Anesthesiology 2006; 104:754–62

Halothane Does Not Inhibit the Functional Coupling between the β_2-Adrenergic Receptor and the $G\alpha_s$ Heterotrimeric G Protein

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Background: **This study investigated whether halothane affects the functional coupling between the β_2 adrenergic receptor and the α subunit of its cognate stimulatory heterotrimeric guanosine-5′-triphosphate (GTP)–binding protein ($G\alpha_s$). The authors hypothesized that halothane does not affect isoproterenol-promoted guanosine nucleotide exchange at $G\alpha_s$ and hence would not affect isoproterenol-induced relaxation of airway smooth muscle.**

Methods: **Halothane effects on isoproterenol-induced inhibition of calcium sensitivity were measured in permeabilized porcine airway smooth muscle. $G\alpha_s$ nucleotide exchange was measured in crude membranes prepared from COS-7 cells transfected to transiently coexpress the human β_1 or β_2 receptor each with human short $G\alpha_s$. A radioactive, nonhydrolyzable analog of GTP, [^{35}S]GTPγS, was used as the reporter for nucleotide exchange at $G\alpha_s$.**

Results: **Halothane (0.75 mM, approximately 2.8 minimum alveolar concentration [MAC] in pigs) did not affect isoproterenol-induced inhibition of calcium sensitivity. Isoproterenol caused a time- and concentration-dependent increase in $G\alpha_s$ nucleotide exchange. Halothane, even at concentrations of 1.5 mM (approximately 5.6 MAC), had no effect on basal $G\alpha_s$ nucleotide exchange in the absence of isoproterenol, whereas halothane inhibited isoproterenol-promoted $G\alpha_s$ nucleotide exchange in both the β_1–$G\alpha_s$ and β_2–$G\alpha_s$ expressing membranes. However, the effect was significantly greater on β_1–$G\alpha_s$ coupling compared with β_2–$G\alpha_s$ coupling, with no effect on β_2–$G\alpha_s$ coupling at 2.8 MAC halothane.**

Conclusion: **Halothane does not inhibit the biochemical coupling between the β_2 receptor and $G\alpha_s$ and hence does not affect the inhibition of calcium sensitivity induced by isoproterenol. Therefore, halothane should not affect the efficacy of β_2 agonists, as suggested by studies of *in vivo* animal models of asthma.**

β_2-ADRENERGIC receptor agonists are used to treat acute perioperative bronchospasm in patients with hyperreactive airway diseases, such as asthma.[1] Volatile anesthetics, particularly halothane, have also proven to be effective and safe therapeutic agents in this clinical setting, even after conventional therapy that included the use of β_2-receptor agonists had failed.[2–5] The mechanisms of this beneficial effect may include inhibition of inflammation,[6–9] attenuation of the airway neural reflex pathways,[10] and direct inhibition of airway smooth muscle constriction. The latter direct effect on the airway smooth muscle cell is produced by both a decrease in intracellular calcium concentration ($[Ca^{2+}]_i$) and a decrease in the force produced for a given $[Ca^{2+}]_i$ (*i.e.*, the Ca^{2+} sensitivity).[10–14]

There are conflicting reports concerning the efficacy of β_2-receptor agonists when administered in the presence of volatile anesthetics. In several studies using a variety of experimental models, halothane impaired β_2 receptor–mediated cell function,[15,16] *e.g.*, halothane markedly attenuated isoproterenol-induced relaxation of vascular smooth muscle.[16] This effect was considered to be due to inhibition of the β_2 receptor and/or its cognate stimulatory heterotrimeric guanosine-5′-triphosphate (GTP)–binding protein (G protein), because smooth muscle relaxation induced by activation of the signaling pathway distal to the G protein was not inhibited by halothane.[16] On the other hand, β_2-receptor agonists relieve bronchospasm during halothane anesthesia in animal models of asthma.[17] In addition, we found that halothane did not affect the ability of isoproterenol to relax isolated canine tracheal smooth muscle contracted with acetylcholine.[18] However, because halothane alters numerous other signaling systems in intact cells, this latter study could not be unambiguously interpreted in the context of direct anesthetic effects on β_2 receptor–$G\alpha_s$ coupling. Because halothane inhibits the coupling between the muscarinic receptor and the heterotrimeric G protein $G_{q/11}$,[19] similar effects on the β_2 receptor–$G\alpha_s$ complex could occur.

The purpose of the current study was to investigate whether halothane affects the functional coupling between the β_2 receptor and its cognate heterotrimeric G protein, G_s. We tested the hypothesis that halothane does not affect isoproterenol-promoted guanosine nucleotide exchange at the α subunit of G_s ($G\alpha_s$) and hence would not affect isoproterenol-induced decreases in Ca^{2+} sensitivity in permeabilized airway smooth muscle. Experiments were also conducted to assess the effect of halothane on the biochemical coupling between the β_1 receptor and $G\alpha_s$ as a positive control, because halothane is known to inhibit isoproterenol binding to this receptor[20] and hence should inhibit β_1–$G\alpha_s$ coupling.

Materials and Methods

Tissue Preparation

After obtaining approval from the Mayo Foundation Institutional Animal Care and Use Committee (Mayo

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Received from the Department of Anesthesiology, Mayo Foundation, Rochester, Minnesota. Submitted for publication April 22, 2005. Accepted for publication December 19, 2005. Supported in part by grant Nos. HL-45532 and HL-54757 from the National Institutes of Health, Bethesda, Maryland, and grants from the Mayo Foundation, Rochester, Minnesota.

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Foundation, Rochester, Minnesota), porcine tracheas were procured by euthanasia of research animals. The animals were first anesthetized by intramuscular injection of Telazol (10 ml/kg) (Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (6 mg/kg) and intravenous injection of Nembutal (400–600 mg) (Ovation Pharmaceuticals Inc., Deerfield, IL), and then killed by exsanguination *via* bilateral transection of the carotid arteries. Then, 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, the tracheal smooth muscle was cut into strips (0.8–1.2 cm long × 0.25–0.5 mm wide) for isometric force measurements.

Isometric Force Measurements and Permeabilization Procedure

Isometric force was measured in permeabilized smooth muscle strips using a previously described superfusion apparatus.[21,22] After setting each muscle strip at optimal length for maximal isometric force development, the strips were permeabilized with 2,500 U/ml *Staphylococcus aureus* α-toxin (20 min, 25°C) as previously described.[21–24] Solutions of varying free Ca^{2+} concentrations were prepared using the algorithm by Fabiato and Fabiato.[25]

Staphylococcus aureus α-toxin creates pores of approximately 26 A° 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 and relaxation 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 remains intact and can be activated, including β_2-adrenergic receptor coupling to adenylate cyclase *via* the $G\alpha_s$. This was confirmed in the permeabilized porcine airway smooth muscle preparation (data not shown) by demonstrating that inhibition of Ca^{2+} sensitivity by the β-adrenergic receptor agonist, isoproterenol, was blocked by the nonhydrolyzable form of guanosine-5′-diphosphate (GDP), GDPβS, and the adenosine 3′,5′-cyclic monophosphate (cAMP)–dependent protein kinase (cAK) inhibitor, adenosine-3′,5′-cyclic monophosphorothioate, Rp isomer (Rp-cAMPS). GDPβS prevents GTP–GDP exchange at Gα, whereas Rp-cAMPS, the nonhydrolyzable analog of cAMP, inhibits cAMP-induced activation of cAK by competing with cAMP for the binding site on cAK. Therefore, inhibition of isometric force by isoproterenol in this preparation is due entirely to inhibition of Ca^{2+} sensitivity (because $[Ca^{2+}]_i$ is "clamped" and does not change)[26] and is mediated by the classic signaling cascade of adenylate cyclase activation *via* $G\alpha_s$ and subsequent activation of cAK by cAMP.[27]

Culture and Transfection of COS-7 Cells

COS-7 cells (American Type Culture Collection, Manassas, VA) 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). The day before transfection, confluent cells were trypsinized and seeded in 10-cm tissue culture dishes so as to reach 90% confluence in 24 h (approximately 4.5×10^6 cells per 10-cm dish). The cells were then transiently transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as per the manufacturer's recommendation. Briefly, for each 10-cm plate transfection, the complementary DNA (cDNA) constructs were each mixed (5 min, room temperature) with 1.5 ml Opt-MEM I (Invitrogen) in a 50-ml falcon tube. For transfection of $G\alpha_s$ only, 5 μg cDNA plus 5 μg of the vector plasmic cDNA 3.1 were used for each 10-cm plate transfection; for cotransfection experiments, 5 μg β_1 or β_2 cDNA and 5 μg $G\alpha_s$ cDNA were used. Lipofectamine 2000 was mixed in another falcon tube with 1.5 ml Opti-MEM (2.5 μg/μl cDNA to be transfected). The two solutions were then mixed and allowed to stand for 20 min at room temperature to promote DNA–Lipofectamine complex formation. Three milliliters of the transfection mixture was added to each 10-cm plate with 5 ml 10% DMEM without penicillin/streptomycin. The transfection mixture was replaced with 7 ml fresh 10% DMEM plus penicillin/streptomycin after 12 h. 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 at 4°C). The cells were flash-frozen in liquid nitrogen and stored at −80°C until they were used to prepare crude membranes.

Crude Membrane Preparation

Crude membrane was prepared as previously described for tissue with several modifications.[19,28] Frozen cells from three 10-cm plates were suspended for 15 min in ice-cold lysis buffer (500 μl per plate) 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 by repeated passage through a 27-gauge needle (approximately 10–12 times). The lysate was then subjected to low-speed centrifugation (400*g*, 10 min, 4°C) to remove intact cells and nuclei and then to ultracentrifugation (87,000*g*, 30 min, 4°C) to pellet the crude membrane. The membrane pellet was washed with lysis buffer and resuspended in assay buffer (100 μl per plate) composed

of 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 4.8 mM $MgCl_2$, and 1 μM GDP, and the membrane protein concentration was determined.[29] The crude membrane suspension was then diluted with assay buffer to a protein concentration of 2 mg/ml, frozen in liquid nitrogen and stored at −70°C until used for the assay.

$G\alpha_s$ Nucleotide Exchange Assay

$G\alpha_s$ nucleotide exchange was assayed using methods originally described by Barr *et al.*[30] and previously used by our laboratory.[28] Reaction mixtures containing 10 μg membrane protein, 100 mM NaCl, 4.8 mM $MgCl_2$, and 1 μM GDP, with or without halothane and with or without isoproterenol, in a total volume of 62 μl were preincubated for 5 min at 30°C. The assay was initiated by the addition 5 μl of the radioactive, nonhydrolyzable form of GTP, [^{35}S]GTPγS, to the reaction mixture (1,250 Ci/mmol; 10 nM final concentration in assay). Reactions were terminated at times according to experimental design, and the supernatant was subjected to the immunoprecipitation step of the assay. Radioactivity was quantified by scintillation counting. 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. The amount of background radioactivity was less than 30% of the radioactivity of the basal $G\alpha_s$ nucleotide exchange measurements. Halothane had no effect on this nonspecific background radioactivity. Data were normalized to the amount of protein and the specific activity of the [^{35}S]GTPγS in the assay, and each experimental condition was assayed in triplicate.

Quantification of β-Adrenergic Receptor Isoforms

β_1 and β_2 were quantified in the crude membranes prepared from COS-7 cells by saturation binding with the tritium-labeled β_1/β_2 receptor antagonist [5,7-^{3}H](−)CGP-12177.[31,32] Specific binding was determined in triplicate assays with a single saturating concentration of [5,7-^{3}H](−)CGP-12177 of 6 nM. Nonspecific binding was determined in the presence of 5 μM propranolol. Aliquots of membranes containing 20 μg protein from COS-7 cells cotransfected with β_1 and $G\alpha_s$, β_2 and $G\alpha_s$, or untransfected COS-7 cells were incubated (90 min, 25°C) in a total volume of 0.5 ml containing 6 nM [5,7-^{3}H](−)CGP-12177, with or without 5 μM propranolol, 50 mM Tris, and 10 mM $MgCl_2$ (pH 7.4). After the 90-min incubation, reactions were applied to prewetted Whatman GF/B filters using a Brandel cell harvester (Gaithersburg, MD) and washed three times with 10 ml Tris, 50 mM, pH 7.4, and 10 mM $MgCl_2$. Bound radioactivity on the filters was quantified by scintillation counting.

Preparation and Delivery of Halothane

For physiologic studies of permeabilized airway smooth muscle strips in the superfusion apparatus, halothane was added to the aqueous buffers *via* a calibrated halothane vaporizer by vigorous aeration of the solutions as previously described.[11,12,33] For biochemical studies of $G\alpha_s$ nucleotide exchange, saturated aqueous stocks of halothane were prepared and added directly to the reaction tubes in volumes that produced the desired final anesthetic concentration.[34–37] To account for the unavoidable rapid loss of halothane 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, Massachusetts, model 5880A) according to the method of Van Dyke and Wood[38] and previously used by our laboratory[24,28,35,39,40] In preliminary studies, we found that after an initial loss of approximately 30% due to transfer and mixing, the amount of halothane in the reaction tube was relatively stable, with less than 5% additional loss during the longest assay time in this report (5 min).

Experimental Protocols

Effect of Halothane on Isoproterenol-induced Inhibition of Ca^{2+} Sensitivity. The permeabilized preparation was chosen as our physiologic model for this study because the potential confounding, independent effects of both isoproterenol[27] and halothane[11,14] on $[Ca^{2+}]_i$ are excluded. We previously documented in this preparation that in contrast to intact tissue where volatile anesthetics inhibit airway smooth muscle contraction in part by inhibiting $[Ca^{2+}]_i$, halothane has no effect on isometric force (and hence, Ca^{2+} sensitivity) when contractions are induced by free Ca^{2+} alone.[24,26,39,41] Therefore, any effects of halothane on relaxation induced by isoproterenol is due only to inhibition of Ca^{2+} sensitivity. We chose to study halothane concentrations that approached the upper range (approximately 3 minimum alveolar concentration [MAC]) previously shown to cause bronchodilation in patients[1–4] and animal models[10,42,43] and inhibit airway smooth muscle contraction and Ca^{2+} sensitivity induced by muscarinic agonists *in vitro*.[11–14,24,26,33,41]

An example of the experimental protocol is shown in figure 1. After permeabilization of a pair of strips obtained from the same animal, one strip was superfused with relaxing solution containing 0.73 ± 0.17 mM halothane (2.7 ± 0.7 MAC for pigs),[37] which was maintained for the duration of the protocol. The second strip was not exposed to halothane and served as a control for the effect of isoproterenol on isometric force. Then, both strips were contracted by superfusion with solution containing 180 nM free Ca^{2+} plus 1 μM GTP for 15 min, which, in preliminary studies, induced contractions (referred to hereafter as "initial force") of 40–50% of max-

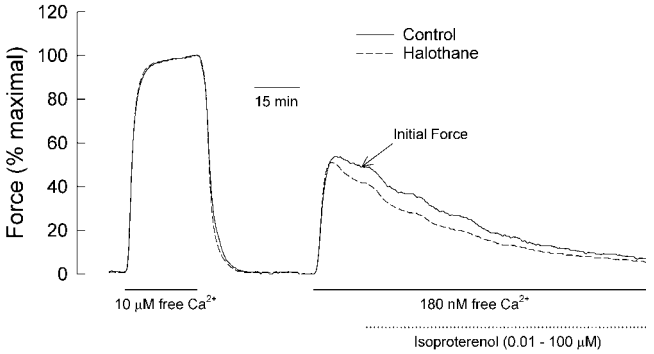

Fig. 1. Representative tracings of the experimental protocol designed to determine the effect of halothane on isoproterenol-induced inhibition of isometric force in porcine tracheal smooth muscle strips permeabilized with *Staphylococcus aureus* α-toxin. See text for description of the experimental protocol.

imal force that were stable for up to 2 h. Concentrations of GTP less than 5 μM do not induce changes in isometric force induced by free Ca^{2+} alone (preliminary data not shown). Finally, concentration–response curves were generated for isoproterenol (0.1–100 μM) with both strips during constant activation with 180 nM free Ca^{2+} plus 1 μM GTP by increasing the isoproterenol concentration in the superfusate every 15 min. The effect of isoproterenol on isometric force is expressed as a percentage of the initial forces induced by 180 nM free Ca^{2+}.

Effect of Isoproterenol on $G\alpha_s$ [^{35}S]GTPγS–GDP Exchange. These studies were conducted to determine the time course for [^{35}S]GTPγS incorporation into $G\alpha_s$ and the concentrations of isoproterenol that produce half-maximal or maximal promotion of $G\alpha_s$ [^{35}S]GTPγS–GDP exchange. These data were then used to guide the design of subsequent protocols to examine the effect of halothane on $G\alpha_s$ [^{35}S]GTPγS–GDP exchange. To determine the time course for $G\alpha_s$ [^{35}S]GTPγS–GDP exchange, crude membrane prepared from β_1–$G\alpha_s$ or β_2–$G\alpha_s$ cotransfected COS-7 cells were incubated for 5 min without (for basal [^{35}S]GTPγS–GDP exchange measurements) or with (for isoproterenol-promoted [^{35}S]GTPγS–GDP exchange) 10 nM isoproterenol, and then the reactions were initiated with [^{35}S]GTPγS. The reactions were terminated after 1, 2, 5, or 10 min, and then the samples were subjected to the immunoprecipitation step of the assay.

To determine the isoproterenol concentration that produced half-maximal and maximal promotion of $G\alpha_s$ [^{35}S]GTPγS–GDP exchange, crude membrane prepared from β_1–$G\alpha_s$ or β_1–$G\alpha_s$ cotransfected COS-7 cells were incubated without or with various concentrations of isoproterenol (0.01–1,000 nM) for 5 min. The reactions were then terminated 10 min after initiation with [^{35}S]GTPγS, and the samples were subjected to the immunoprecipitation step of the assay. The isoproterenol-promoted increase in $G\alpha_s$ [^{35}S]GTPγS–GDP exchange was expressed as a percentage of the difference between the basal exchange values (measured in the absence of isoproterenol) and those measured in the presence of the isoproterenol concentration that produced the maximal effect.

Characterization of $G\alpha_s$ Nucleotide Exchange Assay. To determine the dependence of isoproterenol-promoted $G\alpha_s$ [^{35}S]GTPγS–GDP exchange on expression of the human β_1 or β_2 adrenergic receptor (referred hereafter as β_1 or β_2, respectively), measurements were made using crude membrane prepared from COS-7 cells transfected with cDNA for human $G\alpha_s$ only, or cotransfected with the cDNAs for β_1 and $G\alpha_s$, or β_2 and $G\alpha_s$. The crude membrane was incubated with or without 10 nM isoproterenol for 5 min, and then the reactions were initiated with [^{35}S]GTPγS. The reactions were terminated after 5 min, and the samples were subjected to the immunoprecipitation step of the assay.

Effect of Halothane on [^{35}S]GTPγS–GDP Exchange. Crude membrane prepared from β_1–$G\alpha_s$ or β_2–$G\alpha_s$ cotransfected COS-7 cells were incubated without or with 10 nM isoproterenol for 5 min. Then, the samples were incubated with 0.75 or 1.5 mM halothane (approximately 2.8 and 5.6 MAC for pigs, respectively)[37] for an additional 5 min, and the reactions were initiated with [^{35}S]GTPγS. Finally, the reactions were terminated after 5 min, and the samples were subjected to the immunoprecipitation step of the assay.

Materials

The cDNAs for the human β_1 and β_2 receptors and human $G\alpha_s$ in the expression vector plasmic cDNA 3.1 were obtained from the University of Missouri-Rolla cDNA Resource Center.‖ Adenosine-5′-triphosphate disodium salt was purchased from Research Organics, Inc. (Cleveland, OH). Halothane was purchased from Ayerst Laboratories, Inc. (New York, NY). *Staphylococcus aureus* α-toxin and rabbit nonimmune serums were purchased from Calbiochem (EMD Biosciences, Inc. Affiliate, San Diego, CA). The $G\alpha_s$ rabbit polyclonal antiserum was produced by Covance Research Products (Denver, PA) using a synthetic decapeptide that corresponds to the carboxy-terminal sequence for human $G\alpha_s$ (RMHLRQYELL). This antiserum is highly specific for $G\alpha_s$ and does not cross-react with recombinant, purified $G\alpha_i$ or $G\alpha_q$ protein. Protein A-agarose beads were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Lowry protein assay kits were purchased from Bio-Rad Life Science Research Produces (Hercules, CA). 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.

‖ www.cDNA.org. Posted August 1, 2005.

Data Analysis and Statistics

Data are reported as mean ± SD; n represents the number of animals studied or independent times an assay was performed. For concentration–response curves, EC_{50} and maximal agonist concentrations were determined by nonlinear regression analysis as described by Meddings *et al.* [44] In this method, a dependent variable (y), such as isometric force or $G\alpha_s$ [^{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 $G\alpha_s$ [^{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 $G\alpha_s$ immunoprecipitated from solution (y), the parameter k is the rate of $G\alpha_s$ [^{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 effect of halothane $G\alpha_s$ [^{35}S]GTPγS–GDP exchange. For all statistical comparisons, a value of $P < 0.05$ was considered significant.

Results

Effect of Halothane on Isoproterenol-induced Inhibition of Ca^{2+} Sensitivity

Increasing the free Ca^{2+} concentration in the superfusate from 1 to 180 nM in the presence of 1 μM GTP caused a sustained increase in isometric force to 47.7 ± 9.3% of maximal force induced by 10 μM free Ca^{2+} (fig. 1). The presence 0.73 ± 0.17 mM (2.7 ± 0.7 MAC) halothane in the superfusate had no significant effect on this contraction (41.6 ± 9.6% of maximal isometric force; $P = 0.40$). The subsequent addition of isoproterenol to the superfusate caused a concentration-dependent inhibition of isometric force, which was not significantly attenuated by halothane (figs. 1 and 2). The EC_{50} values for inhibition of Ca^{2+} sensitivity by isoproterenol were 0.38 ± 0.15 and 0.25 ± 0.05 nM for curves generated in the absence and presence of halothane, respectively ($P = 0.13$). Likewise, there was no significant effect of halothane on maximal inhibition of Ca^{2+} sensitivity by 100 μM isoproterenol (17.1 ± 4.3 and 13.2 ± 6.6% of initial force for curves generated in the absence or presence of halothane, respectively; $P = 0.36$).

Effect of Isoproterenol on $G\alpha_s$ [^{35}S]GTPγS–GDP Exchange

In the absence of isoproterenol, membranes prepared from COS-7 cells cotransfected with β_1–$G\alpha_s$ (fig. 3A) or

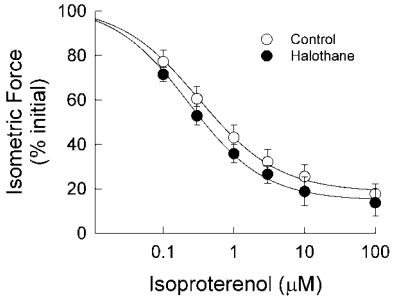

Fig. 2. Effect of halothane (0.73 ± 0.08 mM; 2.7 ± 0.9 minimum alveolar concentration) on isoproterenol-induced inhibition of isometric force in porcine tracheal smooth muscle permeabilized with *Staphylococcus aureus* α-toxin. See text for description of the experimental protocol, which is depicted in figure 1. Effect of isoproterenol on isometric force is expressed as a percentage of the initial force induced by 0.18 μM free Ca^{2+} plus 1 μM GTP. Data are mean ± SD; n = 5.

β_2–$G\alpha_s$ (fig. 3B) incorporated [^{35}S]GTPγS into $G\alpha_s$ in a time-dependent manner with apparent rate constants of k_{app} of 0.045 ± 0.054 and 0.053 ± 0.061 min^{-1}, respectively. There was no significant difference in k_{app} between the two preparations ($P = 0.8$), whereas the maximal exchange value, a, measured at 10 min was significantly greater with membrane prepared from the β_2–$G\alpha_s$ cotransfected cells (fig. 3).

The presence of 10 nM isoproterenol in the assay buffer promoted $G\alpha_s$ [^{35}S]GTPγS–GDP exchange, increasing k_{app} in both preparations by approximately fourfold to fivefold to 0.294 ± 0.024 and 0.351 ± 0.066 fmol/min for membranes prepared from β_1–$G\alpha_s$ and β_2–$G\alpha_s$ cotransfected cells, respectively; these values were not significantly different ($P = 0.25$). The value of a was significantly greater in membrane prepared from the β_2–$G\alpha_s$ cotransfected cells, whereas the relative magnitude of the maximal increase in $G\alpha_s$ [^{35}S]GTPγS–GDP exchange above basal values was similar between the two preparations, approximately threefold. For both preparations, the isoproterenol-promoted increase in

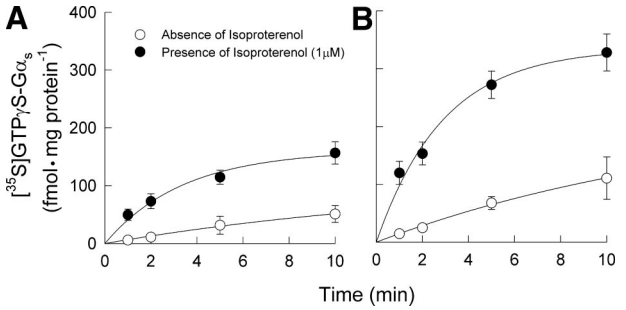

Fig. 3. Time-dependent change in exchange of the radioactive, nonhydrolyzable form of guanosine-5′-triphosphate (GTP), [^{35}S]GTPγS, for guanosine-5′-diphosphate (GDP) ([^{35}S]GTPγS–GDP exchange) at the α subunit of the G_s heterotrimeric G protein. $G\alpha_s$ [^{35}S]GTPγS–GDP exchange was measured in the absence and presence of 10 nM isoproterenol using crude membranes prepared from COS-7 cells cotransfected with the complementary DNAs encoding for β_1 and $G\alpha_s$ (*A*) or β_2 and $G\alpha_s$ (*B*). The reactions were terminated 1, 2, 5, and 10 min after initiation of the assays with [^{35}S]GTPγS. Data are mean ± SD; n = 4.

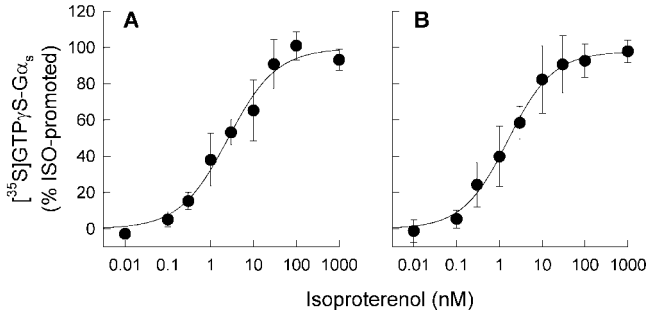

Fig. 4. Concentration-dependent effect of isoproterenol (ISO) on the exchange of the radioactive, nonhydrolyzable form of guanosine-5′-triphosphate (GTP), [^{35}S]GTPγS, for guanosine-5′-diphosphate (GDP) ([^{35}S]GTPγS–GDP exchange) at the α subunit of the G_s heterotrimeric G protein. $G\alpha_s$ [^{35}S]GTPγS–GDP exchange was measured in crude membranes prepared from COS-7 cells cotransfected with the complementary DNAs encoding for β_1 and $G\alpha_s$ (*A*) or β_2 and $G\alpha_s$ (*B*). Assays were performed in the absence and presence of isoproterenol (0.01–1,000 nM), and the reactions were terminated 5 min after initiation of the assays with [^{35}S]GTPγS. The isoproterenol-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 isoproterenol and that measured in the presence of the isoproterenol concentration that produced the maximal effect. Data are mean ± SD; n = 4.

$G\alpha_s$ [^{35}S]GTPγS–GDP exchange above basal exchange was concentration dependent, with EC_{50} values of 2.8 ± 0.4 and 2.4 ± 0.4 nM for membranes prepared from β_1–$G\alpha_s$ (fig. 4A) and β_2–$G\alpha_s$ (fig. 4B) cotransfected cells, respectively; these values were not significantly different ($P = 0.39$).

Characterization of $G\alpha_s$ Nucleotide Exchange Assay

The endogenous levels of β_1/β_2 receptor in the crude membranes prepared from untransfected COS-7 cells were insignificant, expressed at levels barely above the nonspecific background of the assay (< 30 fmol/mg protein). The amount of β_1 or β_2 receptor in the crude membranes prepared from COS-7 cells cotransfected with β_1–$G\alpha_s$ or β_2–$G\alpha_s$ were similar (approximately 15 pmol/mg protein each). In membranes prepared from COS-7 cells transfected with the cDNA for $G\alpha_s$ only, a significant increase in $G\alpha_s$ [^{35}S]GTPγS–GDP exchange was measured above that of the nonspecific background measurements (data not shown). This increase in $G\alpha_s$ [^{35}S]GTPγS–GDP exchange was not promoted by the inclusion of 10 nM isoproterenol in the assay buffer (fig. 5). This was also true in studies using membranes prepared from COS-7 cells transfected with the β_1 or β_2 receptors only (*i.e.*, no $G\alpha_s$ transfection; preliminary data not shown). In membranes prepared from COS-7 cells cotransfected with β_1 and $G\alpha_s$ or β_2 and $G\alpha_s$, $G\alpha_s$ [^{35}S]GTPγS–GDP exchange in the absence of isoproterenol was significantly greater than that measured in membranes prepared from $G\alpha_s$-only transfected cells. The inclusion of 10 nM isoproterenol in the assay buffer caused an additional approximately threefold increase in the magnitude of this exchange with both membrane preparations (fig. 5).

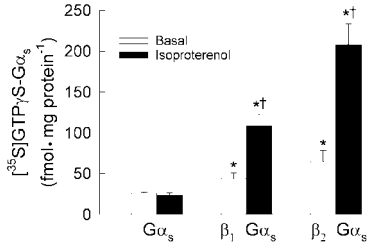

Fig. 5. Effect of isoproterenol on the exchange of the radioactive, nonhydrolyzable form of guanosine-5′-triphosphate (GTP), [^{35}S]GTPγS, for guanosine-5′-diphosphate (GDP) ([^{35}S]GTPγS–GDP exchange) at the α subunit of the G_s heterotrimeric G protein. $G\alpha_s$ [^{35}S]GTPγS–GDP exchange was measured in crude membranes prepared from COS-7 cells transfected with the complementary DNAs encoding for $G\alpha_s$ only, or β_1 and $G\alpha_s$, or β_2 and $G\alpha_s$. $G\alpha_s$ [^{35}S]GTPγS–GDP exchange was measured in the absence or presence of 10 nM isoproterenol. The reactions were terminated 5 min after initiation of the assays with [^{35}S]GTPγS. Data are mean ± SD; n = 3. * Significant difference from $G\alpha_s$ [^{35}S]GTPγS–GDP exchange measured using crude membranes prepared from COS-7 cells transfected with the complementary DNAs encoding for the $G\alpha_s$ only. † Significant difference from $G\alpha_s$ [^{35}S]GTPγS–GDP exchange measured in the absence of isoproterenol (basal exchange).

Effect of Halothane on $G\alpha_s$ Nucleotide Exchange

The presence of 2.8 (fig. 6A) or 5.6 (fig. 6B) MAC halothane in the assay had no significant effect on $G\alpha_s$ [^{35}S]GTPγS–GDP exchange when the assays were performed in the absence of isoproterenol with either the β_1–$G\alpha_s$ or β_2–$G\alpha_s$ membrane preparation. In crude membrane prepared from β_2–$G\alpha_s$ cotransfected cells, the presence of 5.6 MAC halothane in the assay significantly inhibited the increase in [^{35}S]GTPγS–GDP exchange promoted by 10 nM isoproterenol, although only by approximately 15% of control (fig. 7). The presence

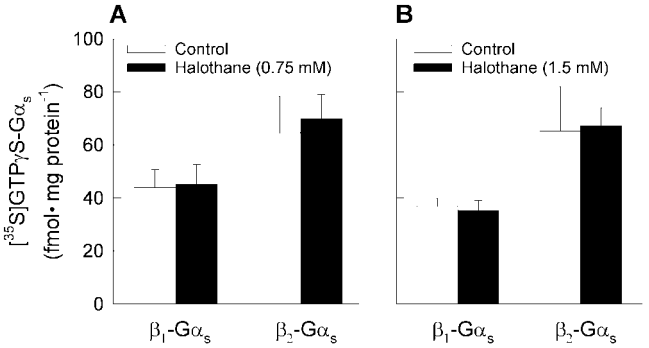

Fig. 6. Effect of 0.75 mM (approximately 2.8 minimum alveolar concentration; *A*) or 1.5 mM (approximately 5.6 minimum alveolar concentration; *B*) halothane on the exchange of the radioactive, nonhydrolyzable form of guanosine-5′-triphosphate (GTP), [^{35}S]GTPγS, for guanosine-5′-diphosphate (GDP) ([^{35}S]GTPγS–GDP exchange) at the α subunit of the G_s heterotrimeric G protein. $G\alpha_s$ [^{35}S]GTPγS–GDP exchange was measured in the absence of isoproterenol using crude membranes prepared from COS-7 cells cotransfected with the complementary DNAs encoding for β_1 and $G\alpha_s$ (*A*) or β_2 and $G\alpha_s$ (*B*). The reactions were terminated 5 min after initiation of the assays with [^{35}S]GTPγS. Data are mean ± SD; n = 9.

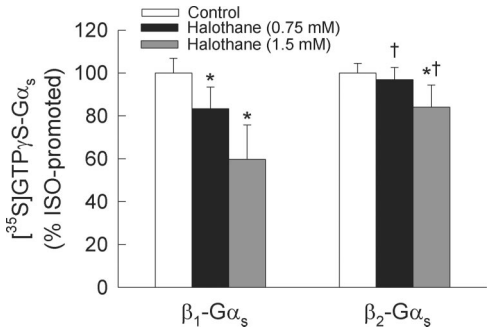

Fig. 7. Effect of 0.75 mM (approximately 2.8 minimum alveolar concentration) or 1.5 mM (approximately 5.6 minimum alveolar concentration) halothane on the exchange of the radioactive, nonhydrolyzable form of guanosine-5′-triphosphate (GTP), [^{35}S]GTPγS, for guanosine-5′-diphosphate (GDP) ([^{35}S]GTPγS–GDP exchange) at the α subunit of the G_s heterotrimeric G protein. $G\alpha_s$ [^{35}S]GTPγS–GDP exchange was measured in the presence of 10 nM isoproterenol (ISO) using crude membranes prepared from COS-7 cells cotransfected with the complementary DNAs encoding for β_1 and $G\alpha_s$, or β_2 and $G\alpha_s$. The isoproterenol-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 isoproterenol or halothane, and that measured in the presence of the 10 nM isoproterenol. The reactions were terminated 5 min after initiation of the assays with [^{35}S]GTPγS. Data are mean ± SD; n = 9. * Significant difference from $G\alpha_s$ [^{35}S]GTPγS–GDP exchange measured in the absence of halothane. † Significant difference from $G\alpha_s$ [^{35}S]GTPγS–GDP exchange measured at the same halothane concentration in membranes prepared from COS-7 cells cotransfected with the complementary DNAs encoding for β_1 and $G\alpha_s$.

of 2.8 MAC halothane in the assay had no significant effect on isoproterenol-promoted $G\alpha_s$ [^{35}S]GTPγS–GDP exchange. By contrast, halothane was significantly more effective in inhibiting the isoproterenol-stimulated incorporation of [^{35}S]GTPγS into $G\alpha_s$ when the assays were performed with the β_1–$G\alpha_s$ membrane preparation. In these assays, halothane caused a concentration-dependent inhibition of isoproterenol-promoted $G\alpha_s$ [^{35}S]GTPγS–GDP exchange (fig. 7). These effects were significantly greater at both 2.8 and 5.6 MAC halothane compared with measurements performed with membrane prepared from β_2–$G\alpha_s$ cotransfected cells.

Discussion

The major finding of this study is that halothane does not inhibit the biochemical coupling of the β_2-receptor to its cognate heterotrimeric G protein, $G\alpha_s$. This finding is consistent with the observation that halothane also had no effect on isoproterenol-induced inhibition of Ca^{2+} sensitivity in airway smooth muscle.

The β-adrenergic receptor is a member of the seven-transmembrane spanning domain family of receptors that regulate cell signaling *via* activation of heterotrimeric G proteins. There are at least three β-adrenergic receptor genes, ADRB1, ADRB2, and ADRB3, which encode the β_1-, β_2-, and β_3-adrenergic receptors, respectively. The β_1-, β_2-, and β_3-adrenergic receptors were classically identified in cardiac tissue, airway smooth muscle, and adipose tissue, respectively.[27,45] These receptors regulate numerous cellular functions through their cognate heterotrimeric G protein, G_s, which is comprised of an α, β, and γ subunit. It is through the GTP-bound $G\alpha_s$ that β receptors are coupled to the signaling pathways that ultimately relax the airway smooth muscle cell both by decreasing $[Ca^{2+}]_i$[46] and Ca^{2+} sensitivity.[47]

There have been conflicting reports regarding whether volatile anesthetics inhibit cell signaling mediated by the β_2 receptor. Halothane impaired β_2 receptor–mediated cell signaling and function in some studies.[15,16] For example, in one *in vitro* study of isolated rat aorta (which predominately expresses the β_2 isoform), decreases in isometric force and $[Ca^{2+}]_i$ and increases in intracellular cAMP levels induced by isoproterenol were significantly attenuated by halothane. When adenylate cyclase was activated directly with forskolin, the effects of halothane were minimal. Furthermore, isoproterenol binding to the β_2 receptor was not affected by halothane. The authors concluded that volatile anesthetics interfered with β_2 receptor–mediated signaling at sites distal to isoproterenol–β_2 receptor binding but proximal to adenylate cyclase activation,[16] such as the G_s heterotrimer. Other studies demonstrated no apparent effect of volatile anesthetics on β_2 receptor–mediated cell function. For example, in a dog model of asthma where bronchoconstriction was induced by histamine, the β_2 receptor–specific agonist albuterol was similarly effective in reducing pulmonary airway resistance and increasing dynamic lung compliance in the presence or absence of halothane.[17] Halothane also had no effect on relaxation of intact, isolated canine airway smooth muscle induced by isoproterenol.[18] However, this study could not be unambiguously interpreted in context of anesthetic effects on the biochemical coupling between the β_2 receptor and $G\alpha_s$, because halothane has several additional confounding effects on signal transduction in intact tissue. For example, halothane inhibits muscarinic coupling to one of its cognate heterotrimeric G proteins, $G\alpha_{q/11}$.[19,28]

To more directly assess whether halothane inhibits β_2 receptor coupling to $G\alpha_s$, we used both a physiologic and a biochemical model (the permeabilized airway smooth muscle model and the $G\alpha_s$ nucleotide exchange assay, respectively). In contrast to studies of living animals or intact, isolated tissue, the experimental protocol using the permeabilized airway smooth muscle model minimized the possible confounding influence of halothane effects on other systems, because halothane has no effect on isometric force in this preparation when induced by free Ca^{2+} alone[19,22,24,26,39,41]; this was confirmed in the current study (fig. 1). Using these experimental models, approximately 2.8 MAC halothane had no significant effect on the decrease in Ca^{2+} sensitivity

induced by isoproterenol. In addition, there was no effect of 2.8 MAC halothane on $G\alpha_s$ [^{35}S]GTPγS–GDP exchange when measured either in the absence or presence of isoproterenol using membranes prepared from β_2–$G\alpha_s$ transfected COS-7 cells. These results are consistent with a ligand binding study of human lymphocytes (which express predominantly β_2 receptors) showing that the β_2 receptor and a measure of its immediate interactions with the G_s heterotrimer (*i.e.*, high-affinity ligand binding) were largely unaffected by halothane.[48] There was a small but statistically significant effect of 5.6 MAC halothane on isoproterenol-promoted $G\alpha_s$ [^{35}S]GTPγS–GDP exchange, but this concentration is beyond that achieved during the management of severe bronchospasm. It is plausible that the conflicting observations on the effects of halothane seen in physiologic studies[15,16] may be due to differences in indirect effects on the β_2 receptor as proposed by Saito *et al.*,[49] including halothane-induced β_2-receptor phosphorylation and desensitization *via* activation of intracellular receptor kinases.

The results of our nucleotide exchange assays showing differences in the sensitivity to halothane of isoproterenol-promoted coupling of β_1 compared with β_2 coupling to $G\alpha_s$ may have mechanistic implications for the effects of volatile anesthetics on heptahelical receptor–heterotrimeric G protein complexes. We observed an approximately threefold greater inhibition of β_1–$G\alpha_s$ coupling at both halothane concentrations. An early radioligand binding study by Bohm *et al.*[50] demonstrated that 1–2 MAC halothane inhibited high-affinity binding (*i.e.*, the active receptor conformation) of isoproterenol to the β_1 receptor in human myocardial membranes but had a minimal effect on low-affinity ligand binding determined in the presence of 100 μM of the GTP analog GPP(NH)P. These results were interpreted as an inhibitory effect on β_1 receptor–G_s heterotrimer interaction without a direct effect on the receptor itself. In a more detailed competitive radioligand binding study on membranes prepared from cardiac myocytes, 1–2.8 MAC halothane, in a concentration-dependent manner, also significantly reduced the percentage of receptors in the high-affinity conformation.[20] However, contrary to the study of Bohm *et al.*, this study found that halothane also inhibited isoproterenol affinity in the presence of GPP(NH)P (*i.e.*, also inhibited low-affinity ligand binding). The fact that an effect was seen in the presence of GPP(NH)P would seem to indicate a direct effect of halothane on the β_1 receptor conformation independent of the G_s heterotrimer. However, care must be taken when interpreting allosteric effects on competitive binding assays, because an unnoticed effect of halothane on the radiolabeled antagonist binding can lead to incorrect conclusions about the contribution of the G-protein heterotrimer to the effect.

Our results clearly demonstrate an inhibitory effect of halothane (approximately 2.8 MAC) on isoproterenol-promoted $G\alpha_s$ [35S]GTPγS–GDP exchange in measurements made using membranes prepared from the β_1–$G\alpha_s$ cotransfected cells, as would be predicted from the previous radiolabeled ligand binding studies that showed halothane effects on high-affinity isoproterenol binding to the β_1 receptor.[20,50] This inhibitory effect may be due to a direct effect on the β_1 receptor, as previously demonstrated for the rhodopsin receptor,[51] or its local membrane environment. This type of receptor-dependent effect would be consistent with the apparent greater effect of halothane on β_1 *versus* β_2 receptor, which are both coupled $G\alpha_s$, although a differential sensitivity of the β_2 receptor to a halothane effect on the G_s heterotrimer could also explain our results. Alternatively, it is possible that a unique anesthetic binding region created at the interface between the β-receptor isoform and $G\alpha_s$ confers sensitivity to halothane.

Pentyala *et al.*[34] demonstrated that volatile anesthetics, even at subanesthetic concentrations, inhibited the steady state [^{35}S]GTPγS–GDP exchange of cholate solubilized, purified bovine brain $G\alpha_s$. Such a direct effect if it persisted in the native membrane environment when $G\alpha_s$ is bound to the G$\beta\gamma$ and the β receptor would be predicted to abolish both basal activity of $G\alpha_s$ and isoproterenol-promoted $G\alpha_s$ nucleotide exchange regardless of which β-receptor isoform was activated. The results of our experiments, namely that halothane, even at very high concentrations (5.6 MAC), did not inhibit basal $G\alpha_s$ nucleotide exchange, and the finding of a marked difference in the magnitude of halothane inhibition of β_1 *versus* β_2 coupling to the same $G\alpha_s$ are both inconsistent with the simple explanation of direct inhibition of $G\alpha_s$. These data suggest that the interactions of $G\alpha_s$ with its native membrane environment, the G$\beta\gamma$ dimer, or receptor prevent a direct effect on halothane $G\alpha_s$.[34]

The clinical significance of the findings of this study is readily evident. The relative balance between the neurologic and hormonal mediators that induce airway smooth muscle constriction and relaxation determines airway smooth muscle tone. Numerous types of receptors mediate airway smooth muscle constriction, whereas the β-adrenergic receptor is the best-characterized receptor that mediates airway smooth muscle relaxation. Combined with the previous findings that halothane inhibits muscarinic receptor–heterotrimeric G protein coupling[19,28] and hence the increase in Ca^{2+} sensitivity and contraction of isolated airway smooth muscle induced by muscarinic receptor agonists,[12–14,24,26,33,39–41] the current findings that β_2 receptor coupling to $G\alpha_s$ and hence the downstream signaling pathways that decreases Ca^{2+} sensitivity is spared make volatile anesthetics an ideal therapeutic agent for the treatment of perioperative bronchospasm. Signaling systems that induce bronchoconstriction are attenuated, whereas β_2 receptor–mediated signaling induced by bronchodilators are unaffected by halothane.

In summary, the current study provides evidence that concentrations of halothane typically achieved during treatment of acute perioperative bronchospasm or exacerbation of asthma do not prevent the biochemical coupling between the β_2 receptor and its associated heterotrimeric G protein, $G\alpha_s$. This is supported by the physiologic studies showing that halothane had no effect on the inhibition of Ca^{2+} sensitivity induced by isoproterenol and indicate that halothane would not prevent the efficacy of β_2 agonists when used to treat perioperative bronchospasm.

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