reliable assessment of a possible anesthetic effect, because the background radioactivity was only 10% or less of the radioactivity of this measurement. The low level of $G\alpha_{q11}$ nucleotide exchange observed in the absence of exogenous acetylcholine also optimized our ability to examine anesthetic effects on receptor-G-protein coupling, because the relative difference in the magnitude of the acetylcholine-promoted *versus* basal $G\alpha_{q11}$ nucleotide exchange was approximately fourfold to fivefold (fig. 3). The magnitude of this difference is similar to that reported in previous work.^{36,38,39}

The observation that acetylcholine significantly increased $G\alpha_{q11}$ nucleotide exchange demonstrated functional coupling between the muscarinic receptor and $G\alpha_{q11}$ in porcine ASM. The time course for agonist-promoted nucleotide exchange measured in the current study was similar to that reported by others using a similar crude membrane preparation.³⁷ However, the rate of acetylcholine-promoted nucleotide exchange was slower than that anticipated based on kinetic measurements of other heterotrimeric G-protein-mediated signals obtained in intact, undisturbed cells or tissue, such as free calcium concentration. The explanation for this difference is most likely the loss of soluble, heterotrimeric G-protein regulatory proteins, known as GTPase-

activating proteins, caused by cell membrane disruption in our preparation. These proteins, which are present in intact smooth muscle cells,⁴⁰ accelerate G-protein nucleotide exchange by approximately 200-fold and thus confer the speed needed for reliable intracellular signaling.⁴¹⁻⁴³ Nevertheless, the crude membrane preparation is a well-established model that provides direct, unambiguous measurement of membrane receptor coupling to heterotrimeric G proteins and thus is appropriate for examining the hypothesis proposed in the current study.

In the absence of receptor stimulation, neither halothane nor hexanol had an effect on basal, intrinsic $G_{\alpha_{q/11}}$ [³⁵S]GTP γ S/GDP exchange (figs. 4 and 5). By contrast, Pentylala *et al.*¹⁶ found that halothane and other volatile anesthetics modulated the binding of guanine nucleotides to recombinant $G\alpha$ subunits in aqueous solution, thereby inhibiting the exchange of GDP for GTP γ S. They did not study $G\alpha_q$ subfamily proteins because nucleotide exchange is not detectable in these purified subunits, unlike in membrane preparations as demonstrated by the current and previous studies.^{44,45} However, for reasons that we have not been able to elucidate, we have not been able to duplicate their findings on intrinsic, basal nucleotide exchange using either purified, recombinant $G\alpha_i$ protein or endogenous $G\alpha_i$ in a porcine ASM membrane preparation.¹⁷

In contrast to basal measurements, both halothane and hexanol significantly inhibited acetylcholine-promoted [³⁵S]GTP γ S/GDP exchange in concentrations that produce anesthesia *in vivo* and ASM relaxation *in vitro*.^{7,32,46} This effect is consistent with functional studies of heterotrimeric G-protein-mediated processes in cells³ and expression systems.^{1,5,47} In studies of ASM, we found in previous work that hexanol and halothane inhibited activation of downstream effectors induced by stimulation of muscarinic receptors with acetylcholine and by direct stimulation of heterotrimeric G proteins using tetrafluoroaluminate.^{9,27} Tetrafluoroaluminate directly activates heterotrimeric G proteins by binding to the α subunit next to the β phosphate of GDP and mimicking the γ phosphate of GTP.⁴⁸ Because the magnitude of these effects was similar for both methods of activation, we interpreted these findings as demonstrating a direct interaction of anesthetics with the heterotrimer, acting to stabilize the heterotrimer and inhibit its dissociation. However, these interpretations must now be revised based on the current finding that the anesthetics did not affect intrinsic, basal $G_{\alpha_{q/11}}$ nucleotide exchange. We now propose that some anesthetics stabilize the entire GPCR, rather than just the heterotrimeric G protein, thereby attenuating muscarinic receptor coupling to the heterotrimeric G protein $G_{q/11}$.

The experimental techniques used in the current study can provide only a functional assessment of the interaction between the membrane delimited muscarinic receptor and the associated heterotrimeric G protein $G_{q/11}$.

They cannot ascertain with which of the possible protein targets, either the muscarinic receptor or the $G_{q/11}$ heterotrimer subunits, the anesthetic molecules interacted to produce the observed effects. For example, it is possible that $G_{\alpha_{q/11}}$ possesses an anesthetic binding region that is at its receptor binding domain, which could interfere with receptor coupling but have no effect on basal $G_{\alpha_{q/11}}$ nucleotide exchange. Another plausible interpretation of our data is that the anesthetic molecules interacted directly with the muscarinic receptor only, as previously demonstrated for the rhodopsin receptor,^{18,19} thereby only interfering with the ability of acetylcholine to activate $G_{\alpha_{q/11}}$ nucleotide exchange. The techniques used in this study also cannot distinguish between binding of anesthetic molecules directly to a protein or at a hydrophobic binding site created at the interface between one of the possible protein targets and the lipid membrane. Determining the target sites (*e.g.*, protein, protein-protein interface, or lipid-protein interface) would involve the use of biophysical methods that directly assess binding interactions between the anesthetic and these possible targets. Nevertheless, regardless of the biophysical mechanisms, the current work provides compelling evidence for an anesthetic effect on muscarinic receptor-heterotrimeric G-protein coupling. The experiments in the current study in isolation do not exclude actions on other components of the heterotrimeric G-protein-mediated signaling pathway *in situ*, but our previous work suggests that such effects are not present, at least in ASM.³¹

In summary, to our knowledge, these are the first data to demonstrate, using a direct measurement technique, that anesthetics inhibit receptor-promoted nucleotide exchange at the $G\alpha$ subunit of heterotrimeric G proteins. The preponderance of previous work indicates that anesthetics bind to membrane-delimited receptors but not G proteins and have no effect on basal, intrinsic nucleotide exchange at $G\alpha$ in the absence of receptor activation. Taken together with these previous observations, our findings suggest that halothane and hexanol interact either with the receptor itself or at the interface between receptor and G protein. Accordingly, these interactions prevent the ability of acetylcholine to promote $G_{\alpha_{q/11}}$ nucleotide exchange and its subsequent dissociation from the muscarinic receptor and the $G\beta\gamma$ dimer. These data are consistent with the ability of anesthetics to interfere with cellular processes mediated by heterotrimeric G proteins,¹⁻⁵ including our previous observations of anesthetic effects on muscarinic receptor-G-protein regulation of ASM contraction. These findings also have implications in other organ systems, including the central nervous system, given the ubiquitous nature of heterotrimeric G proteins in mediating signaling pathways and cell function.

References

1. Anthony BL, Dennison RL, Aronstam RS: Disruption of muscarinic receptor-G protein coupling is a general property of liquid volatile anesthetics. *Neurosci Lett* 1989; 99:191-6
2. Dennison RL, Jr., Anthony BL, Narayanan TK, Aronstam RS: Effects of halothane on high affinity agonist binding and guanine nucleotide sensitivity of muscarinic acetylcholine receptors from brainstem of rat. *Neuropharmacology* 1987; 26:1201-5
3. Magyar J, Szabo G: Effects of volatile anesthetics on the G protein-regulated muscarinic potassium channel. *Mol Pharmacol* 1996; 50:1520-8
4. Nietgen GW, Honemann CW, Chan CK, Kamatchi GL, Durieux ME: Volatile anaesthetics have differential effects on recombinant m1 and m3 muscarinic acetylcholine receptor function. *Br J Anaesth* 1998; 81:569-77
5. Durieux ME: Halothane inhibits signaling through m1 muscarinic receptors expressed in *Xenopus* oocytes. *ANESTHESIOLOGY* 1995; 82:174-82
6. Kai T, Takahashi S, Kanaide H: Halothane counteracts acetylcholine-induced increase in Ca²⁺ sensitivity of the contractile apparatus in airway smooth muscle. *Eur J Pharmacol* 1996; 315:313-8
7. Sakihara C, Jones KA, Lorenz RR, Perkins WJ, Warner DO: Effects of primary alcohols on airway smooth muscle. *ANESTHESIOLOGY* 2002; 96:428-37
8. Hanazaki M, Jones KA, Perkins WJ, Warner DO: Halothane increases smooth muscle protein phosphatase in airway smooth muscle. *ANESTHESIOLOGY* 2001; 94:129-36
9. Yoshimura H, Jones KA, Perkins WJ, Warner DO: Dual effects of hexanol and halothane on the regulation of calcium sensitivity in airway smooth muscle. *ANESTHESIOLOGY* 2003; 98:871-80
10. Pabelick CM, Prakash YS, Kannan MS, Warner DO, Sieck GC: Effects of halothane on sarcoplasmic reticulum calcium release channels in porcine airway smooth muscle cells. *ANESTHESIOLOGY* 2001; 95:207-15
11. Kamatchi GL, Durieux ME, Lynch C III: Differential sensitivity of expressed L-type calcium channels and muscarinic M(1) receptors to volatile anesthetics in *Xenopus* oocytes. *J Pharmacol Exp Ther* 2001; 297:981-90
12. Yamakage M: Direct inhibitory mechanisms of halothane on canine tracheal smooth muscle contraction. *ANESTHESIOLOGY* 1992; 77:546-53
13. Bohm M, Schmidt U, Gierschik P, Schwinger RH, Bohm S, Erdmann E: Sensitization of adenylate cyclase by halothane in human myocardium and S49 lymphoma wild-type and cyc- cells: Evidence for inactivation of the inhibitory G protein Gi alpha. *Mol Pharmacol* 1994; 45:380-9
14. Schotten U, Schumacher C, Sigmund M, Karlein C, Rose H, Kammermeier H, Sivarajan M, Hanrath P: Halothane, but not isoflurane, impairs the beta-adrenergic responsiveness in rat myocardium. *ANESTHESIOLOGY* 1998; 88:1330-9
15. Schmidt U, Schwinger RH, Bohm M: Interaction of halothane with inhibitory G-proteins in the human myocardium. *ANESTHESIOLOGY* 1995; 83:353-60
16. Pentylala SN, Sung K, Chowdhury A, Rebecchi MJ: Volatile anesthetics modulate the binding of guanine nucleotides to the alpha subunits of heterotrimeric GTP binding proteins. *Eur J Pharmacol* 1999; 384:213-22
17. Streiff J, Jones K, Perkins WJ, Warner DO, Jones KA: Effect of halothane on the guanosine 5' triphosphate binding activity of G-protein α subunits. *ANESTHESIOLOGY* 2003; 99:105-11
18. Ishizawa Y, Sharp R, Liebman PA, Eckenhoff RG: Halothane binding to a G protein coupled receptor in retinal membranes by photoaffinity labeling. *Biochemistry* 2000; 39:8497-502
19. Ishizawa Y, Pidikiti R, Liebman PA, Eckenhoff RG: G protein-coupled receptors as direct targets of inhaled anesthetics. *Mol Pharmacol* 2002; 61:945-52
20. Croxton TL, Lande B, Hirshman CA: Role of G proteins in agonist-induced Ca²⁺ sensitization of tracheal smooth muscle. *Am J Physiol* 1998; 275:L748-55
21. Cuq P, Zumbihl R, Fischer T, Rouot B, Bali JP, Magous R: Involvement of G alpha q/11 in the contractile signal transduction pathway of muscarinic M3 receptors in caecal smooth muscle. *Eur J Pharmacol* 1996; 315:213-9
22. Barr AJ, Manning DR: Agonist-promoted [³⁵S]GTPγS-binding as a problem of receptor-G protein communication in reconstituted Sf9 cells, *G Proteins: Techniques of Analysis*. Edited by Manning DR. Boca Raton, CRC Press, 1999, pp 227-46
23. Chen CA, Manning DR: Immunological techniques of G protein analysis, *G Proteins: Techniques of Analysis*. Edited by Manning DR. Boca Raton, CRC Press, 1999, pp 99-121
24. Lowry AH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin reagent. *J Biol Chem* 1951; 193:265-75
25. Van Dyke RA, Wood CL: Binding of radioactivity from 14 C-labeled halothane in isolated perfused rat livers. *ANESTHESIOLOGY* 1973; 38:328-32
26. Jones KA, Wong GY, Lorenz RR, Warner DO, Sieck GC: Effects of halothane on the relationship between cytosolic calcium and force in airway smooth muscle. *Am J Physiol* 1994; 266:L199-204
27. Kai T, Jones KA, Warner DO: Halothane attenuates calcium sensitization in airway smooth muscle by inhibiting G-proteins. *ANESTHESIOLOGY* 1998; 89:1543-52
28. Shah MV, Hirshman CA: Mode of action of halothane on histamine-induced airway constriction in dogs with reactive airways. *ANESTHESIOLOGY* 1986; 65:170-4
29. Somlyo AP, Somlyo AV: Signal transduction by G-proteins, rho-kinase and protein phosphatase to smooth muscle and non-muscle myosin II. *J Physiol* 2000; 522(pt 2):177-85
30. Ali N, Agrawal DK: Guanine nucleotide binding regulatory proteins: Their characteristics and identification. *J Pharmacol Toxicol Methods* 1994; 32:187-96
31. Bremerich DH, Hirasaki A, Jones KA, Warner DO: Halothane attenuation of calcium sensitivity in airway smooth muscle: Mechanisms of action during muscarinic receptor stimulation. *ANESTHESIOLOGY* 1997; 87:94-101
32. Jones KA, Housmans PR, Warner DO, Lorenz RR, Rehder K: Halothane alters cytosolic calcium transient in tracheal smooth muscle. *Am J Physiol* 1993; 265:L80-6
33. Jones KA, Hirasaki A, Bremerich DH, Jankowski C, Warner DO: Halothane inhibits agonist-induced potentiation of rMLC phosphorylation in permeabilized airway smooth muscle. *Am J Physiol* 1997; 273:L80-5
34. Akao M, Hirasaki A, Jones KA, Wong GY, Bremerich DH, Warner DO: Halothane reduces myofilament Ca²⁺ sensitivity during muscarinic receptor stimulation of airway smooth muscle. *Am J Physiol* 1996; 271:L719-25
35. Nietgen GW, Honemann CW, Durieux ME: Influence of anesthetics on endogenous and recombinantly expressed G protein-coupled receptors in the *Xenopus* oocyte. *Toxicol Lett* 1998; 100-101:319-27
36. Akam EC, Challiss RA, Nahorski SR: G(q/11) and G(i/o) activation profiles in CHO cells expressing human muscarinic acetylcholine receptors: Dependence on agonist as well as receptor-subtype. *Br J Pharmacol* 2001; 132:950-8
37. Cao WB, Harnett KM, Chen Q, Jain MK, Behar J, Biancani P: Group I secreted PLA2 and arachidonic acid metabolites in the maintenance of cat LES tone. *Am J Physiol* 1999; 277:G585-98
38. Hepler JR, Kozasa T, Gilman AG: Purification of recombinant Gq alpha, G11 alpha, and G16 alpha from Sf9 cells. *Methods Enzymol* 1994; 237:191-212
39. Kozasa T: Purification of recombinant G protein α and $\beta\gamma$ subunits from Sf9 cells, *G Proteins: Techniques of Analysis*. Edited by Manning DR. Boca Raton, CRC Press, 1999, pp 23-38
40. Cho H, Harrison K, Schwartz O, Kehrl JH: The aorta and heart differentially express RGS (regulators of G-protein signalling) proteins that selectively regulate sphingosine 1-phosphate, angiotensin II and endothelin-1 signalling. *Biochem J* 2003; 371:973-80
41. Doupnik CA, Davidson N, Lester HA, Kofuji P: RGS proteins reconstitute the rapid gating kinetics of Gbetagamma-activated inwardly rectifying K⁺ channels. *Proc Natl Acad Sci U S A* 1997; 94:10461-6
42. He W, Cowan CW, Wensel TG: RGS9, a GTPase accelerator for phototransduction. *Neuron* 1998; 20:95-102
43. Saitoh O, Kubo Y, Miyatani Y, Asano T, Nakata H: RGS8 accelerates G-protein-mediated modulation of K⁺ currents. *Nature* 1997; 390:525-9
44. Selkirk JV, Price GW, Nahorski SR, Challiss RA: Cell type-specific differences in the coupling of recombinant mGlu1alpha receptors to endogenous G protein sub-populations. *Neuropharmacology* 2001; 40:645-56
45. Barr AJ, Brass LF, Manning DR: Reconstitution of receptors and GTP-binding regulatory proteins (G proteins) in Sf9 cells: A direct evaluation of selectivity in receptor G protein coupling. *J Biol Chem* 1997; 272:2223-9
46. Yamamoto K, Morimoto N, Warner DO, Rehder K, Jones KA: Factors influencing the direct actions of volatile anesthetics on airway smooth muscle. *ANESTHESIOLOGY* 1993; 78:1102-11
47. Kamatchi GL, Tiwari SN, Durieux ME, Lynch C III: Effects of volatile anesthetics on the direct and indirect protein kinase C-mediated enhancement of alpha1E-type Ca(2+) current in *Xenopus* oocytes. *J Pharmacol Exp Ther* 2000; 293:360-9
48. Bigay J, Deterre P, Pfister C, Chabre M: Fluoride complexes of aluminium or beryllium act on G-proteins as reversibly bound analogues of the gamma phosphate of GTP. *Embo J* 1987; 6:2907-13