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Differential Effects of Volatile Anesthetics on M_3 Muscarinic Receptor Coupling to the $G\alpha_q$ Heterotrimeric G Protein

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Background: **Halothane inhibits airway smooth muscle contraction in part by inhibiting the functional coupling between muscarinic receptors and one of its cognate heterotrimeric G proteins, $G\alpha_q$. Based on previous studies indicating a more potent effect of halothane and sevoflurane on airway smooth muscle contraction compared with isoflurane, the current study hypothesized that at anesthetic concentrations of 2 minimum alveolar concentration (MAC) or less, halothane and sevoflurane but not isoflurane inhibit acetylcholine-promoted $G\alpha_q$ guanosine nucleotide exchange.**

Methods: **$G\alpha_q$ guanosine nucleotide exchange was measured in crude membranes prepared from COS-7 cells transiently coexpressing the human M_3 muscarinic receptor and human $G\alpha_q$. A radioactive, nonhydrolyzable analog of guanosine-5′-triphosphate, [^{35}S]GTPγS, was used as a reporter for nucleotide exchange at $G\alpha_q$.**

Results: **Acetylcholine caused a concentration-dependent increase in $G\alpha_q$ [^{35}S]GTPγS–GDP exchange. Neither anesthetic affected constitutive $G\alpha_q$ [^{35}S]GTPγS–GDP exchange in the absence of acetylcholine. Conversely, each anesthetic caused a concentration-dependent and reversible inhibition of $G\alpha_q$ [^{35}S]GTPγS–GDP exchange when promoted by acetylcholine. At concentrations of 3 MAC or less, the effect of halothane and sevoflurane were significantly greater than that of isoflurane, with only a minimal inhibition by isoflurane observed at 2 MAC.**

Conclusion: **The differential effects of volatile anesthetics on acetylcholine-promoted guanosine nucleotide exchange at $G\alpha_q$ are consistent with the apparent more potent direct effect of halothane and sevoflurane compared with isoflurane on muscarinic receptor–mediated contraction of isolated airway smooth muscle. These differential effects also suggest a mode of anesthetic action that could be due to anesthetic–protein interactions and not simply anesthetic accumulation in the lipid membrane.**

VOLATILE anesthetics are effective and safe therapeutic agents for acute perioperative bronchospasm in patients with hyperreactive airway diseases, such as asthma.[1–4] For example, even after intensive therapy that included inhalation of β_2-adrenergic receptor agonists, tracheal intubation, and mechanical ventilation had failed, inhalation of halothane reversed persistent bronchospasm and markedly improved arterial blood gases in a series of 12 patients with status asthmaticus.[5] The mechanism of this beneficial effect is multifactorial but is known to include a direct inhibitory effect on the airway smooth muscle (ASM) cell.[6,7] This direct effect is due in part to inhibition of the increase in cytoplasmic calcium (Ca^{2+}) concentration ($[Ca^{2+}]_c$) and the signaling proteins that regulate the amount of force at a given $[Ca^{2+}]_c$ (*i.e.*, Ca^{2+} sensitivity) induced by physiologic agonists, including acetylcholine[6–12] and endothelin 1.[13]

Contractile agonists induce ASM contraction by activating heterotrimeric guanosine 5′-triphosphate (GTP) binding protein (G protein)–dependent mechanisms that increase both $[Ca^{2+}]_c$ and Ca^{2+} sensitivity *via* binding to their cognate receptors.[10,14–17] The preponderance of evidence indicates that the GTP-bound form of the α subunit ($G\alpha$) of the heterotrimer activates the signaling pathway(s) that mediates Ca^{2+} sensitivity,[18,19] whereas both $G\alpha$ and the $\beta\gamma$ dimer ($G\beta\gamma$) activate signaling proteins that regulate $[Ca^{2+}]_c$.

There are considerable differences in the relative potency of volatile anesthetics to inhibit ASM contraction. For example, in studies of isolated ASM, halothane was more potent than both isoflurane and sevoflurane in inhibiting the increase in $[Ca^{2+}]_c$ [7] and Ca^{2+} sensitivity[20] induced by muscarinic receptor agonists. In fact, in the study of Ca^{2+} sensitivity, concentrations of isoflurane equivalent to approximately 2 minimum alveolar concentration (MAC), the highest concentration examined, produced no significant inhibition of Ca^{2+} sensitivity, and the effect of halothane was greater than that of sevoflurane.

Our previous work showed that the mechanism by which halothane and the anesthetic hexanol inhibit the acetylcholine-induced increase in Ca^{2+} sensitivity is due, at least in part, to an effect on signaling mediated by pertussis toxin–insensitive heterotrimeric G proteins, such as those belonging to the $G\alpha_q$ subfamily.[21] This study also indicated that this effect could be due to a direct action on the muscarinic receptor–heterotrimeric G protein complex.[22] This assertion has been supported by recent studies of crude membrane prepared from porcine ASM, which showed that both halothane and hexanol inhibited guanosine nucleotide exchange at $G\alpha_q$ when promoted by muscarinic receptor stimulation.[13,23] These findings,[13,23] when interpreted in context of the apparent greater potency of halothane to inhibit acetylcholine-induced increases in $[Ca^{2+}]_c$ [7] and Ca^{2+} sensitivity,[20] raise the intriguing question of whether concentrations of halothane, sevoflurane, and

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isoflurane used to treat acute bronchospasm differ in their ability to inhibit functional coupling between the muscarinic receptor and the $G\alpha_q$ heterotrimeric G protein. To address this question, this study first characterized a mammalian cell model in which the human muscarinic-3 (M_3) receptor and the human $G\alpha_q$ were transiently coexpressed and biochemically coupled. Then, two hypotheses were tested using crude membranes prepared from these cells. The first hypothesis was that halothane and hexanol each inhibit acetylcholine-promoted guanosine nucleotide exchange at $G\alpha_q$ without effecting constitutive exchange, similar to that proven in studies of crude membranes derived from ASM,[13,23] thus validating the use of this model to elucidate mechanisms operating in ASM. We then tested the hypothesis that at volatile anesthetic concentrations up to 2 MAC, halothane and sevoflurane but not isoflurane inhibit acetylcholine-promoted $G\alpha_q$ guanosine nucleotide exchange.

Materials and Methods

Tissue Preparation and Isometric Force Measurements in Permeabilized Tissue

After obtaining approval from the Mayo Foundation Institutional Animal Care and Use Committee, porcine tracheas were procured by euthanasia of research animals as previously described.[13] After removal of fat, connective tissue, and epithelium, the tracheal smooth muscle was cut into strips (0.8–1.2 cm long × 0.25–0.5 mm wide), mounted in a superfusion apparatus, and set at optimal length for isometric force development as previously described.[13,17,21] The strips were permeabilized with 2,500 U/ml *Staphylococcus aureus* α-toxin (see Nakayama *et al.*,[13] Yoshimura *et al.*,[17,21] and Kai *et al.*[24] for detailed description). *Staphylococcus aureus* α-toxin creates pores of approximately 26 A˚ in the ASM cell membrane, thereby allowing substances of small molecular weight, such as Ca^{2+}, to freely diffuse across the cell membrane, whereas proteins necessary for contraction and relaxation are retained within the ASM cells. Thus, $[Ca^{2+}]_c$ can be manipulated and controlled by changing the concentration of Ca^{2+} in the buffer bathing the cells.[24] In addition, coupling of the membrane receptors to the heterotrimeric G protein–mediated signaling proteins that regulate Ca^{2+} sensitivity remain intact and can be activated, including the muscarinic receptors.[24] Solutions of varying free Ca^{2+} concentrations were prepared using the algorithm by Fabiato and Fabiato.[25]

Culture and Transfection of COS-7 Cells

COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (10% DMEM), and penicillin and streptomycin (50 U/ml each). Transient transfections were performed with 5 μg M_3 complementary DNA (cDNA) plus 5 μg $G\alpha_q$ cDNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as described previously.[26] Twenty-four hours after transfection, the cells were washed twice with phosphate-buffered saline, scraped in ice-cold phosphate-buffered saline, transferred to 1.5-ml microfuge tubes, and pelleted by centrifugation at 500*g* (2 min, 4°C). The cells were flash-frozen in liquid nitrogen and stored at −70°C until they were used to prepare crude membranes.

Measurement of Cytoplasmic Ca^{2+} Concentration

COS-7 cells were plated on 8-chambered Lab-Tek slides (Nalge Nunc International, Naperville, IL) and grown to approximately 80% confluence in 10% DMEM containing penicillin and streptomycin.[27] Cells were then made quiescent by washing in Ca^{2+}-free Hanks buffered saline solution (HBSS) and replacing the medium with DMEM lacking serum or growth factors for 2 h. The composition of the Ca^{2+}-free HBSS was as follows: 137 mM NaCl, 5.5 mM KCl, 0.3 mM K_2HPO_4, 0.3 mM Na_2HPO_4, and 1 mM $MgCl_2$ (pH 7.4 at room temperature, 1 mg/ml glucose). The cells were incubated (1 h, room temperature) with Ca^{2+}-free HBSS containing 5 μM of the acetoxymethyl ester of fura-2 (fura-2 AM). After fura-2 AM loading, the cells were washed three times with HBSS without fura-2, and the medium was replaced with 200 μl HBSS containing 2 mM $CaCl_2$. Finally, the slides were transferred to an inverted Nikon Diaphot microscope (Melville, NY) equipped with a Nikon ×20/0.75 objective lens and a 12-bit digital charge-coupled device camera that was controlled by a personal computer workstation (Princeton Instruments, Trenton, NJ). All images were acquired at 720 × 540 pixels.

To measure fura-2 fluorescence, light from a xenon lamp was filtered to restrict excitation light to 340- and 380-nm wavelengths. This light was focused to alternately excite the fura-2 within the cells with a λ-10 filter wheel (Sutter Instruments, Novato, CA). Emitted fluorescence was filtered at 510 ± 5 nm and acquired for 100 ms at each excitation wavelength. The emission fluorescence intensities due to excitation at 340-nm (F_{340}) and 380-nm (F_{380}) wavelengths were measured from 50 individual cells, and the F_{340}/F_{380} ratio was used as an index of $[Ca^{2+}]_c$. $[Ca^{2+}]_c$ was quantified with Metamorph software (Jandel Scientific, Santa Barbara, CA) by applying the equation

$$Ca^{2+} = K_d(F_{min}/F_{max}) \cdot ((R - \eta \cdot R_{min})/\eta \cdot R_{max} - R),$$

where K_d is the apparent dissociation constant of fura-2 for free Ca^{2+} (224 nM at 25°C); F_{min} and F_{max}, and R_{min} and R_{max} are the minimum (absence of free Ca^{2+}) and maximum (saturating free Ca^{2+}) fluorescence values of

F_{380} and the F_{340}/F_{380} ratios, respectively; R is the difference in F_{340}/F_{380} ratio and the background fluorescence of F_{380}; and η is the viscosity value of the cytoplasm and is assumed to equal 1.

Measurement of IP_3 Levels

COS-7 cells (4×10^5 cells) were seeded in 10-cm tissue culture plates for 24 h and then transfected with the vector pcDNA3.1 only, or the cDNA constructs encoding for $G\alpha_q$ only, or M_3 and $G\alpha_q$. Twenty-four hours after transfection, the cells were incubated in 1 ml serum-free DMEM for 2 h before the experiments were conducted. After the experimental protocol, the plates were placed on ice and immediately lysed with 250 μl ice-cold trichloroacetic acid, 1 M, and the lysates were transferred to 2-ml plastic tubes and centrifuged (12,000*g*, 2 min, 4°C). The supernatant was allowed to warm to room temperature over 15 min, and the trichloroacetic acid was extracted from the supernatant with 2 ml trioctylamine and trichloro-trifluoroethane (1:3 vol/vol). Finally, inositol-1,4,5-triphosphate (IP_3) levels were determined in the cell-free extract using a commercially available radioreceptor binding assay kit (Perkin Elmer, Boston, MA) as described in a previous study from our laboratory.[28] Data are expressed as picomoles of IP_3 in the extract per 4×10^5 cells.

Crude Membrane Preparation

COS-7 cell membranes were prepared as previously described,[26] suspended at 3 mg/ml in assay buffer (50 mM Tris-HCl [pH 7.4], 100 mM NaCl, 4.8 mM $MgCl_2$, and 1 μM GDP), snap frozen in liquid nitrogen, and stored at −70°C until used for the assay A portion of the crude membrane suspension was solubilized in 6 ml NaOH, 0.1 N, and heated (3 min) to determine protein concentration.[29]

$G\alpha_q$ Guanosine Nucleotide Exchange Assay

$G\alpha_q$ nucleotide exchange was assayed using methods originally described by Barr and Manning[30] and previously used by our laboratory.[13,23] Reaction mixtures containing 10 μg membrane protein, 100 mM NaCl, 4.8 mM $MgCl_2$, and 1 μM GDP, with or without halothane, isoflurane, sevoflurane, or hexanol, with or without acetylcholine in a total volume of 62 μl were preincubated for 5 min at 30°C. Reactions were performed in narrow 0.25-ml polypropylene tubes to minimize loss of volatile anesthetics. The assays were initiated by the addition of 5 μl of the radioactive, nonhydrolyzable form of GTP, [^{35}S]GTPγS, to the reaction mixture (1,250 Ci/mmol; 20 nM final concentration in assay). Reactions were terminated at times according to experimental design with 600 μl ice-cold immunoprecipitation buffer containing 50 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 150 mM NaCl, 0.5% (vol/vol) IGEPAL CA-630, 100 μM GDP, 100 μM GTP, and 1% (wt/vol) bovine serum albumin. Reaction tubes were then vortex mixed, gently rotated (10 min, 4°C) and centrifuged at 12,000*g* (10 min, 4°C). The supernatant containing detergent solubilized $G\alpha_q$ was transferred to 1.5-ml microfuge tubes and incubated (1 h, 4°C) with rabbit polyclonal antiserum specific for $G\alpha_q$ (1:800 vol/vol dilution). Then, 40 μl protein A-agarose beads were added to each sample for an additional hour (4°C). The beads were then washed four times by repeated pelleting (2,000*g*, 1 min, 4°C) followed by resuspension in 1 ml immunoprecipitation buffer and rotated on an orbital rocker (15 min, 4°C). Finally, the washed beads were placed in 4 ml Ultima 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 with the same amount of protein except that the assay was immediately terminated with 600 μl ice-cold immunoprecipitation buffer. In preliminary work, halothane had no effect on this nonspecific background radioactivity. Data were normalized to the amount of protein and the specific activity of [^{35}S]GTPγS in the assay, and each experimental condition was assayed in triplicate.

Immunoblotting of M_3 Muscarinic Receptor and $G\alpha_q$ Proteins

Crude membrane samples (10 μl) were mixed with 20 μl sodium dodecyl sulfate sample buffer (62.5 mM Tris-HCl, 2% sodium dodecyl sulfate, 25% glycerol, and 0.01% bromophenol blue, pH 6.8). Samples used for immunoblotting of $G\alpha_q$ were then boiled for 5 min, and samples for immunoblotting of the M_3 muscarinic receptor were heated to 60°C for 10 min. The samples were then subjected to polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane. The polyvinylidene difluoride membranes were then probed (60 min, room temperature) with rabbit polyclonal antibodies directed against a peptide mapping at the carboxy terminus of the M_3 muscarinic receptor (1:10,000 vol/vol) or native rat brain $G\alpha_q$ protein (1:1,000 vol/vol). The antibodies were diluted in blotting buffer composed of 10 mM Tris, 150 mM NaCl, and 1% bovine serum albumin (pH 7.4). The primary antibodies were then probed (30 min, room temperature) using horseradish peroxidase–conjugated goat anti-rabbit immunoglobulin G diluted in blotting buffer (1:10,000 vol/vol), which was then detected by enhanced chemiluminescence.

Quantification of M_3 Muscarinic Receptor and $G\alpha_q$ Expression

M_3 muscarinic receptor was quantified in the crude membranes prepared from COS-7 cells by saturation binding with the muscarinic antagonist tritium-labeled L-[benzilic-4,4′-^{3}H]-quinuclidinyl benzilate (^{3}H-QNB).[31,32] Specific

binding was determined in triplicate assays with a single saturating concentration of ^{3}H-QNB of 5 nM. Nonspecific binding was determined by performing triplicate assays in the presence of 5 μM atropine. Aliquots of crude membranes containing 20 μg protein from COS-7 cells cotransfected with M_3 and $G\alpha_q$, or untransfected COS-7 cells were incubated (90 min, room temperature) in 0.5 ml buffer containing 5 nM ^{3}H-QNB, with or without 5 μM atropine, 50 mM Tris, and 10 mM $MgCl_2$ (pH 7.4). After the 90-min incubation period, reactions were applied to prewetted Whatman GF/C filters (Whatman plc, Brentford, United Kingdom) and washed three times with 5 ml buffer containing 50 mM Tris and 10 mM $MgCl_2$ (pH 7.4). Bound radioactivity on the filters was quantified by liquid scintillation counting. The amount of $G\alpha_q$ in the crude membranes was determined by comparative Western blotting using a dilution series of purified, recombinant rat $G\alpha_q$ (1.25–20 ng in each lane) as the standard.

Preparation of Anesthetic Solutions

Saturated aqueous stocks of halothane, sevoflurane, and isoflurane were prepared by stirring (30 min, room temperature) 5 ml liquid anesthetic with 10 ml assay buffer in an airtight, ground-glass flask as previously described.[13,23,33,34] After the stirring had been stopped for 5 min, 5 ml of the anesthetic saturated assay buffer was transfer to a 5-ml glass vial. Saturated aqueous concentration stocks of halothane, sevoflurane, and isoflurane were 12.0–13.0, 2.6–3.8, and 7.0–7.9 mM, respectively. Aliquots of the stocks were added directly to the reaction tubes in volumes that produced the desired final anesthetic concentration. To account for the unavoidable rapid loss of anesthetic upon mixing in the assay tubes, tandem experiments were conducted under the same assay conditions where anesthetic concentrations were measured after hexane extraction by gas chromatography using an electron capture detector (Hewlett-Packard, Waltham, MA; model 5880A).[35] In preliminary studies, we found that after an initial loss of approximately 20% due to transfer and mixing, the amount of volatile anesthetic in the reaction tubes was relatively stable, with less than 10% additional loss over the longest assay time in this report (60 min).

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 as expected on the basis of its density and molecular weight.[23,36,37]

Materials

COS-7 cells were purchased from American Type Culture Collection (Manassas, VA). The cDNAs for the human M_3 muscarinic receptor and human $G\alpha_q$ in the mammalian expression vector pcDNA3.1 were purchased from the University of Missouri-Rolla cDNA Resource Center, Rolla, MO.# The M_3 muscarinic receptor was tagged with 3×-hemagglutinin at the amino-terminus. All chemicals and supplies required for cell culture and cDNA transfection of COS-7 cells, and pCDNA3.1 plasmid were purchased from (Carlsbad, CA). EndoFree maxi-prep kits used for cDNA preparation were purchased from Qiagen Science (Valencia, CA). Halothane was purchased from Ayerst Laboratories, Inc. (New York, NY), and sevoflurane and isoflurane were purchased from Abbott Laboratories (Chicago, IL). Fura-2 AM was purchased from Molecular Probes, Inc. (Eugene, OR). ^{3}H-QNB (42 Ci/mmol) and [^{35}S]GTPγS (1,250 Ci/mmol) were purchased from Perkin-Elmer (Boston, MA). *Staphylococcus aureus* α-toxin, rabbit nonimmune serum, rabbit polyclonal antiserum generated against recombinant native rat brain $G\alpha_q$ protein, and recombinant native rat brain $G\alpha_q$ protein were purchased from Calbiochem (EMD Biosciences, Inc. Affiliate, San Diego, CA). Protein A-agarose beads, rabbit affinity-purified immunoglobulin G generated against a synthetic peptide sequence corresponding to the carboxy terminus of the human M_3 muscarinic receptor, and horseradish peroxidase–conjugated goat anti-rabbit immunoglobulin G were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The chemiluminescence kits were purchased from Amersham Biosciences (Piscataway, NJ). Stock solutions of running and transfer buffers, Laemmli buffer, polyvinylidene difluoride membrane, precast sodium dodecyl sulfate polyacrylamide gels, and Lowry protein assay kits were purchased from Bio-Rad Life Science Research Products (Hercules, CA). Adenosine triphosphate disodium salt was purchased from Research Organics, Inc. (Cleveland, OH). All other chemicals were purchased from Sigma Chemical Company (St. Louis, MO). All drugs and chemicals were prepared in distilled, filtered water.

Data Analysis and Statistics

Data are reported as mean ± SD; n represents the number of independent times an assay was performed. Time- and concentration-dependent effects on acetylcholine on $G\alpha_q$ [^{35}S]GTPγS–GDP exchange were determined by nonlinear regression analysis using Prism 4 (GraphPad Software, San Diego, CA). The parameters for the concentration-dependent effects of halothane, isoflurane, and sevoflurane on acetylcholine-promoted [^{35}S]GTPγS–GDP exchange were determined by using the four-parameter, logistic Hill equation and compared by analysis of variance with *post hoc* comparisons using unpaired *t* tests (SigmaPlot 8; Systat Software, Point Richmond, CA). All other comparisons were made by paired Student *t* test. For all statistical comparisons, a value of $P < 0.05$ was considered significant.

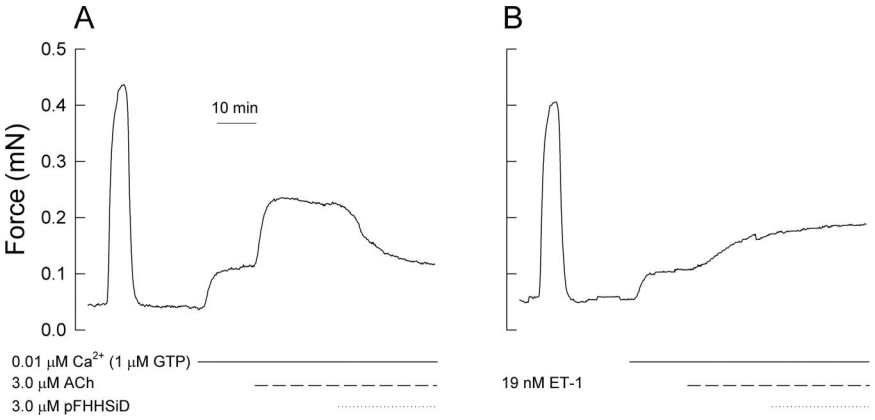

Fig. 1. Representative tracings showing the effects of the M_3 muscarinic receptor antagonist p-F-hexahydro-sila-difenidol (pFHHSiD) on the increase in Ca^{2+} sensitivity induced by acetylcholine (ACh; *A*) or endothelin 1 (ET-1; *B*) in porcine tracheal smooth muscle permeabilized with *Staphylococcus aureus* α-toxin. Each tracing is representative of at least four experiments. See text for details of experimental protocol.

Results

Effect of M_3-selective Antagonist pFHHSiD on the Increase in Ca^{2+} Sensitivity Induced by Acetylcholine

In previous reports, we established that volatile anesthetics relax permeabilized tracheal smooth muscle when contracted with acetylcholine.[10,11,16,20–22] However, these previous studies did not directly address which member of the muscarinic receptor family was responsible for the acetylcholine-induced increase in Ca^{2+} sensitivity. This protocol determined the effect of the M_3 muscarinic receptor competitive antagonist p-F-hexahydro-sila-difenidol (pFHHSiD) on the increase in Ca^{2+} sensitivity induced by acetylcholine in permeabilized porcine ASM.

An example of the experimental protocol is shown in figure 1. Two pairs of permeabilized strips were prepared from the same animal, one pair to study acetylcholine and the second pair to study endothelin 1. Increasing the free Ca^{2+} concentration in the superfusate from 1 to 100 nM in the presence of 1 μM GTP caused sustained increases in isometric force of 14.6 ± 2.3% of the maximal values. The subsequent addition of 3 μM acetylcholine (fig. 1A) or 19 nM endothelin 1 (fig. 1B) to this superfusate caused additional increases in isometric force at a constant Ca^{2+} concentration (*i.e.*, increase Ca^{2+} sensitivity) of 35.9 ± 4.3 and 35.9 ± 3.1% of the maximal values, which were sustained for the duration of the protocol (data not shown). Finally, 3 μM pFHHSiD was added to the superfusate of one strip of each pair; the second strip of each pair did not receive pFHHSiD and served as a control for the time-dependent stability of the agonist-induced increase in Ca^{2+} sensitivity. The addition of 3 μM pFHHSiD to the superfusate during constant activation with 100 nM free Ca^{2+}, 1 μM GTP, and 19 nM endothelin 1 had no effect on isometric force, demonstrating that pFHHSiD had no apparent nonspecific or inverse agonist effects in this tissue. By contrast, during constant activation with 100 nM free Ca^{2+}, 1 μM GTP, and 3 μM acetylcholine, the addition of 3 μM pFHHSiD caused a sustained inhibition of isometric force of 82.1 ± 4.2%. These results indicate that the M_3 muscarinic receptor is largely responsible for acetylcholine-induced increase in Ca^{2+} sensitivity in tracheal smooth muscle and thus support the rationale for studying the effects of volatile anesthetics on the expressed M_3 muscarinic receptor.

Characterization of Cotransfected COS-7 Cells

Immunoblots of crude membranes prepared from vector pcDNA3.1 transfected and M_3–Gα_q cotransfected COS-7 cells were performed to document expression of the M_3 muscarinic receptor and Gα_q using isoform specific rabbit polyclonal antibodies (fig. 2A). In membranes prepared from vector only (pcDNA3.1)–transfected cells, the antiserum raised against Gα_q native protein detected an immunoreactive protein band that migrated just below the 43-kd molecular weight marker (fig. 2A, left). This protein band had a molecular weight of 41 kd and thus seemed to represent a Gα_q-like protein and not the slightly larger protein Gα_{11}. This assertion was strengthened by the observation that when overexpressed in these cells, Gα_q exhibited a migratory pattern similar to that of the endogenous protein. Comparative immunoblotting using a dilution series of purified, recombinant Gα_q revealed an approximately fivefold greater expression of Gα_q in the M_3–Gα_q cotransfected compared with vector pcDNA3.1 transfected cells.

In membranes prepared from vector pcDNA3.1 transfected COS-7 cells, antiserum raised against a carboxy-terminal peptide sequence of the M_3 muscarinic receptor did not detect an immunoreactive protein band (fig. 2A, right). In addition, no muscarinic receptor could be detected in these membranes by radioligand binding using ^{3}H-QNB. Conversely, in membranes prepared from M_3–Gα_q cotransfected cells, a prominent, broad immunoreactive band that migrated at the 90-kd molecular weight marker and an additional protein band with a molecular mass of approximately 75 kd were detected. These two bands likely represent the disulfide-linked dimer and the amino-terminal proteolytic fragment of the 45-kd M_3 muscarinic receptor.[38] Likewise, ^{3}H-QNB

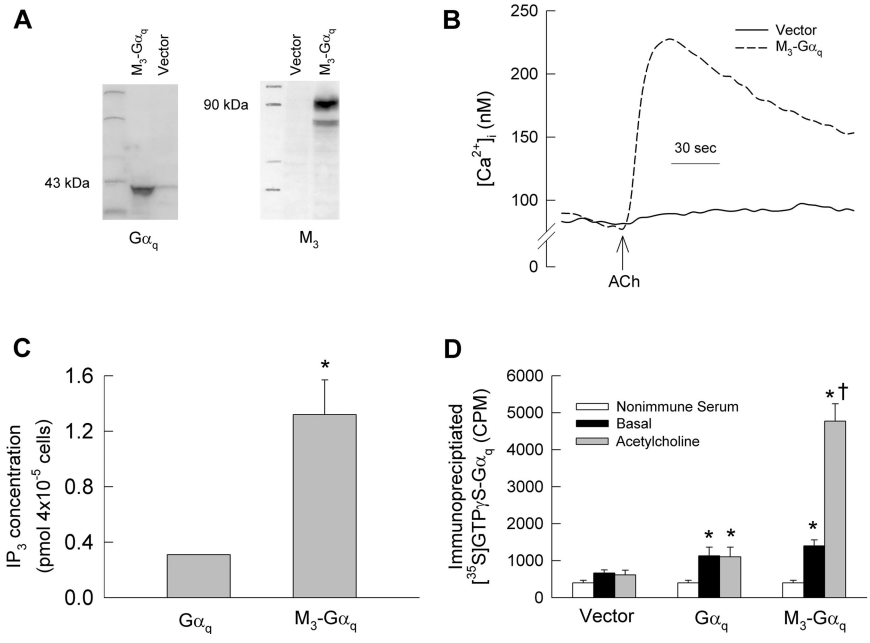

Fig. 2. (*A*) Immunoblots of crude membranes prepared from COS-7 cells transfected with vector pcDNA3.1 only or cotransfected with the complementary DNA (cDNA) constructs encoding for the human M_3 muscarinic receptor and human $G\alpha_q$ (M_3–$G\alpha_q$ cotransfected). Each lane was loaded with 10 μg membrane protein. (*B*) Representative tracings showing the effect of acetylcholine (ACh) on cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_c$) in fura-2–loaded COS-7 transfected with vector pcDNA3.1 only or cotransfected with the cDNA constructs encoding for the human M_3 muscarinic receptor and human $G\alpha_q$. (*C*) Effect of ACh on inositol triphosphate (IP_3) levels in COS-7 cells expressing human $G\alpha_q$ only, or human M_3 muscarinic receptor and human $G\alpha_q$. (*D*) Effect of acetylcholine on the exchange of the radioactive, nonhydrolyzable form of guanosine-5′-triphosphate (GTP), [^{35}S]GTPγS, for guanosine-5′-diphosphate (GDP) ([^{35}S]GTPγS–GDP exchange) at the α subunit of the G_q heterotrimeric G protein ($G\alpha_q$). Assays were performed using crude membranes prepared from COS-7 cells expressing human $G\alpha_q$ only, or human M_3 muscarinic receptor and human $G\alpha_q$. Measurements were made in the absence (constitutive exchange) and presence of 10 μM ACh. The immunoprecipitation step of the assays was performed using nonimmune serum (for nonspecific background measurements) or antiserum specific for $G\alpha_q$. Data are mean ± SD; n = 4. * Significant difference from background radioactivity. † Significant difference from constitutive Gα [^{35}S]GTPγS-GDP exchange. CPM = counts per minute.

binding studies revealed expression levels of the M_3 muscarinic receptor of 3–5 pmol/mg protein.

To establish that the M_3 muscarinic receptor and $G\alpha_q$ transiently coexpressed in the COS-7 cells are functionally coupled to downstream signaling molecules, we examined the ability of acetylcholine to stimulate an increase in $[Ca^{2+}]_c$ and IP_3 levels. For $[Ca^{2+}]_c$ measurements, fura-2 AM–loaded cells were superfused with HPSS containing 1 μM acetylcholine. Because COS-7 cells express endogenous H_1 histamine receptors,[39] vector pcDNA3.1 only transfected cells were also stimulated with 1 μM histamine as a positive control for adequate fura-2 AM loading in living cells. For IP_3 measurements, cells were incubated with 1 ml serum-free DMEM containing 0 (control) or 10 μM acetylcholine for 20 min (37°C). The cells were then placed on ice, and IP_3 was extracted and quantified.

In vector pcDNA3.1 only transfected and M_3–$G\alpha_q$ cotransfected COS-7 cells loaded with fura-2, the addition of 1 μM histamine to the HBSS superfusate stimulated a biphasic, sustained increase in $[Ca^{2+}]_c$ from a constitutive level of 116 ± 6 nM to a peak concentration of 338 ± 45 nM. Consistent with the lack of expression of endogenous M_3 muscarinic receptors in COS-7 cells, 1 μM acetylcholine had no significant effect on $[Ca^{2+}]_c$ (fig. 2B) or IP_3 concentration (fig. 2C) in cell transfected with vector pcDNA3.1 only. By contrast, in the M_3–$G\alpha_q$ cotransfected cells, 1 μM acetylcholine induced a biphasic, sustained increase in $[Ca^{2+}]_c$ from a constitutive level of 86 ± 4 nM to a peak concentration of 226 ± 17 nM (fig. 2B) and induced an approximately threefold increase in IP_3 levels compared with cells transfected with $G\alpha_q$ alone (fig. 2C). These observations demonstrate that the M_3 muscarinic receptor and $G\alpha_q$ transiently coexpressed in the COS-7 cells were properly assembled as a functional receptor–heterotrimer complex at the plasmalemma.

Characterization of the $G\alpha_q$ Guanosine Nucleotide Exchange Assay

To determine the dependence of the $G\alpha_q$ [^{35}S]GTPγS–GDP exchange measurements on M_3 muscarinic receptor and $G\alpha_q$ coexpression and activation by acetylcholine, assays were performed using crude membranes prepared from COS-7 cells transfected with the vector pcDNA3.1 only, or COS-7 cells transfected with the cDNA constructs encoding for $G\alpha_q$ only, or both the M_3 muscarinic receptor and $G\alpha_q$. The crude membranes were incubated without (for constitutive $G\alpha_q$ [^{35}S]GTPγS–GDP exchange measurements) or with (for acetylcholine-promoted $G\alpha_q$ [^{35}S]GTPγS–GDP exchange) 10 μM acetylcholine for 5 min, and then the reactions were initiated with [^{35}S]GTPγS. The reactions were terminated after 10 min, and the samples were subjected to the immunoprecipitation step of the assay using either nonimmune rabbit serum (for nonspecific background measurements) or rabbit antiserum raised against recombinant native rat brain $G\alpha_q$ protein. These data were not subjected to nonspecific background subtraction and are expressed as absolute values of counts per minute.

In crude membranes prepared from COS-7 cells transfected with the vector pcDNA3.1 only, no $G\alpha_q$

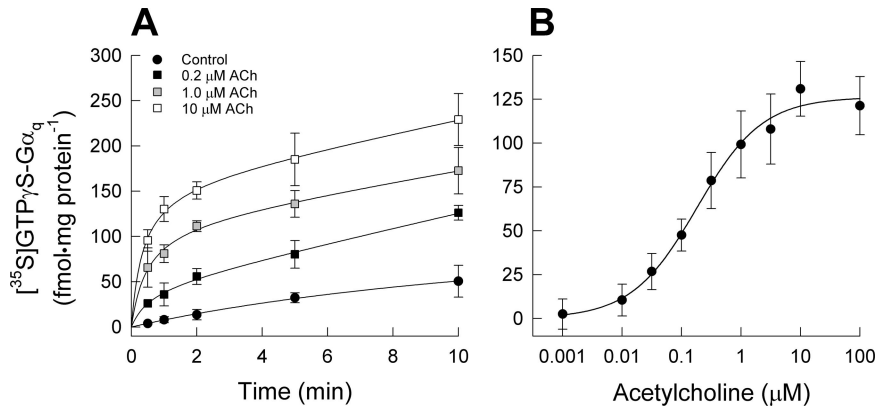

Fig. 3. (*A*) Concentration-dependent effect of acetylcholine (ACh) on the exchange of the radioactive, nonhydrolyzable form of guanosine-5′-triphosphate (GTP), [^{35}S]GTPγS, for guanosine-5′-diphosphate (GDP) ([^{35}S]GTPγS–GDP exchange) at the α subunit of the G_q heterotrimeric G protein ($G\alpha_q$). Assays were performed using crude membranes prepared from COS-7 cells expressing human M_3 muscarinic receptor and human $G\alpha_q$. Data are expressed as the absolute increase in $G\alpha_q$ [^{35}S]GTPγS–GDP exchange values induced by ACh above the constitutive exchange values. Data are mean ± SD; n = 4. (*B*) Time-dependent change in $G\alpha_q$ [^{35}S]GTPγS–GDP exchange measured in the absence or presence of 0.2, 1, or 10 μM ACh. Data are mean ± SD; n = 6.

[^{35}S]GTPγS–GDP exchange could be detected above that of the nonspecific background measurements (fig. 2D). By contrast, in crude membranes prepared from M_3–$G\alpha_q$ cotransfected cells, there was a small but significant increase in $G\alpha_q$ [^{35}S]GTPγS–GDP exchange above that of the nonspecific background in the absence of acetylcholine (*i.e.*, constitutive exchange). The amount of background radioactivity was less than 50% of the radioactivity of the constitutive $G\alpha_q$ nucleotide exchange. The inclusion of 10 μM acetylcholine in the assay buffer caused a further approximately fourfold increase in the magnitude of this exchange. When the receptor was omitted from the transfection (*i.e.*, crude membranes prepared from $G\alpha_q$ alone transfected cells), acetylcholine did not promote an increase in $G\alpha_q$ [^{35}S]GTPγS–GDP exchange above the constitutive exchange levels (fig. 2D).

Effect of Acetylcholine on $G\alpha_q$ Guanosine Nucleotide Exchange

Time course and concentration response curves were conducted to help guide the experimental design of subsequent protocols that examined possible volatile anesthetic effects on $G\alpha_q$ [^{35}S]GTPγS–GDP exchange. To determine the time course for $G\alpha_q$ [^{35}S]GTPγS–GDP exchange, crude membrane prepared from M_3–$G\alpha_q$ cotransfected cells were incubated for 5 min with or without 0.2, 1, or 10 μM acetylcholine, and then the reactions were initiated with [^{35}S]GTPγS. These acetylcholine concentrations are approximately the EC_{50}, EC_{80}, and EC_{100} values for isometric force development in permeabilized porcine ASM.[13] The reactions were terminated after 0.5, 1, 2, 5, or 10 min, and then the samples were subjected to the immunoprecipitation step of the assay. To generate concentration–response curves, crude membranes were incubated with or without various concentrations of acetylcholine (0.001–100 μM) for 5 min. The reactions were then terminated 5 min after initiation with [^{35}S]GTPγS, and the samples were subjected to the immunoprecipitation step of the assay. The acetylcholine-promoted $G\alpha_q$ [^{35}S]GTPγS–GDP exchange was quantified by subtracting the constitutive exchange values from those measured in the presence of acetylcholine.

In the absence of acetylcholine in the assay, membranes incorporated [^{35}S]GTPγS into $G\alpha_q$ in a time-dependent manner (fig. 3A). The presence of acetylcholine in the assay promoted a significant increase in the magnitude of $G\alpha_q$ [^{35}S]GTPγS–GDP exchange at each time point. The curves were biphasic even after subtracting the constitutive values measured in the absence of acetylcholine. All of the time course curves could be fit adequately ($r^2 > 0.95$) with a biexponential equation with rate constants K_1 and K_2 of 2.4 and 0.1 min^{-1}, respectively. Both components of the acetylcholine-promoted $G\alpha_q$ [^{35}S]GTPγS–GDP exchange were completely blocked by the muscarinic receptor antagonist atropine (1 mM, preliminary data not shown). Y_{max} values measured at 1 min with 0, 0.2, 1, and 10 μM acetylcholine in the assay were 8.0 ± 3.3, 35.9 ± 12.8, 81.0 ± 9.8, and 130 ± 13.8 fmol/min. The acetylcholine-promoted increase in $G\alpha_q$ [^{35}S]GTPγS–GDP exchange above constitutive exchange levels was concentration dependent, with an EC_{50} value of 0.24 ± 0.09 μM acetylcholine (fig. 3B). Based on these initial investigations, subsequent experiments examining the effects of anesthetics on $G\alpha_q$ [^{35}S]GTPγS–GDP exchange were performed using 1 μM acetylcholine to optimize the ability to detect modest shifts in acetylcholine-promoted $G\alpha_q$ [^{35}S]GTPγS–GDP exchange, and were confined to 1 min when the time course was largely monophasic.

Effect of Halothane and Hexanol on $G\alpha_q$ Nucleotide Exchange

Crude membranes prepared from M_3–$G\alpha_q$ cotransfected cells were incubated for 5 min with or without

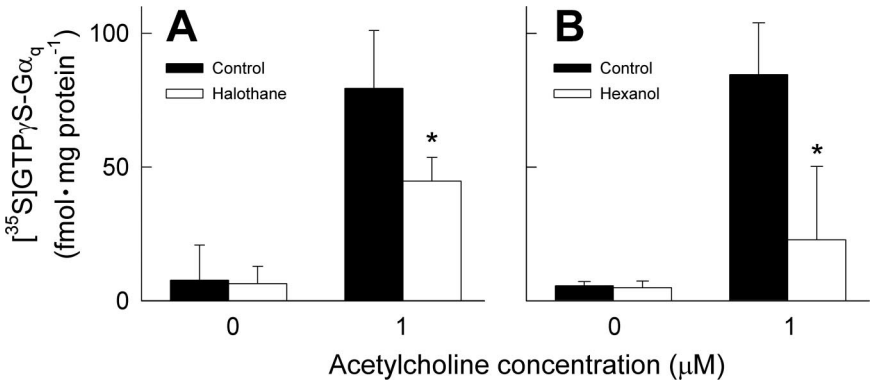

Fig. 4. Effect of 0.55 mM halothane (*A*) and 10 mM hexanol (*B*) on acetylcholine-promoted exchange of the radioactive, nonhydrolyzable form of guanosine-5′-triphosphate (GTP), [^{35}S]GTPγS, for guanosine-5′-diphosphate (GDP) ([^{35}S]GTPγS–GDP exchange) at the α subunit of the G_q heterotrimeric G protein ($G\alpha_q$). Assays were performed using crude membranes prepared from COS-7 cells expressing the human M_3 muscarinic receptor and human $G\alpha_q$. Measurements were made in the absence and presence of acetylcholine. Data are mean ± SD; n = 6. * Significant difference from control.

0.5 mM halothane or 10 mM hexanol, and with or without 1 μM acetylcholine. The anesthetic concentrations used are similar to those studied in our previous work with crude membranes prepared from porcine tracheal smooth muscle, thereby allowing comparisons with these previous data.[13,21,23,37] The reactions were terminated at 1 min after initiation with [^{35}S]GTPγS, and then the samples were subjected to the immunoprecipitation step of the assay.

Neither halothane (fig. 4A) nor hexanol (fig. 4B) affected constitutive $G\alpha_q$ [^{35}S]GTPγS–GDP exchange in the absence of acetylcholine. Conversely, the presence of approximately 0.55 mM halothane (equivalent to approximately 3 MAC) or 10 mM hexanol in the assay significantly inhibited the increase in $G\alpha_q$ [^{35}S]GTPγS–GDP exchange promoted by acetylcholine by 48 ± 8.9 and 22.8 ± 22.1%, respectively. The presence of anesthetic in the immunoprecipitation buffer only had no effect on acetylcholine-promoted $G\alpha_q$ [^{35}S]GTPγS–GDP exchange (data not shown), indicating that the anesthetic had no effect on detergent extraction of $G\alpha_q$ from the membrane or any component immunoprecipitation step of the assay.

Concentration-dependent Effect of Volatile Anesthetics on $G\alpha_q$ Guanosine Nucleotide Exchange

Crude membranes prepared from M_3–$G\alpha_q$ cotransfected cells were incubated for 5 min with or without halothane, isoflurane, or sevoflurane concentrations equivalent to 0.5, 1, 2, 3, 5, and 8 MAC for humans,[40] and with and without 1 μM acetylcholine. The anesthetic concentrations in the assay reactions were 0.09–1.5, 0.15–1.5, and 0.13–2.4 mM for halothane, sevoflurane, and isoflurane, respectively. These anesthetic concentrations included those previously shown to cause bronchodilation *in vivo* [41,42] and inhibit Ca^{2+} sensitivity and ASM contraction in isolated tissue.[6–12,22] The reactions were terminated at 1 min after initiation with [^{35}S]GTPγS, and then the samples were subjected to the immunoprecipitation step of the assay.

The presence of each volatile anesthetic in the assay caused a concentration-dependent inhibition of $G\alpha_q$ [^{35}S]GTPγS–GDP exchange when promoted by 1 μM acetylcholine (fig. 5). The EC_{50} values for halothane, isoflurane, and sevoflurane were 0.66 ± 0.23 mM (3.5 ± 1.2 MAC), 1.34 ± 0.17 mM (4.96 ± 0.62 MAC), and 1.05 ± 0.37 mM (3.5 ± 1.2 MAC), respectively. At anesthetic concentrations equivalent to 2 MAC (designated by the dashed lines), the inhibition by halothane and sevoflurane of acetylcholine-promoted $G\alpha_q$ [^{35}S]GTPγS–GDP exchange was significantly greater than that caused by isoflurane; no significant difference was found between halothane and sevoflurane (P = 0.3). These findings were also true at each anesthetic concentration up to and including 3 MAC. However, there was no significant difference in the magnitude of the anesthetic effects on acetylcholine-promoted $G\alpha_q$ [^{35}S]GTPγS–GDP exchange among the three anesthetics at concentrations equivalent to 5 MAC. The Hill slope coefficients for the con-

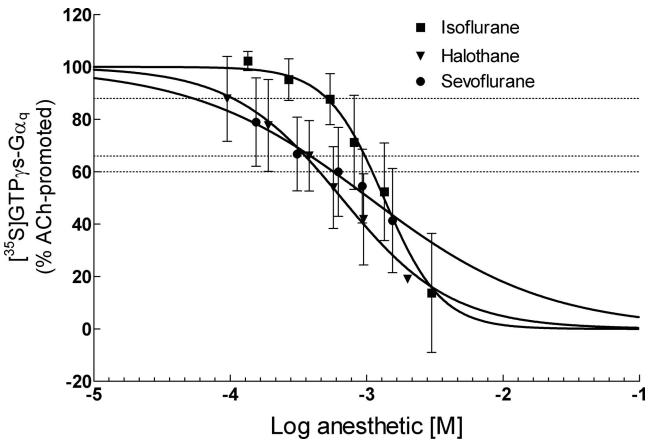

Fig. 5. Concentration-dependent effect of halothane, sevoflurane, and isoflurane on acetylcholine (ACh)–promoted exchange of the radioactive, nonhydrolyzable form of guanosine-5′-triphosphate (GTP), [^{35}S]GTPγS, for guanosine-5′-diphosphate (GDP) ([^{35}S]GTPγS–GDP exchange) at the α subunit of the G_q heterotrimeric G protein ($G\alpha_q$). Assays were performed using crude membranes prepared from COS-7 cells expressing the human M_3 muscarinic receptor and human $G\alpha_q$. The ACh-promoted increase in $G\alpha_q$ [^{35}S]GTPγS–GDP exchange was expressed as the percentage of the difference between the values measured in the absence of acetylcholine or halothane, and the values measured in the presence of ACh (% ACh-promoted). The *dashed lines* identify the values measured at anesthetic concentrations equivalent to 2 minimum alveolar concentration. Data are mean ± SD; n = 9.

centration–response curves generated by halothane, sevoflurane, and isoflurane were 1.09 ± 0.36, 0.67 ± 0.36, and 2.07 ± 0.72, respectively; each of these values was significantly different from the other.

Reversible Effect of Anesthetics on $G\alpha_q$ Guanosine Nucleotide Exchange

To determine whether inhibition of acetylcholine-promoted $G\alpha_q$ [^{35}S]GTPγS–GDP exchange by the anesthetics was reversible, crude membranes prepared from M_3–$G\alpha_q$ cotransfected cells were incubated for 5 min with approximately 0.7, 1.3, or 1.0 mM halothane, isoflurane, or sevoflurane, respectively, and then the anesthetics were allowed to dissipate from the assay tubes before initiating the nucleotide exchange reactions. To ensure complete dissipation of the volatile anesthetics from the reaction mixture, the tube caps were opened, and a stream of nitrogen was directed into the headspace above the mixture, which was agitated at 5-min intervals by pipetting, for a total of 1 h. In preliminary studies, this protocol resulted in complete dissipation of the anesthetics from the tubes as measured by gas chromatography. Negative control reactions were incubated with acetylcholine only (not exposed to volatile anesthetic) and subjected to the same protocol, and positive control (for volatile anesthetic effect) reactions were incubated with acetylcholine and anesthetic but the tubes remained closed. A fourth reaction was not exposed to volatile anesthetic or acetylcholine to provide the constitutive exchange measurements. After the 1-h incubation period, 1-min reactions were initiated with [^{35}S]GTPγS, and the samples were subjected to the immunoprecipitation step of the assay. The acetylcholine-promoted increase in $G\alpha_q$ [^{35}S]GTPγS–GDP exchange was expressed as a percentage of the difference between the values measured in the absence of acetylcholine or halothane (constitutive exchange) and that measured in the presence of acetylcholine but not halothane (negative control).

Halothane, isoflurane, or sevoflurane caused the expected approximately 50–60% inhibition of acetylcholine-promoted $G\alpha_q$ [^{35}S]GTPγS–GDP exchange. The inhibitory effects of all three anesthetics were completely reversible when the anesthetics were allowed to fully dissipate from the assay mixtures, because there were no significant differences in the acetylcholine-promoted [^{35}S]GTPγS–GDP exchange values measured in the reversal tubes compared to the control measurements (fig. 6).

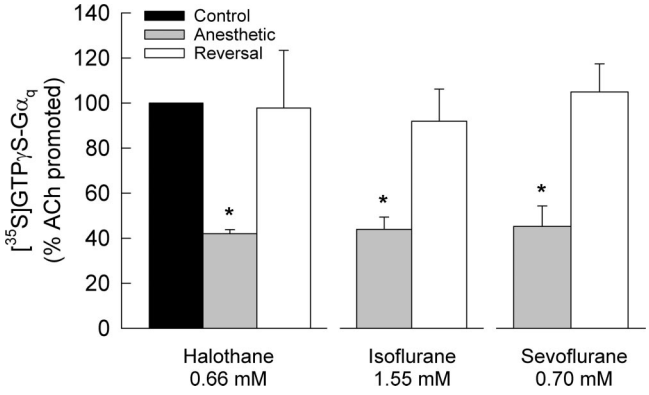

Fig. 6. Effect of volatile anesthetic removal on acetylcholine (ACh)–promoted exchange of the radioactive, nonhydrolyzable form of guanosine-5′-triphosphate (GTP), [^{35}S]GTPγS, for guanosine-5′-diphosphate (GDP) ([^{35}S]GTPγS-GDP exchange) at the α subunit of the G_q heterotrimeric G protein ($G\alpha_q$). Assays were performed using crude membranes prepared from COS-7 cells expressing the human M_3 muscarinic receptor and human $G\alpha_q$. Measurement were made before (control), during, and after (reversal) exposure to halothane, isoflurane, or sevoflurane for 5 min (see Results for a detailed protocol description). The ACh-promoted increase in $G\alpha_s$ [^{35}S]GTPγS–GDP exchange was expressed as the percentage of the difference between the values measured in the absence of ACh or volatile anesthetic, and that measured in the presence of the 1 μM ACh (% ACh-promoted). Data are mean ± SD; n = 4. * Significant difference from control.

Discussion

The major finding of this study is that concentrations of halothane and sevoflurane less than or equal to 3 MAC were more effective than isoflurane in inhibiting the biochemical coupling between the M_3 muscarinic receptor and the $G\alpha_q$ heterotrimeric G protein. These findings are consistent with the apparent more potent direct effect of halothane and sevoflurane compared with isoflurane on muscarinic receptor–mediated contraction of isolated ASM from several species,[7,20] including humans.[43]

The muscarinic receptor is a member of a superfamily of receptors that have seven transmembrane spanning domains and regulate cell signaling *via* activation of heterotrimeric G proteins. There are at least five isoforms of the receptor, of which two, the M_2 and M_3 muscarinic receptor, are functionally expressed in ASM.[44] Studies of isolated, intact tracheal smooth muscle obtained from M_2 and M_3 muscarinic receptor knockout mice indicate that tracheal smooth muscle contraction induced by acetylcholine is mediated approximately equally by each receptor.[45,46] M_3 muscarinic receptors are coupled to phospholipase Cβ *via* $G\alpha_q$ subfamily proteins, thereby stimulating phosphoinositide hydrolysis and intracellular Ca^{2+} mobilization. The observed acetylcholine-stimulated increases in both IP_3 levels and $[Ca^{2+}]_c$ in the COS-7 cells transiently expressing the M_3 muscarinic receptor and $G\alpha_q$ (fig. 2) are consistent with this known mechanism and indicate properly assembled and functional receptor–heterotrimer complexes at the plasmalemma.

In the simplest two-state model for heterotrimeric G protein–coupled receptors, the M_3 muscarinic receptor exists as two conformations, active and inactive (see

Roberts and Waelbroeck[47] for review). In the absence of ligand binding, these two forms are in dynamic equilibrium, with the inactive form being predominant. Receptor agonists induce or select for the active conformation, which promotes or stabilizes the nucleotide-free $G\alpha_q$ subunit. The high intracellular ratio of GTP to GDP ensures that activated receptors promote GTP binding to $G\alpha_q$, which ultimately reduces its affinity for the receptor and the $G\beta\gamma$ dimer, resulting in its dissociation from the receptor–ligand complex. The GTP-bound $G\alpha_q$ then is thought to be coupled to the downstream signaling proteins that increase Ca^{2+} sensitivity in ASM.[15,48] Hydrolysis of the bound GTP by the intrinsic GTPase activity of $G\alpha_q$ permits reassociation of the subunits into a heterotrimer and terminates the activation of the signaling cascade.

Our findings are consistent with this two-state receptor model. Acetylcholine increased the fraction of receptors in the active conformation, as demonstrated by a concentration-dependent increase in the magnitude, but not the rate, of $G\alpha_q$ [^{35}S]GTPγS–GDP exchange. The slow component of the acetylcholine-promoted $G\alpha_q$ [^{35}S]GTPγS–GDP exchange likely reflects coupling of some of the expressed M_3 muscarinic receptors to endogenous heterotrimers other than the expressed $G\alpha_q$, because this slower component is typically not observed when the receptor is expressed alone in mammalian cells.[49] In this setting, the endogenous $G\alpha$ subunits must first dissociate from this fraction of M_3 muscarinic receptors before the receptor is available for coupling to the expressed $G\alpha_q$.

Several lines of evidence have established the rationale for studying anesthetic effects specifically on the biochemical coupling between the M_3 muscarinic receptor and $G\alpha_q$ using the mammalian protein expression model used in the current study. First, the involvement of the M_3 muscarinic receptor in mediating the acetylcholine-induced increase in Ca^{2+} sensitivity was demonstrated by our data showing inhibition of such increases by the relatively specific M_3 muscarinic receptor antagonist, pFHHSiD. Second, the acetylcholine-induced increase in Ca^{2+} sensitivity is mediated, at least in part, by $G\alpha_q$ subfamily proteins, as demonstrated by a partial inhibition of such increases by the $G\alpha_q$ peptide inhibitor 2A.[15] This observation is supported by the recent identification of the guanine nucleotide exchange factor responsible for $G\alpha_q$-mediated activation of RhoA,[48] one of the signaling proteins responsible for mediating agonist-induced increases in Ca^{2+} sensitivity in ASM.[22,50] Third, our previous studies demonstrated that an effect involving pertussis toxin insensitive heterotrimeric G proteins are responsible in part for anesthetic inhibition of Ca^{2+} sensitivity, a finding consistent the role for $G\alpha_q$.[21] This assertion was recently confirmed by studies of crude membrane prepared from porcine ASM, which showed that halothane and hexanol inhibit guanosine nucleotide exchange at $G\alpha_{q/11}$ when promoted by acetylcholine.[13,23] Finally, our current data showed that concentrations of halothane and hexanol similar to those used in the aforementioned studies of crude membranes prepared from porcine ASM[13,23] produced comparable observations. Whereas neither anesthetic had an effect on the constitutive $G\alpha_q$ guanosine nucleotide exchange, both caused marked inhibition when acetylcholine was present in the assay. These data, when evaluated in the context of the aforementioned supporting observations,[10,11,13,15,20–23,48] strongly support the use of the M_3–$G\alpha_q$ coexpression model used in the current study for comparing the three volatile anesthetics.

Whereas no significant difference was observed in the EC_{50} values between the three anesthetics, the Hill slopes were markedly different with sevoflurane $<$ halothane $\ll$ isoflurane. These findings suggest a mechanistic difference in the effects of volatile anesthetics on M_3–$G\alpha_q$ coupling. One possibility to explain this difference is two inhibitory sites for isoflurane with cooperativity and a single inhibitory site for halothane and sevoflurane. A second possibility is that the Hill slope of 2 for isoflurane reflects activation at low concentrations of isoflurane combined with inhibition at higher concentrations of isoflurane. Indeed, the data shown in figure 5 suggest a slight activation of acetylcholine-promoted $G\alpha_q$ [^{35}S]GTPγS–GDP exchange by 0.15 mM (0.5 MAC) isoflurane. In support of this assertion, stimulation of G protein–coupled receptor activity at less than 1 MAC isoflurane has been observed for the human D_2 dopamine receptor expressed in Chinese hamster ovarian cells using a guanosine nucleotide exchange assay similar to that presented in the current study.[51] A similar finding was observed with less than 1 MAC desflurane for the rat M_1 muscarinic receptor expressed in *Xenopus* oocytes using a calcium signaling assay.[52] Therefore, the most parsimonious model to explain the differential effects observed between the anesthetics is one in which all three anesthetics have similar affinity for inhibition but different abilities to activate M_3–$G\alpha_q$ coupling at lower concentrations. The difference in the Hill slopes between sevoflurane and halothane likely reflects the difficulty we encountered in reliably and reproducibly achieving the higher concentrations of sevoflurane in the assays.

Although the concentration–response curves for a measure of clinical anesthesia, such as lack of movement to an incision, are very steep (Hill slopes > 10),[53] this is likely the result of cumulative anesthetic effects at multiple protein and/or membrane targets, rather than a cooperative effect on a single target such as the classic case of cooperative binding of oxygen to hemoglobin.[53] Therefore, it is not surprising that the Hill slopes for volatile anesthetic inhibition of M_3–$G\alpha_q$ coupling were 0.7–2.0. Although concentration–response curves to volatile anesthetics while monitoring output of airway re-

laxation have not been performed with sufficient data to establish an accurate Hill slope, they do not seem to be highly cooperative. Indeed, Revich *et al.*[54] examined 15 case studies where concentrations of halothane up to approximately 4 MAC (approximately 0.8 mM) have been administered clinically to successfully reverse refractory status asthmaticus; there was not an obvious concentration–response relation as seen when a measure of anesthetic state is used as a measure of effect.

In summary, this study shows that volatile anesthetics, at concentrations that are used in the treatment of severe bronchospasm, reversibly and in a concentration-dependent manner inhibit acetylcholine-promoted exchange of [^{35}S]GTPγS for GDP at the nucleotide binding site of Gα_q. The finding that at these concentrations halothane and sevoflurane were more effective than isoflurane in inhibiting M_3–Gα_q coupling is consistent with the more potent direct effect of these anesthetics compared with isoflurane on muscarinic receptor– mediated contraction of isolated ASM.[7,20,43] These differential effects also suggest that a direct interaction of anesthetics with the proteins or protein interfaces of this receptor–heterotrimer complex could be responsible for these observations, rather than simply the accumulation of anesthetic molecules in the cell membrane. Finally, these findings are consistent with those demonstrated in crude membrane prepared from differentiated ASM, indicating that this expression system is a relevant model for future mechanistic studies that could use techniques such as domain switching, site-directed mutagenesis, or chimeras to ascertain a greater understanding of this anesthetic action.

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