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Anesthetics Inhibit Membrane Receptor Coupling to the $G_{q/11}$ Heterotrimeric G Protein in Airway Smooth Muscle

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Background: **Some anesthetics relax airway smooth muscle in part by inhibiting acetylcholine-induced increases in Ca^{2+} sensitivity, an effect associated with inhibition of guanosine nucleotide exchange at the α subunit of the $G_{q/11}$ ($G\alpha_{q/11}$) heterotrimeric G protein. This study tested the hypothesis that these anesthetic effects are not unique to the muscarinic receptor but are a general property of the heptahelical receptors that increase Ca^{2+} sensitivity in airway smooth muscle.**

Methods: **Anesthetic effects on agonist-induced increases in Ca^{2+} sensitivity were measured in porcine airway smooth muscle strips permeabilized with *S. aureus* α-toxin. Anesthetic effects on basal (without agonist stimulation) and agonist-promoted $G\alpha_{q/11}$ guanosine nucleotide exchange were determined in crude membranes prepared from porcine airway smooth muscle. The nonhydrolyzable, radioactive form of guanosine 5′-triphosphate was used as the reporter for nucleotide exchange at $G\alpha_{q/11}$.**

Results: **Acetylcholine, endothelin-1, and histamine caused a concentration-dependent increase in Ca^{2+} sensitivity. Halothane (0.67 ± 0.07 mM) and hexanol (10 mM) significantly inhibited the increase in Ca^{2+} sensitivity induced by each agonist. Each agonist also caused a time- and concentration-dependent increase in $G\alpha_{q/11}$ nucleotide exchange. Neither anesthetic had an effect on basal $G\alpha_{q/11}$ nucleotide exchange, whereas halothane and hexanol significantly inhibited the increase in $G\alpha_{q/11}$ nucleotide exchange promoted by each agonist.**

Conclusion: **These data suggest that inhibition of agonist-promoted guanosine nucleotide exchange at $G\alpha_{q/11}$ by some anesthetics may be a general property of heptahelical receptors involved cellular processes mediated by $G\alpha_{q/11}$, including muscarinic, endothelin-1, and histamine receptor activation of Ca^{2+} sensitivity.**

VOLATILE anesthetics are potent bronchodilators, relaxing airway smooth muscle (ASM) in part by depressing the reflex neural pathways innervating the airways[1] and by a direct inhibitory effect on the ASM cell.[1–3] This latter direct effect is due to actions on several key intracellular second messengers, including intracellular calcium[2–5] and those that regulate the amount of force at a given intracellular calcium concentration ($[Ca^{2+}]_i$) (*i.e.*, that regulate Ca^{2+} sensitivity).[6,7] In aggregate, these anesthetic effects ultimately converge to inhibit ASM contraction, an effect that has been exploited clinically to treat bronchospasm in patients with hyperreactive airway disease.[8,9]

Several endogenous contractile agonists regulate ASM tone *in situ*, including acetylcholine, endothelin-1, and histamine.[10] These agonists activate heterotrimeric guanosine 5′-triphosphate (GTP) binding protein (G protein)–dependent mechanisms that increase both $[Ca^{2+}]_i$ and Ca^{2+} sensitivity *via* the muscarinic, endothelin, and histamine receptors, respectively.[6,7,11–17] The preponderance of evidence indicates that it is the GTP-bound form of the α subunit ($G\alpha$) of the heterotrimer that activates the signaling pathway that mediates Ca^{2+} sensitivity and not the $\beta\gamma$ dimer ($G\beta\gamma$).[18,19] Several subfamilies of heterotrimeric G proteins are known to mediate acetylcholine-, endothelin-1-, and histamine-induced increases in Ca^{2+} sensitivity in ASM, such as those belonging to the G_i and G_q subfamilies.[13,20,21]

Our previous work shows that the anesthetics halothane and hexanol relax ASM in part by inhibiting the increase in Ca^{2+} sensitivity induced by muscarinic receptor activation.[11,12,21,22] This action was due in part to effects on signaling mediated by pertussis toxin–insensitive heterotrimeric G proteins, such as those belonging to the G_q subfamily.[21] Our previous work also indicated that these effects could be due to a direct action on the muscarinic receptor–heterotrimeric G-protein complex.[16] This hypothesis was recently supported by a study of crude membrane prepared from porcine ASM, which showed that both halothane and hexanol inhibited guanosine nucleotide exchange at $G\alpha_{q/11}$ when activated by muscarinic receptor stimulation.[23] These observations are in contrast to those made in studies of intravenous anesthetics, which indicate that these compounds relax ASM by mechanisms that do not involve effects on the membrane receptor–heterotrimeric G-protein complex. The intravenous anesthetics ketamine, midazolam, and propofol each had no effect on acetylcholine-induced increases in Ca^{2+} sensitivity.[24] Whereas each intravenous agent inhibited ASM contraction in intact tissue, these effects were due entirely to effects on Ca^{2+} homeostasis,[24–26] probably *via* inhibition of Ca^{2+} influx *via* voltage-gated Ca^{2+} channels.[27,28]

Although the mechanism responsible for the ability of volatile anesthetics to inhibit Ca^{2+} sensitivity is not fully known, the preponderance of evidence suggests that they may interact directly with the receptor rather than the G proteins.[29] This raises the possibility that the observed anesthetic effects on the coupling between the muscarinic receptor and $G\alpha_{q/11}$ may be specific to this receptor, rather than a general property of G protein–coupled receptors (GPCRs). Furthermore, it is not known whether anesthetics inhibit the increase in Ca^{2+} sensitivity in ASM produced by other physiologic ago-

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nists. This study tested the hypothesis that the ability of halothane and hexanol to inhibit receptor-induced increases in Ca^{2+} sensitivity and guanosine nucleotide exchange at Gα is not unique to the muscarinic receptor, but rather a property shared by other GPCRs that increase Ca^{2+} sensitivity in ASM. To achieve this goal, we first characterized the ability of endothelin-1 and histamine to increase Ca^{2+} sensitivity in porcine ASM and promote guanosine nucleotide exchange at $G\alpha_{q/11}$ in crude membrane prepared from porcine ASM, as previously demonstrated for acetylcholine.[16,23] Then, we examined the effect of hexanol, a prototypical alkane anesthetic, and that of clinically relevant concentrations of halothane on these measurements. If this hypothesis were true, the data would suggest that the salient protein target might be the G protein rather that the receptor.

Materials and Methods

Tissue Preparation

After obtaining approval from the Mayo Foundation Institutional Animal Care and Use Committee (Mayo Foundation, Rochester, Minnesota) porcine tracheas were procured either from a local abattoir or by euthanasia of research animals. In preliminary work, we have found no physiologic difference in the tracheal smooth muscle obtained from these two tissue sources (KA Jones, M.D., DO Warner, M.D., T Nakayama, M.D., H Yoshimura, M.D., unpublished observations, 2000–2004). The research animals were first anesthetized by intramuscular injection of tiletamine (10 ml/kg) and xylazine (6 mg/kg) and intravenous injection of pentobarbital (400–600 mg) and then killed by exsanguination *via* bilateral 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 of the following composition: 110.5 mM NaCl, 25.7 mM $NaHCO_3$, 5.6 mM dextrose, 3.4 mM KCl, 2.4 mM $CaCl_2$, 1.2 mM KH_2PO_4, and 0.8 mM Mg_2SO_4. After removal of fat, connective tissue, and epithelium, tracheal smooth muscle was cut into strips (1.5 cm long × 0.25 cm wide), frozen in liquid nitrogen, and stored at −70°C until it was used to prepare crude membranes for investigation.

Isometric Force Measurements and Permeabilization Procedure

Isometric force was measured in permeabilized smooth muscle strips using a previously described superfusion apparatus.[17,21] After setting each muscle strip at optimal length for maximal isometric force development, the strips were permeabilized by incubation in relaxing solution containing 2,500 U/ml *Staphylococcus aureus* α-toxin (20 min, 25°C).[16] *S. aureus* α-toxin creates pores of approximately 26 Å in the smooth muscle 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 are retained within the smooth muscle cells. Thus, $[Ca^{2+}]_i$ can be manipulated and controlled by changing the concentration of Ca^{2+} in the buffer bathing the smooth muscle cells. 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. Therefore, changes in isometric force induced by a contractile agonist or anesthetic are due entirely to changes in Ca^{2+} sensitivity, because $[Ca^{2+}]_i$ is "clamped" and not allowed to change.[11]

The composition of the relaxing solution was as follows: 7.5 mM magnesium adenosine 5′-triphosphate, 4 mM EGTA, 20 mM imidazole, 10 mM creatinine phosphate, 0.1 mg/ml creatine phosphokinase, 1 nM free Ca^{2+}, and 1 mM free Mg^{2+}. The ionic strength was kept constant at 0.20 M by adjusting the concentration of potassium acetate. The pH was buffered to 7.0 (25°C) with potassium hydroxide. After treatment with α-toxin, the permeabilized strips were washed with relaxing solution without α-toxin for 5 min. Calcium ionophore A23187 (10 μM) was added to the relaxing solution and all subsequent experimental solutions to disrupt the sarcoplasmic reticulum and deplete intracellular Ca^{2+} stores.[15,30] Solutions of varying free Ca^{2+} concentrations were prepared using the algorithm by Fabiato and Fabiato.[31]

Crude Membrane Preparation

A crude membrane fraction of porcine ASM homogenate was prepared according to previously described methods from our laboratory.[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 phenylmethysulfonyl fluoride, 10 μg/ml leupeptin, and 2 μg/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 87,000*g* (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 in the preceding sentence. A portion of the crude membrane emulsion was solubilized in 6 ml of 0.1 N NaOH and heated (3 min) to determine protein concentration.[32] The homogenate was then diluted with assay buffer to a protein concentration of 2.5 mg/ml.

Immunoblotting of Gα Proteins

Membrane samples (10 μl) were mixed with 20 μl Laemmli sample buffer (62.5 mM Tris-HCl, 2% sodium dodecyl sulfate, 25% glycerol, and 0.01% bromophenol blue [pH 6.8]) and boiled for 5 min. The samples were then subjected to polyacrylamide gel electrophoresis (200 V, 30 min) on a 10% acrylamide separating–4% acrylamide stacking gel. The running buffer was composed of 25 mM Tris, 192 mM glycine, and 0.1% sodium dodecyl sulfate (pH 8.3). The proteins were then transferred (100 V, 45 min) to polyvinylidene diflouride membrane using a semidry apparatus. The composition of the transfer buffer was 25 mM Tris, 192 mM glycine, and 20% methanol (pH 8.3). The polyvinylidene diflouride membranes were then probed (60 min, 25°C) with Gα subfamily–specific affinity-purified immunoglobulin G (IgG; 1:1,000 vol/vol dilution) or immune antiserum (1:10,000 vol/vol dilution) diluted in blotting buffer (10 mM Tris, 150 mM NaCl, and 1% BSA [pH 7.4]). The primary antibodies were then probed (30 min, 25°C) using horseradish peroxidase–conjugated goat anti-rabbit IgG (1:10,000 vol/vol dilution). The horseradish peroxidase–conjugated secondary antibody was detected by chemiluminescence that was captured on x-ray film.

Gα Nucleotide Exchange Assay

The assay was performed as previously described.[23] Briefly, the reactions were initiated by the addition of 29 nM (final concentration) [^{35}S]GTPγS (specific activity1.25 μCi/pmol) to the crude membrane emulsion (containing 125 μg protein) at 30°C. Reactions were terminated according to the experimental protocol (see Experimental Protocols section) 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 μg/ml aprotinin, 0.5% (vol/vol) IGEPAL CA-630, 1% (wt/vol) bovine serum albumin, 100 μM GDP, and 100 μM GTP. All of the reaction tubes were then briefly vortex mixed, gently rotated (5 min, 4°C) and centrifuged at 12,500*g* (10 min, 4°C). The soluble fractions were transferred into fresh tubes and incubated (1 h, 4°C) with 40 μl protein A-agarose beads that had been precoated with rabbit anti-$G\alpha_{q/11}$, anti-$G\alpha_i$ (isoforms 1–3) or nonimmune antiserum (for nonspecific background radioactivity measurements), or affinity purified anti-$G\alpha_{12}$ or anti-$G\alpha_s$ IgG antibody. Then, the beads were washed four times by repeated pelleting and centrifugation at 3,260*g* (10 min, 4°C), followed by resuspension in immunoprecipitation buffer (30 min, 1 ml). 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). The amount of radioactivity above the background radioactivity was taken to indicate the amount of [^{35}S]GTPγS-bound Gα subunit dissociated from the membrane into the soluble fraction due to the exchange of [^{35}S]GTPγS for GDP at the nucleotide binding site. Values were normalized to the total amount of protein in the assay tubes.

Precoating the beads with antiserum or antibody was accomplished by incubating the beads in immunoprecipitation buffer containing 1:200 (vol/vol) antiserum or 1:1,000 (vol/vol) antibody, respectively, for at least 2 h (4°C) before the performing assay. The coated beads were then washed four times as described in the previous paragraph.

Preparation of Anesthetic Solutions

Stock solutions of assay buffer with saturating concentrations of halothane were prepared by mixing halothane in the assay buffer over night in a glass flask.[33,34] These stocks were diluted with fresh assay buffer to achieve the desired concentration of halothane. Assay tubes were capped with polytetrafluoroethylene-coated rubber stoppers immediately after the addition of halothane-containing solutions. Halothane concentrations in solution under assay conditions were measured by gas chromatography according to the method of Van Dyke and Wood.[35] 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.[22,36]

Experimental Protocols

Concentration-dependent Effect of Agonists on Ca^{2+} Sensitivity. These studies were conducted to determine the concentrations of agonist that produce half-maximal or maximal activation of Ca^{2+} sensitivity in porcine tissue, because our previous work was conducted using permeabilized canine tracheal smooth muscle.[2,3,6,7,12,16,21,22,37] These data were then used to guide the design of subsequent protocols to examine anesthetic effects on agonist-induced increases in Ca^{2+} sensitivity and agonist-promoted Gα [^{35}S]GTPγS–GDP exchange. Two protocols were conducted using tissue obtained from separate sets of animals, depending on the contractile agonist studied. For both protocols, permeabilized muscle strips were first maximally activated with 10 μM free Ca^{2+}; all subsequent isometric force measurements were normalized to this maximal value. For studies of acetylcholine and histamine, strips were superfused with solution containing 100 nM free Ca^{2+} plus 1 μM GTP for 10 min. Preliminary studies demonstrated that concentrations of GTP less than 5 μM did not induce increases in Ca^{2+} sensitivity (*i.e.*, isometric force at constant Ca^{2+} concentration) in the absence of receptor agonist. Then, in the continued presence of 100 nM free Ca^{2+} plus 1 μM GTP, concentration–response curves (0.01–100 μM) were generated for acetylcholine

or histamine by increasing the agonist concentration in superfusate. This protocol was not feasible for generating concentration–response curves for endothelin-1, because the endothelin-1-induced increase in Ca^{2+} sensitivity in permeabilized porcine tracheal smooth muscle is prohibitively slow. To construct concentration–response curves for endothelin-1, seven of eight permeabilized strips prepared from the same animal were superfused with relaxing solution containing one of seven concentrations of endothelin-1 (0.01–100 nM) plus 1 μM GTP. Then, all strips were activated with 100 nM free Ca^{2+} (seven strips in the presence of each endothelin-1 concentration plus 1 μM GTP). For both protocols, the agonist-induced increases in Ca^{2+} sensitivity were quantified by subtracting the isometric force induced by 100 nM free Ca^{2+} in the absence of agonist and normalized to the maximal isometric force induced by 10 μM free Ca^{2+}.

Effect of Anesthetics on Agonist-induced Increases in Ca^{2+} Sensitivity. Although we have published extensively regarding the effects of anesthetics on Ca^{2+} sensitivity in canine tissue,[6,7,11,12,16,21] we have not conducted similar studies with porcine tissue. Therefore, we performed a few experiments to confirm qualitatively that anesthetics also inhibit Ca^{2+} sensitivity in porcine ASM. This protocol was not conducted for histamine, because the results obtained using the aforementioned protocol showed that the histamine-induced increases in Ca^{2+} sensitivity were typically not sustained. A pair of permeabilized muscle strips prepared from the same animal were superfused with solution containing 80 nM free Ca^{2+} plus 1 μM GTP for 10 min, followed by the addition of the EC_{50} concentration of acetylcholine (10 min) or endothelin-1 (20 min). Then, one strip of each pair was exposed to halothane (0.67 ± 0.07 mM) or 10 mM hexanol; the second strip of each pair was not exposed to anesthetic and served as a control for the effect of time on the stability of the contractions. Preliminary studies demonstrated that neither halothane nor hexanol inhibited isometric force induced by free Ca^{2+} alone, which is consistent with observations previously reported for canine tracheal smooth muscle.[11,16]

Effect of Exogenous Agonist on Gα [^{35}S]GTPγS–GDP Exchange. Three experimental protocols were conducted, each using crude membranes prepared from a separate set of animals. To determine the effect of receptors agonists on [^{35}S]GTPγS–GDP exchange at $G\alpha_{q/11}$, $G\alpha_s$, $G\alpha_i$, and $G\alpha_{12}$, crude membrane samples were incubated without (basal nucleotide exchange) or with agonist concentrations demonstrated to produce maximal increase in Ca^{2+} sensitivity determined in the above protocol. The reactions were terminated 10 min after activation with [^{35}S]GTPγS. To determine the agonist concentrations that produced half-maximal and maximal increases in $G\alpha_{q/11}$ [^{35}S]GTPγS–GDP exchange, crude membrane samples were incubated without or with various concentrations of acetylcholine (0.1–300 μM), endothelin-1 (0.3–300 nM), or histamine (0.01–100 μM). The reactions were then terminated 20 min after initiation with [^{35}S]GTPγS. The agonist-promoted increases in $G\alpha_{q/11}$ [^{35}S]GTPγS–GDP exchange were quantified by subtracting the basal values from those measured in the presence of agonist. To determine the effect of receptor agonist on the time course for $G\alpha_{q/11}$ [^{35}S]GTPγS–GDP exchange, crude membrane samples were incubated without or with the agonist concentration determined in the above protocol that produced maximal agonist-promoted $G\alpha_{q/11}$ [^{35}S]GTPγS–GDP exchange. The reactions were terminated at 1, 3, 5, 10, 20, and 30 min after initiation with [^{35}S]GTPγS.

Effect of Anesthetics on Agonist-promoted $G\alpha_{q/11}$ [^{35}S]GTPγS–GDP Exchange. Assays were performed in the presence or absence of either halothane or 10 mM hexanol. Aqueous halothane concentrations were 0.31 ± 0.05 mM, which did not vary significantly over the duration of an experiment (preliminary data not shown) and are within the range previously shown to inhibit Ca^{2+} sensitivity and ASM contraction.[2–5,11,12,16,37] Hexanol (10 mM) produces maximal functional effects on ASM and was chosen so that the current results could be compared to our previous work.[21,22] The effects of halothane and hexanol on $G\alpha_{q/11}$ [^{35}S]GTPγS–GDP exchange were determined in separate experiments using samples incubated without (to assess effects on basal nucleotide exchange) or with the EC_{50} (acetylcholine or enothelin-1) or EC_{100} (acetylcholine, endotheline-1, or histamine) concentrations of agonist (to assess anesthetic effects on agonist-promoted $G\alpha_{q/11}$ [^{35}S]GTPγS–GDP exchange). The effect of anesthetics at EC_{50} of histamine was not examined because the increase in $G\alpha_{q/11}$ [^{35}S]GTPγS–GDP exchange above basal measurements was not reproducible. All reactions were terminated at 20 min after initiating the assay reactions. Each condition was assayed in triplicate.

Materials

Adenosine 5′-triphosphate disodium salt was purchased from Research Organics, Inc. (Cleveland, OH). Halothane was purchased from Ayerst laboratories, Inc. (New York, NY). *S. aureus* α-toxin, rabbit polyclonal antiserum generated against recombinant native rat brain $G\alpha_q$ protein, rabbit nonimmune serum, and affinity-purified IgG antibody generated against synthetic peptides corresponding to C-terminal sequences for $G\alpha_{i\text{-}3}$ (KNNLKECGLY), $G\alpha_s$ (RMHLRQYELL), $G\alpha_{12}$ (RYLVQCFDRKRRNRSK), and $G\alpha_{13}$ (LHDNLKQLMLQ) were purchased from Calbiochem (EMD Biosciences, Inc. Affiliate, San Diego, CA). The $G\alpha_{i\text{-}3}$ antiserum is only relatively specific for the $G\alpha_{i\text{-}3}$; as we have shown in preliminary work, it also cross-reacts with recombinant, purified $G\alpha_{i\text{-}1}$ and $G\alpha_{i\text{-}2}$. This antiserum was produced by Covance Research Products (Denver, PA) using recombinant native human $G\alpha_{i\text{-}3}$ that was expressed and puri-

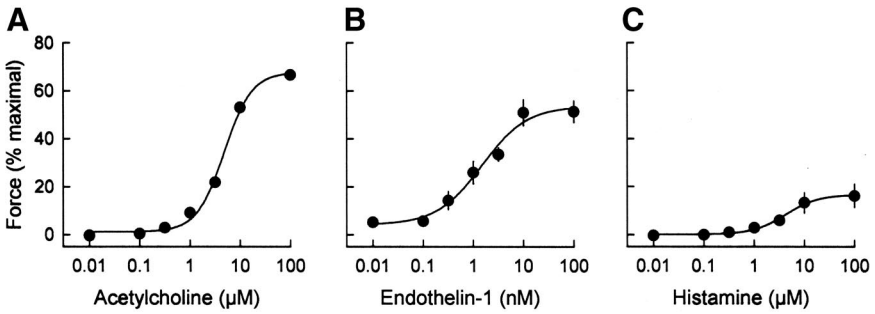

Fig. 1. Concentration-dependent effect of acetylcholine (*A*), endothelin-1 (*B*), or histamine (*C*) on isometric force in permeabilized porcine tracheal smooth muscle strips. The agonist-induced increase in Ca^{2+} sensitivity was quantified by subtracting the isometric force induced by 100 nM free calcium plus 1 μM guanosine 5′-triphosphate in the absence of agonist and normalized to the maximal isometric force induced by 10 μM free calcium. See text for detailed description of the experimental protocol. Data are presented as mean ± SD; n = 4.

fied in our laboratory as previously described.[38] This antiserum detects all three isoforms of $G\alpha_i$ but displays no crossreactivity for native $G\alpha_s$ or $G\alpha_{q/11}$. Protein A-agarose beads were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Stock solutions of running and transfer buffers, Laemmli buffer, polyvinylidene diflouride membrane, and the Lowry protein assay kits were purchased from Bio-Rad Life Science Research Produces (Hercules, CA). The enhanced chemiluminescence kits for detection of horseradish peroxidase–conjugated antibodies and [^{35}S]GTPγS were purchased from Amersham Biosciences (Piscataway, NJ). All other chemicals were purchased from Sigma Chemical Company (St. Louis, MO). A23187 was dissolved in dimethyl sulfoxide (0.05% final concentration). All other drugs and chemicals were prepared in distilled, filtered water.

Statistical Analysis

Data are reported as mean ± SD; n represents the number of animals studied. For concentration–response curves, EC_{50} and maximal agonist concentrations were determined by nonlinear regression analysis as described by Meddings *et al.* [39] In this method, a dependent variable (y), such as isometric force or $G\alpha_{q/11}$ [^{35}S]GTPγS–GDP exchange, for any concentration of drug (c) is given by the equation $y = vc/(EC_{50} + c)$, where v represents the maximal response and EC_{50} represents the concentration that produces a half-maximal response for that drug. Nonlinear regression analysis was used to fit values of v and EC_{50} to data for y and c for each condition studied. For the time course curves, the data for $G\alpha_{q/11}$ [^{35}S]GTPγS–GDP exchange were fit with the equation $y = a(1 - e^{-kt})$ using nonlinear least squares fitting. The independent variable is time (t), the dependent variable is the amount of [^{35}S]GTPγS-bound $G\alpha_{q/11}$ immunoprecipitated from solution (y), the parameter k is the rate of $G\alpha_{q/11}$ [^{35}S]GTPγS–GDP exchange, and the parameter a vertically scales the curve and is the maximal value. Repeated-measures analysis of variance with *post hoc* testing performed using the Student-Newman-Keuls test was used to compare values of k and a and to determine the effects of halothane or hexanol on $G\alpha_{q/11}$ [^{35}S]GTPγS–GDP exchange. For all statistical comparisons, a value of $P < 0.05$ was considered significant.

Results

Concentration-dependent Effect of Agonists on Ca^{2+} Sensitivity

Increasing the free Ca^{2+} concentration in the superfusate from 1 nM to 100 nM in the presence of 1 μM GTP caused a sustained increase in isometric force to 23.9 ± 1.1% of maximal force induced by 10 μM free Ca^{2+}. Adding acetylcholine, endothelin-1, or histamine to this superfusate caused further increases in isometric force, indicative of an increase in Ca^{2+} sensitivity (fig. 1). The EC_{50} values for increases in Ca^{2+} sensitivity were 2.2 ± 0.4 μM, 18.7 ± 7.3 nM, and 2.5 ± 0.6 μM for acetylcholine, endothelin-1, and histamine, respectively.

Effect of Anesthetics on Agonist-induced Increases in Ca^{2+} Sensitivity

Increasing the free Ca^{2+} concentration in the superfusate from 1 to 80 nM in the presence of 1 μM GTP caused a sustained increase in isometric force (fig. 2). The addition of acetylcholine or endothelin-1 to this superfusate caused a sustained, additional increase in isometric force, indicating an increase in Ca^{2+} sensitivity. Finally, the subsequent addition of halothane or hexanol (data not shown) to the superfusate caused a reproducible decrease in the additional isometric force induced by either acetylcholine (fig. 2A) or endothelin-1 (fig. 2B). When quantified as a percentage change from sustained increase in isometric force produced by the agonists above that produced by free Ca^{2+} alone, halothane caused 45.9 ± 9.4 and 37.2 ± 8.2% inhibitions of Ca^{2+} sensitivity induced by acetylcholine and endothelin-1, respectively. Likewise, 10 mM hexanol caused 62.2 ± 6.2 and 36.8 ± 6.7% inhibitions of Ca^{2+} sensitivity induced by acetylcholine and endothelin-1, respectively.

Effect of Exogenous Agonist on Gα [^{35}S]GTPγS–GDP Exchange

Immunoblots of the porcine tracheal smooth muscle crude membrane preparation are shown in figure 3A. Proteins corresponding to $G\alpha_{q/11}$, the two splice variants for the short form of $G\alpha_s$, $G\alpha_i$ (isoforms 1–3), and $G\alpha_{12}$ were detected; no protein corresponding to $G\alpha_{13}$ could be detected. The nonspecific background radioactivity

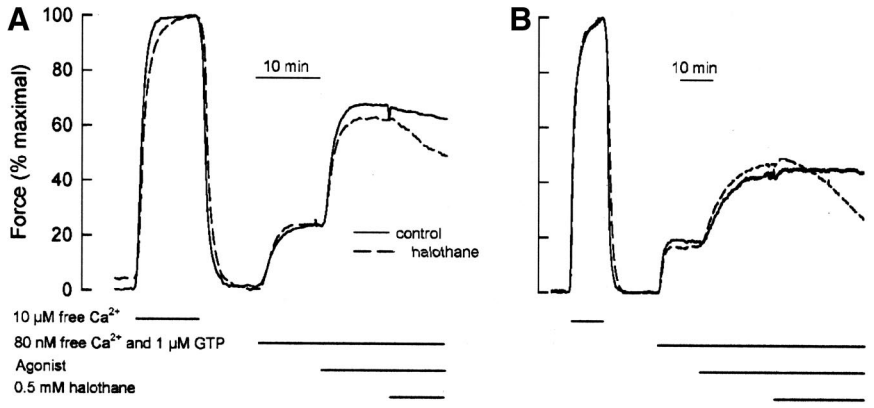

Fig. 2. Representative tracings showing the effect of halothane (approximately 0.5 mM) on isometric force induced by 10 μM acetylcholine (*A*) or 10 nM endothelin-1 (*B*) during constant free calcium plus guanosine 5′-triphosphate (GTP) concentrations in permeabilized porcine tracheal smooth muscle strips (each tracing is representative of at least three experiments). See text for details of experimental protocol.

was approximately 50–60% of the radioactivity of the basal, unstimulated specific, $G\alpha_{q/11}$ nucleotide exchange measurements (fig. 3B) and was not affected by the receptor agonists (data not shown). In the absence of agonist stimulation (basal [^{35}S]GTPγS–GDP exchange), there was a significant increase in [^{35}S]GTPγS–GDP exchange at $G\alpha_{q/11}$ above the nonspecific background radioactivity, but no nucleotide exchange could be detected above background with immunoprecipitation for $G\alpha_s$, $G\alpha_i$, or $G\alpha_{12}$ (fig. 3B). Acetylcholine, endothelin-1, and histamine each caused a significant, additional increase in $G\alpha_{q/11}$ [^{35}S]GTPγS–GDP exchange above basal levels. However, there was no detectable effect of the agonists on [^{35}S]GTPγS–GDP exchange with $G\alpha_s$, $G\alpha_i$, or $G\alpha_{12}$ immunoprecipitation. The increase in $G\alpha_{q/11}$ [^{35}S]GTPγS–GDP exchange was time dependent, reaching a maximal value between 10 and 20 min (fig. 4), and had an apparent rate constant (k_{app}) of 0.09 ± 0.01 fmol/min. Acetylcholine and endothelin-1 each significantly increased both k_{app} (0.20 ± 0.04 and 0.23 ± 0.02 fmol/min, respectively) and maximal $G\alpha_{q/11}$ [^{35}S]GTPγS–GDP exchange compared with basal exchange values (figs. 4A and B, respectively). Conversely, whereas histamine significantly increased maximal $G\alpha_{q/11}$ [^{35}S]GTPγS–GDP exchange, k_{app} was not significantly different from that measured in the absence of agonist (0.11 ± 0.01 *vs.* 0.09 ± 0.01 fmol/min for histamine-promoted and basal exchange measurements, respectively). The agonist-promoted increase in $G\alpha_{q/11}$ [^{35}S]GTPγS–GDP exchange above basal exchange was concentration dependent for all three agonists (fig. 5), with EC_{50} values of 3.6 ± 0.1 μM, 18.5 ± 3.7 nM, and 2.9 ± 2.6 μM for acetylcholine, endothelin-1, and histamine, respectively.

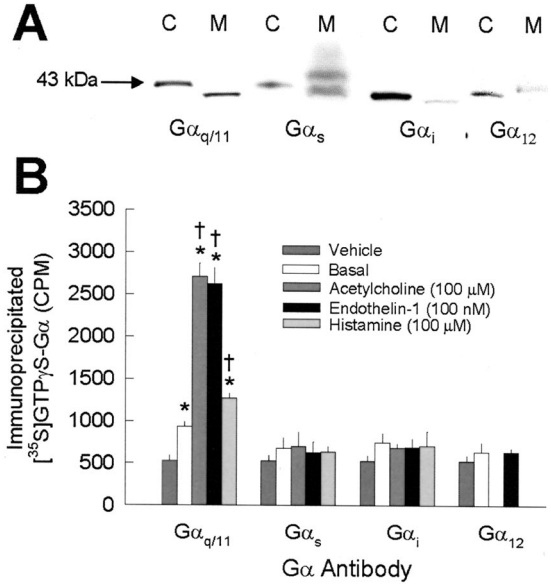

Fig. 3. (*A*) Immunoblots of the heterotrimeric G-protein α subunits (Gα) $G\alpha_{q/11}$, $G\alpha_s$, $G\alpha_i$ (isoforms 1–3), and $G\alpha_{12}$ control proteins (C) and the proteins present in the porcine tracheal smooth muscle crude membrane preparation (M). Total amounts of membrane protein loaded in the lanes were 25, 50, 75 and 100 μg for $G\alpha_{q/11}$, $G\alpha_s$, $G\alpha_i$, and $G\alpha_{12}$ immunoblots, respectively. (*B*) Effect of contractile agonists on the exchange of the nonhydrolyzable, radioactive form of guanosine 5′-triphosphate (GTP), [^{35}S]GTPγS, for guanosine 5′-diphosphate (GDP) ([^{35}S]GTPγS–GDP exchange) at Gα of heterotrimeric G proteins. [^{35}S]GTPγS–GDP exchange was measured in the absence (basal exchange) or presence of maximal stimulation (agonist-promoted exchange) with acetylcholine (100 μM), endothelin-1 (100 nM), or histamine (100 μM). Immunoprecipitation was performed using nonimmune serum (for background measurements), antiserum specific for $G\alpha_{q/11}$ or $G\alpha_i$ (isoforms 1-3), or immunoglobulin G antibody specific for $G\alpha_s$ or $G\alpha_{12}$. Agonist effects on $G\alpha_{12}$ [^{35}S]GTPγS–GDP exchange were conducted only for endothelin-1, because functional coupling of this protein and muscarinic and histamine-1 receptors has not been demonstrated. Data are normalized to the total protein in the assay and are presented as mean ± SD; n = 3. * Significant difference from background radioactivity. † Significant difference from basal Gα [^{35}S]GTPγS–GDP exchange. CPM = counts per minute.

Effect of Anesthetics on Agonist-promoted $G\alpha_{q/11}$ [^{35}S]GTPγS–GDP Exchange.

Neither anesthetic had an effect on the nonspecific background radioactivity (data not shown). Neither halothane nor hexanol affected basal $G\alpha_{q/11}$ [^{35}S]GTPγS–GDP exchange (*i.e.*, in the absence of agonist), which was 5.2 ± 0.5 fmol/mg protein in the absence of anesthetic. Basal $G\alpha_{q/11}$ [^{35}S]GTPγS–GDP exchange was 5.1 ± 0.8 or 5.9 ± 0.3 fmol/mg protein in the presence of halothane or hexanol, respectively. Hexanol significantly inhibited the increase in $G\alpha_{q/11}$ [^{35}S]GTPγS–GDP

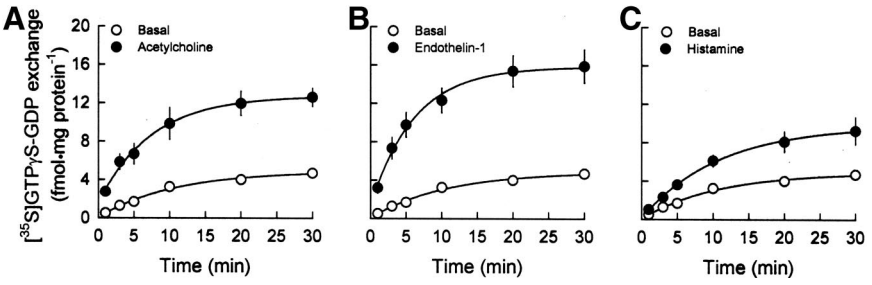

Fig. 4. Time-dependent change in exchange of the nonhydrolyzable, radioactive form of guanosine 5′-triphosphate (GTP), [^{35}S]GTPγS, for guanosine 5′-diphosphate (GDP) ([^{35}S]GTPγS–GDP exchange) at the α subunit of the $G_{q/11}$ heterotrimeric G protein. [^{35}S]GTPγS–GDP exchange was measured in the absence (basal exchange) or presence of maximal stimulation (agonist-promoted exchange) with acetylcholine (*A*), endothelin-1 (*B*), or histamine (*C*). Data are presented as mean ± SD; n = 5.

exchange induced by all three agonists (fig. 6). At maximal activation, hexanol inhibited acetylcholine-, endothelin-1–, and histamine-promoted $G\alpha_{q/11}$ [^{35}S]GTPγS–GDP exchange by 39, 31, and 52%, respectively (fig. 6A). At half maximal activation, hexanol caused a 91 or 50% inhibition of acetylcholine- or endothelin-1–promoted $G\alpha_{q/11}$ [^{35}S]GTPγS–GDP exchange, respectively (fig. 6B). Likewise, halothane significantly inhibited the increase in $G\alpha_{q/11}$ [^{35}S]GTPγS–GDP exchange induced by half-maximal activation with acetylcholine or endothelin-1, or maximal activation with histamine by 66, 31, or 78%, respectively (fig. 7).

Discussion

The major findings of this study are that halothane and hexanol inhibit increases in Ca^{2+} sensitivity and $G\alpha_{q/11}$ [^{35}S]GTPγS–GDP exchange induced by activation of all three GPCRs. Therefore, susceptibility to these anesthetic effects seems to be a general property of these seven-transmembrane-domain receptor–$G\alpha_{q/11}$ heterotrimeric G-protein complexes rather than specific to the muscarinic receptor as previously reported.[23] These observations have important mechanistic implications suggesting that the salient protein target might be the heterotrimeric G protein rather that the receptor, although the possibility of direct anesthetic effects on all three receptors has not been eliminated.

G protein–coupled receptors, including muscarinic, endothelin, and histamine receptors, mediate ASM contraction and bronchospasm in patients with hyperreactive airway diseases. Ligand binding to these receptors induces this contraction not only by increasing $[Ca^{2+}]_i$, but also by a heterotrimeric G protein–mediated signaling cascade that increases Ca^{2+} sensitivity (see Somlyo and Somlyo[18,19] for review). We have shown that some anesthetics inhibit canine ASM contraction in part by attenuating the increase in Ca^{2+} sensitivity induced by muscarinic receptor agonists.[6,7] We subsequently localized the mechanism of this anesthetic effect to the muscarinic receptor–heterotrimeric G-protein complex,[16,21] because anesthetics had no effect when Ca^{2+} sensitivity was induced by direct activation of the signaling cascade distal to the heterotrimeric G proteins.[11,12,16,21] We recently confirmed that volatile anesthetics inhibit muscarinic receptor coupling to the $G_{q/11}$ heterotrimeric G protein using the same model presented in the current study.[23] This observation raises the question of whether the receptor or G protein is the biochemically important target for anesthetic effects. To address this issue, this study determined whether the observed inhibition of receptor–G protein coupling was specific to the muscarinic–$G\alpha_{q/11}$ complex, or whether similar effects can be observed with other receptors that couple to $G\alpha_{q11}$, particularly those that also happen to be important mediators of bronchospasm and ASM contraction *in vivo*.

The considerable heterogeneity among cell types that express GPCRs and heterotrimeric G-protein isotypes confers specificity of GPCR–heterotrimeric G-protein coupling to permit precise intracellular signaling. Porcine tracheal smooth muscle cells express muscarinic-2, muscarinic-3, endothelin-A, endothelin-B, and histamine-1 receptors.[40–43] The heterotrimeric G proteins expressed in porcine ASM include those belonging to the $G\alpha_q$, $G\alpha_{i/o}$, and $G\alpha_{12/13}$ subfamilies. $G\alpha_i$ subfamily proteins are functionally coupled to the muscarinic-2[44] and the two endothelin receptors,[45] whereas the muscarinic-3,[44] both endothelin,[45] and the histamine-1[46,47] receptors are functionally coupled to $G\alpha_q$ subfamily proteins. Accordingly, it is presumed in the current study of

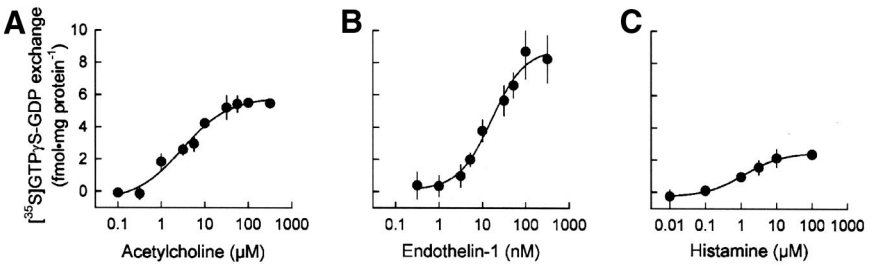

Fig. 5. Concentration-dependent effect of exogenous acetylcholine (*A*), endothelin-1 (*B*), or histamine (*C*) on the extent of exchange of the nonhydrolyzable, radioactive form of guanosine 5′-triphosphate (GTP), [^{35}S]GTPγS, for guanosine 5′-diphosphate (GDP) ([^{35}S]GTPγS–GDP exchange) at the α subunit of the $G_{q/11}$ heterotrimeric G protein. Data are expressed as the increase in $G\alpha_{q/11}$ [^{35}S]GTPγS–GDP exchange above the basal exchange values. Data are presented as mean ± SD; n = 4.

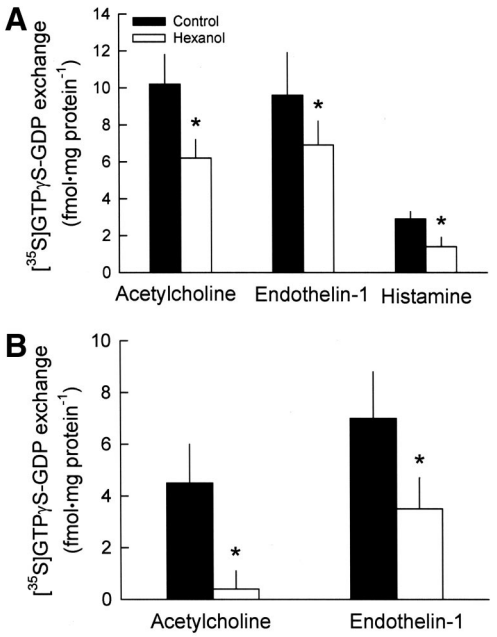

Fig. 6. Effect of hexanol (10 mM) on agonist-promoted exchange of the nonhydrolyzable, radioactive form of guanosine 5′-triphosphate (GTP), [^{35}S]GTPγS, for guanosine 5′-diphosphate (GDP) ([^{35}S]GTPγS–GDP exchange) at the α subunit of the $G_{q/11}$ heterotrimeric G protein. [^{35}S]GTPγS–GDP exchange was measured in the absence (basal exchange) or presence of maximal (*A*) or half-maximal (*B*) stimulation with exogenous acetylcholine, endothelin-1, or histamine. Data are expressed as the increase in $G\alpha_{q/11}$ [^{35}S]GTPγS–GDP exchange above the basal exchange values. Data are presented as mean ± SD; n = 4. * Significant difference from control.

receptor coupling to $G\alpha_{q/11}$ that the receptor subtypes examined include the muscarinic-3, endothelin-A, endothelin-B, and histamine-1 isoforms. Of the GPCRs examined in the current study, only the endothelin receptors have been demonstrated to couple to $G\alpha_{12/13}$.[48]

In porcine ASM, acetylcholine-induced increases in Ca^{2+} sensitivity are mediated by both $G\alpha_i$ and $G\alpha_q$ subfamily proteins, as demonstrated by a partial inhibition of such increases by pertussis toxin[13,20] and the $G\alpha_q$ peptide inhibitor 2A,[13] respectively. By contrast, increases in Ca^{2+} sensitivity induced by endothelin-1 seem to be mediated entirely by pertussis toxin–insensitive Gα subfamily proteins, such as G_q and $G_{12/13}$ subfamily proteins, because adenosine 5′-diphosphate ribosylation of $G\alpha_i$ has no effect on such increases[13,20]; however, the relative physiologic importance of $G\alpha_q$ and $G\alpha_{12/13}$ subfamily proteins in mediating this effect has not been determined. Although the Gα subfamily protein mediating Ca^{2+} sensitivity induced by histamine has not been examined, it is presumed to be a $G\alpha_q$ subfamily protein, because functional coupling between the histamine-1 receptor and $G\alpha_i$ or $G\alpha_{12/13}$ has not been demonstrated.[47]

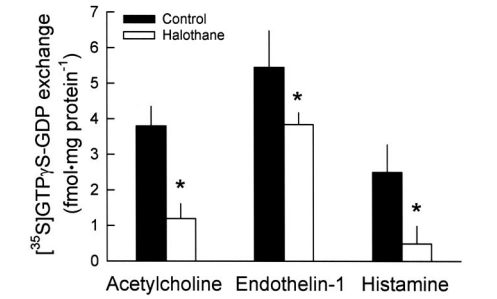

Fig. 7. Effect of halothane (0.39 ± 0.09 mM) on agonist-promoted exchange of the nonhydrolyzable, radioactive form of guanosine 5′-triphosphate (GTP), [^{35}S]GTPγS, for guanosine 5′-diphosphate (GDP) ([^{35}S]GTPγS–GDP exchange) at the α subunit of the $G_{q/11}$ heterotrimeric G protein. [^{35}S]GTPγS–GDP exchange was measured in the absence (basal exchange) or presence of half-maximal stimulation with exogenous acetylcholine (3.6 μM) or endothelin-1 (18.5 nM), or maximal stimulation with histamine (100 μM). Data are expressed as the increase in $G\alpha_{q/11}$ [^{35}S]GTPγS–GDP exchange above the basal exchange values. Data are presented as mean ± SD; n = 5. * Significant difference from control.

Assessment of nucleotide exchange at Gα in cellular membrane preparations from specific tissues provides a direct biochemical measure of the coupling between GPCRs and their associated heterotrimeric G proteins.[49] Using the technique described in the current study, the exchange of [^{35}S]GTPγS for GDP at Gα can be quantified, with subfamily specificity determined by the epitope to which the antibody is raised in the immunoprecipitation step. However, this experimental approach is limited by the amount of endogenous Gα subfamily protein of interest expressed in the tissue and the extent to which the protein dissociates from its associated GPCR and Gβγ dimer into solution with agonist binding. The original goal of the current study was to investigate anesthetic effects on the coupling between GPCRs and both $G\alpha_{q/11}$ and $G\alpha_{12/13}$ subfamily proteins. However, the expression level of $G\alpha_{12/13}$ in the porcine tracheal smooth muscle membrane preparation was very low, such that only small amounts of $G\alpha_{12}$ could be detected ($G\alpha_{13}$ could not be detected). Accordingly, the magnitude of the radioactivity of the basal and agonist-promoted nucleotide exchange measurements for $G\alpha_{12/13}$ was within the variability of the background radioactivity, thereby obfuscating our ability to test this hypothesis. Likewise, our inability to detect [^{35}S]GTPγS–GDP exchange at $G\alpha_s$ and $G\alpha_i$ is most likely due to insufficient G-protein expression levels.

The observation that acetylcholine, endothelin-1, and histamine each significantly increased $G\alpha_{q/11}$ nucleotide exchange demonstrated functional coupling between the muscarinic-3, endothelin-A and endothelin-B, and histamine-1 receptors with $G\alpha_{q/11}$ in porcine tracheal smooth muscle. 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[49] and was consistent with our previous observations using acetylcholine.[23] The rate of agonist-promoted nucleotide exchange was slower than that anticipated based on kinetic measurements of other heterotrimeric G protein–mediated signals obtained in intact, undisrupted cells or tissue, such as $[Ca^{2+}]_i$,[2,50]

and on measurements of isometric force in the permeabilized smooth muscle strips used in the current study. This was because the normally high ratio of GTP to GDP present in intact biologic systems (typically at least 100:1) was markedly reduced in the current biochemical assay, which markedly slows down the rate of GTP (or in this case, [^{35}S]GTPγS) exchange for GDP.[51] This compromise was necessary to detect receptor stimulation of a very small fraction of the $G\alpha_{q/11}$ coupled to the receptor of interest within a background of a substantially higher amount of free $G\alpha_{q/11}$ and $G\alpha_{q/11}$ coupled to other receptors.

The rate of basal, intrinsic nucleotide exchange at $G\alpha_{q/11}$ measured using either recombinant, pure protein[52,53] or crude membrane prepared from mammalian cells in which the receptor and the heterotrimer G-protein subunits have been enriched[44] is low. Although this was also true in the current study, the basal $G\alpha_{q/11}$ nucleotide exchange was still sufficient to conduct a reliable assessment of a possible anesthetic effect, even though the nonspecific background radioactivity was approximately 50% of the radioactivity of this measurement. Consistent with our previous work,[23] in the absence of receptor stimulation, neither halothane nor hexanol had an effect on basal, intrinsic $G\alpha_{q/11}$ [^{35}S]GTPγS–GDP exchange. This finding is in contrast to the work of Pentyala *et al.*,[34] who found that halothane and other volatile anesthetics modulated the binding of guanine nucleotides to recombinant Gα in aqueous solution, thereby inhibiting the exchange of GTPγS for GDP. They did not study $G\alpha_q$ subfamily proteins, because nucleotide exchange cannot be detectable in these purified subunits, unlike in membrane preparations as demonstrated by the current and previous studies.[23,44,46,54–56] 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 $G\alpha_{i\text{-}1}$ protein or endogenous $G\alpha_i$ in a porcine ASM membrane preparation.[33,36]

In contrast to the lack of an effect on basal exchange measurements, both halothane and hexanol significantly inhibited the increase in $G\alpha_{q/11}$ [^{35}S]GTPγS–GDP exchange induced by all three agonists in concentrations that produce anesthesia *in vivo* and ASM relaxation *in vitro*.[2,22] The experimental techniques used in the current study can provide only a functional assessment of the interaction between the GPCRs examined and $G\alpha_{q/11}$, and cannot directly ascertain with which of the possible protein targets, either receptor or $G_{q/11}$ heterotrimer subunit, the anesthetic molecules interacted to produce the observed effects. However, the data do enable us to formulate several plausible hypotheses. For example, it is possible that the anesthetic molecules interacted directly with the receptor only, as previously demonstrated for the rhodopsin receptor,[29,57] thereby only interfering with the ability of the contractile agonist to activate $G\alpha_{q/11}$ nucleotide exchange. If so, the similar effects observed for all three receptors implies that such an interaction would occur at a consensus site (or structure) common to all three receptors, rather than a site unique to the muscarinic receptor. Another interpretation of our data is that $G\alpha_{q/11}$ possesses an anesthetic binding region, such as at the receptor binding domain, which could interfere with receptor coupling, but has no effect on basal $G\alpha_{q/11}$ nucleotide exchange. The fact that the anesthetics had similar effects on agonist-promoted $G\alpha_{q/11}$ [^{35}S]GTPγS–GDP exchange regardless of which receptor was activated makes this potential mechanism more plausible than the former.

Clinically, bronchospasm may result from reflexes (such those activated by tracheal intubation) causing activation of muscarinic receptors by neurally released acetylcholine and from the release of mediators such as histamine and endothelin-1. The fact that the anesthetic effects on Ca^{2+} sensitivity and agonist-promoted nucleotide exchange are not limited to the muscarinic receptor suggests that they should have beneficial effects on bronchospasm induced by either category of mechanism.

In summary, halothane and hexanol decrease Ca^{2+} sensitivity in ASM at least in part by inhibiting receptor-promoted nucleotide exchange at $G\alpha_{q/11}$. Susceptibility to these anesthetic effects seems to be a general property of the GPCR–$G_{q/11}$ heterotrimeric G-protein complexes examined in the current study, because the effects on Ca^{2+} sensitivity and $G\alpha_{q/11}$ [^{35}S]GTPγS–GDP exchange were observed with activation of muscarinic, endothelin, and histamine receptors. These data suggest that the salient protein target might be the heterotrimeric G protein rather that the receptor, although anesthetic effects on receptors have not been ruled out. Therefore, during contraction of ASM with agonists that increase Ca^{2+} sensitivity, inhibition of agonist-induced guanosine nucleotide exchange contributes to the ability of anesthetics to relax ASM.

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