

## Effect of Halothane on $G\alpha_{i-3}$ and Its Coupling to the $M_2$ Muscarinic Receptor

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**Background:** Halothane is an effective bronchodilator and inhibits airway smooth muscle contraction in part by inhibiting intracellular signaling pathways activated by the  $M_2$  muscarinic receptor and its cognate inhibitory heterotrimeric guanosine-5'-triphosphate (GTP)-binding protein (G protein),  $G_i$ . This study hypothesized that halothane inhibits nucleotide exchange at the  $\alpha$  isoform-3 subunit of  $G_i$  ( $G\alpha_{i-3}$ ), but only when regulated by the  $M_2$  muscarinic receptor.

**Methods:** GTP hydrolysis by  $G\alpha_{i-3}$  and the  $G\alpha_{i-3}\beta_1\gamma_{2HF}$  heterotrimer expressed in *Spodoptera frugiperda* cells was measured using a phosphohydrolase assay with [ $\gamma^{32}P$ ]GTP. Anesthetic binding to  $G\alpha_{i-3}$  was measured by saturation transfer difference nuclear magnetic resonance spectroscopy.  $G\alpha_{i-3}$  nucleotide exchange was measured in crude membranes prepared from COS-7 cells transiently coexpressing the  $M_2$  muscarinic receptor and  $G\alpha_{i-3}$ . A radioactive analog of GTP, [ $^{35}S$ ]GTP $\gamma$ S, was used as a reporter for  $G\alpha_{i-3}$  nucleotide exchange.

**Results:** Although spectroscopy demonstrated halothane binding to  $G\alpha_{i-3}$ , this binding had no effect on [ $\gamma^{32}P$ ]GTP hydrolysis by the  $G\alpha_{i-3}\beta_1\gamma_{2HF}$  heterotrimer expressed in *Spodoptera frugiperda* cells, nor basal  $G\alpha_{i-3}$  nucleotide exchange measured in crude membranes when the muscarinic receptor agonist acetylcholine was omitted from the assay. Conversely, halothane caused a concentration-dependent inhibition of  $G\alpha_{i-3}$  nucleotide exchange with acetylcholine included in the assay.

**Conclusion:** These data indicate that despite halothane binding to  $G\alpha_{i-3}$ , halothane has no direct inhibitory effect on the intrinsic activity of the  $G\alpha_{i-3}\beta_1\gamma_{2HF}$  heterotrimer but inhibits  $M_2$  muscarinic receptor regulation of the heterotrimer. This novel effect is consistent with the ability of halothane to inhibit airway smooth muscle contraction and bronchoconstriction induced by acetylcholine.

VOLATILE anesthetics are potent bronchodilators that can effectively reverse severe perioperative bronchospasm in patients with hyperreactive airway diseases, such as asthma.<sup>1-4</sup> This beneficial effect is due in significant part to a direct inhibitory effect on the airway smooth muscle (ASM) cell.<sup>5-7</sup> Although the mechanisms of this direct effect is not fully known, considerable evidence indicates that volatile anesthetics inhibit the increase in cytoplasmic calcium ( $Ca^{2+}$ ) concentration ( $[Ca^{2+}]_c$ )<sup>8-13</sup> and activation of the signaling proteins that regulate the amount of force at a given  $[Ca^{2+}]_c$  (i.e.,  $Ca^{2+}$

sensitivity)<sup>9,11,14-19</sup> induced by physiologic agonists, including acetylcholine.

Agonists mediate ASM contractile state by activating signaling cascades *via* their cognate heterotrimeric guanosine 5'-triphosphate (GTP) binding protein (G-protein)-coupled receptors. The GTP-bound form of the  $\alpha$  subunit ( $G\alpha$ ) of the heterotrimer activates the signaling pathways that mediate  $Ca^{2+}$  sensitivity, whereas both  $G\alpha$  and the  $\beta\gamma$  dimer ( $G\beta\gamma$ ) activate signaling proteins that regulate  $[Ca^{2+}]_c$ .<sup>20,21</sup>

Acetylcholine induces ASM contraction by reversibly binding and activating two of the five isoforms of the muscarinic receptor, the  $M_2$  and  $M_3$  muscarinic receptors.<sup>22</sup> Studies of isolated, intact tracheal smooth muscle obtained from  $M_2$  and  $M_3$  muscarinic receptor knockout mice indicate that acetylcholine-induced ASM contraction is mediated approximately equally by each receptor.<sup>23,24</sup> In porcine ASM, acetylcholine-induced increases in  $Ca^{2+}$  sensitivity are mediated by the  $\alpha$  subunit of both  $G_i$  and  $G_q$  subfamily proteins ( $G\alpha_i$  and  $G\alpha_q$ , respectively),<sup>19,25</sup> which are thought to couple to  $M_2$  and  $M_3$  muscarinic receptors, respectively.

Our previous work showed that halothane and hexanol each inhibit acetylcholine-induced increases in  $Ca^{2+}$  sensitivity mediated by both pertussis toxin-sensitive and pertussis toxin-insensitive heterotrimeric G proteins,<sup>17,19</sup> such as  $G\alpha_i$  and  $G\alpha_q$  subfamily proteins, respectively. Recent studies of crude membranes prepared from porcine ASM showed that these anesthetics inhibit acetylcholine-promoted guanosine nucleotide exchange at  $G\alpha_q$ .<sup>14,26</sup> In another study of recombinant  $G\alpha_i$  isoform-1 ( $G\alpha_{i-1}$ )- or  $G\alpha_i$  heterotrimer-derived ASM membranes, halothane had no effect on either GTP binding or hydrolysis, suggesting that halothane does not directly interact with  $G\alpha_i$  subunits in a functionally significant manner.<sup>27</sup> These experiments did not examine preparations in which the  $M_2$  muscarinic receptor was coupled to the  $G_i$  heterotrimer. Therefore, it is unknown whether halothane affects the ability of the  $M_2$  muscarinic receptor to promote guanosine nucleotide exchange at  $G\alpha_i$ .

The current study tested the hypothesis that clinically relevant concentrations of halothane ( $\leq 1$  mM) inhibit acetylcholine-promoted guanosine nucleotide exchange at  $G\alpha_i$  isoform-3 ( $G\alpha_{i-3}$ ) but not the intrinsic, basal  $G\alpha_{i-3}$  activity. This hypothesis was tested using recombinant  $G\alpha_{i-3}$  and  $G\alpha_{i-3}\beta_1\gamma_2$  heterotrimer (isoforms expressed in ASM) and crude membranes prepared from mammalian cells transfected to transiently coexpress the human  $M_2$  muscarinic receptor and human  $G\alpha_{i-3}$ . Based on these

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Received from the Department of Anesthesiology, Mayo Foundation, Rochester, Minnesota. Submitted for publication June 8, 2005. Accepted for publication August 4, 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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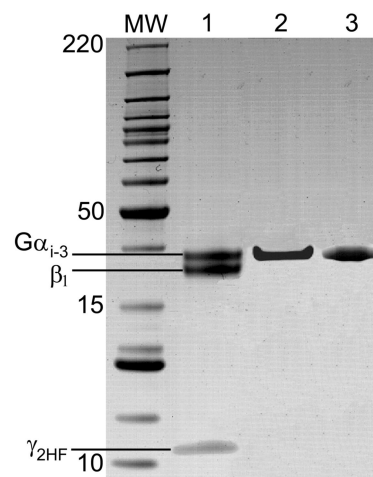
results and additional experiments using a novel technique to detect anesthetic-protein interactions,<sup>28</sup> we make inferences regarding the salient mechanism for the anesthetic action on the  $M_2$ - $G\alpha_{i-3}\beta\gamma$  heterotrimer complex.

## Materials and Methods

### Expression and Purification of Heterotrimeric G-protein Subunits

**Expression and Purification of  $G\alpha_{i-3}$  in *E. coli* Bacteria.** A plasmid encoding human  $G\alpha_{i-3}$  in the mammalian expression vector pcDNA3.1 was obtained from the University of Missouri-Rolla cDNA Resource Center (Rolla, Missouri).<sup>||</sup>  $G\alpha_{i-3}$  was expressed in *Escherichia coli* and purified by conventional chromatography as previously described,<sup>29</sup> with minimal modifications. Briefly, the  $G\alpha_{i-3}$  insert was excised from the pcDNA3.1:  $G\alpha_{i-3}$  construct and ligated to pET28a plasmid. pET28a:  $G\alpha_{i-3}$  was then transformed into *E. coli* BL21(DE3). After growing a single colony (4–5 h, 30°C) in 80 ml Luria-Bertani medium containing 50  $\mu$ g/ml kanamycin, protein expression was induced with 30  $\mu$ M isopropyl thiogalactoside (16 h, 30°C). The cells were pelleted (14,000g, 15 min, 4°C) and then lysed in 40 ml ice-cold lysis buffer composed of 20 mM HEPES (pH 8.0), 1 mM EDTA, 6.25 mM  $MgCl_2$ , 3 mM dithiothreitol, 10  $\mu$ M guanosine 5'-diphosphate (GDP), 0.5 ml protease inhibitor cocktail P8849, 0.2 mg/ml lysozyme, and 0.15 mg/ml DNase I. The lysate was centrifuged (35,000g, 45 min, 4°C), filtered, and subjected to sequential chromatography with Q sepharose, hydroxyapatite, and then phenyl sepharose columns.<sup>29</sup> Purified  $G\alpha_{i-3}$  was dialyzed, concentrated, and stored (–70°C) in 50 mM HEPES (pH 8.0), 1 mM EDTA, and 2 mM dithiothreitol. The yield was approximately 4 mg/l of culture, with purity greater than 95% as estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (fig. 1).

**Construction of Recombinant Baculoviruses.** Baculoviruses containing untagged G-protein  $\beta_1$  ( $\beta_1$ ) and amino-terminally hexahistidine-tagged, Flag-tagged G-protein  $\gamma_2$  ( $\gamma_{2HF}$ ) were generous gifts from James C. Garrison, Ph.D. (Professor, Department of Pharmacology, University of Virginia, Charlottesville, Virginia). To generate a baculovirus containing  $G\alpha_{i-3}$ , the pcDNA3.1:  $G\alpha_{i-3}$  construct was amplified by polymerase chain reaction, and *Pst*I and *Xba*I restriction sites were incorporated into the 5' and 3' ends, respectively. The resulting polymerase chain reaction product was gel purified and subcloned into the pCR-BluntII TOPO vector. The pCR-BluntII:  $G\alpha_{i-3}$  was digested with *Pst*I and *Xba*I, and the resulting  $G\alpha_{i-3}$  insert was gel purified and ligated into the baculovirus transfer vector pVL1392. Recombinant bacu-



**Fig. 1.** Coomassie brilliant blue stains of sodium dodecyl sulfate polyacrylamide gels of the  $\alpha$  isoform-3 G-protein subunit ( $G\alpha_{i-3}$ ) expressed in and purified from *Escherichia coli* bacteria (lane 3) or *Spodoptera frugiperda* (*Sf9*) insect cells (lane 2). Lane 1 shows the same for the heterotrimer composed of  $G\alpha_{i-3}$ , and the untagged  $\beta$  isoform-1 ( $\beta_1$ ) and amino-terminally hexahistidine-tagged, Flag-tagged  $\gamma$  isoform-2 ( $\gamma_{2HF}$ ) G-protein subunits ( $G\alpha_{i-3}\beta_1\gamma_{2HF}$ ) expressed in and purified from *Sf9* insect cells. The molecular weight (MW) marker ladder is shown in the left lane.

loviruses were generated by cotransfection of *Spodoptera frugiperda* (*Sf9*) insect cells with the transfer vector and Sapphire baculoviral DNA. After amplification, the titer of recombinant baculovirus was determined by immunocytochemistry of infected *Sf9* cells with a monoclonal antibody directed against the baculovirus-encoded gp64 (FastPlax; Novagen, Madison, WI).

**Expression of  $G\alpha_{i-3}$  and  $G\alpha_{i-3}\beta_1\gamma_{2HF}$  in *Sf9* Cells.** *Sf9* cells were coinfecting with the recombinant baculoviruses encoding  $G\alpha_{i-3}$ ,  $\beta_1$ , and  $\gamma_{2HF}$ . Typically, cells were grown to a density of  $2 \times 10^6$  cells/ml in 250 ml Hinks TNM-FH media supplemented with 10% fetal bovine serum, 10  $\mu$ g/ml gentamicin, and 0.1% Pluronic F-68. The cells were infected at a multiplicity of infection of 3, 1.5, and 1 for  $G\alpha_{i-3}$ ,  $\beta_1$ , and  $\gamma_{2HF}$ , respectively. Forty-eight hours after infection, the cells were harvested by centrifugation (400g, 15 min), flash-frozen in liquid nitrogen, and stored at –70°C.

**Purification of  $G\alpha_{i-3}$  and  $G\alpha_{i-3}\beta_1\gamma_{2HF}$  from *Sf9* Cell Membrane.** Membrane preparation and protein purification were performed, with minor modifications, according to the methods of Kozasa.<sup>30</sup> Cell pellets were thawed and lysed by nitrogen cavitation (Parr Instruments, Moline, IL) in 37.5 ml ice-cold lysis buffer composed of 20 mM HEPES-NaOH (pH 8.0), 100 mM NaCl, 0.1 mM EDTA, 2 mM  $MgCl_2$ , 9.8 mM 2-mercaptoethanol, 10  $\mu$ M GDP, 1 mM phenylmethylsulfonyl fluoride and the protease inhibitor cocktail P8849. The cell lysates were centrifuged (400g, 10 min, 4°C) to remove intact cells and nuclei, and the supernatant was subjected to ultracentrifugation (100,000g, 30 min, 4°C) to pellet cell membranes. The membranes were resuspended in 18.75 ml wash buffer composed of 20 mM HEPES-NaOH

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(pH 8.0), 100 mM NaCl, 1 mM MgCl<sub>2</sub>, 9.8 mM 2-mercaptoethanol, and 10 μM GDP, homogenized (8–10 strokes) with a Potter-Elvehjem homogenizer (Pierce Biotechnology, Rockford, IL) and ultracentrifuged (100,000g, 30 min, 4°C). Washed membranes were resuspended to a concentration of 1–2 mg membrane protein/ml in wash buffer containing fresh protease inhibitors.

To extract the G protein from the membranes, sodium cholate was added to the preparation at a concentration of 1% and the mixture was stirred on ice for 1 h. After ultracentrifugation (100,000g, 30 min, 4°C), the supernatant was diluted fourfold with nickel-nitrilotriacetic acid (Ni-NTA) loading buffer composed of 20 mM HEPES-NaOH (pH 8.0), 100 mM NaCl, 1 mM MgCl<sub>2</sub>, 9.8 mM 2-mercaptoethanol, 10 μM GDP, and 0.5% polyoxyethylene 10-laurylether and then loaded onto an Ni-NTA resin column preequilibrated with the Ni-NTA loading buffer (4°C). The column was washed with 20 column volumes of Ni-NTA salt wash composed of 20 mM HEPES-NaOH (pH 8.0), 300 mM NaCl, 3 mM MgCl<sub>2</sub>, 9.8 mM 2-mercaptoethanol, 10 μM GDP, 10 mM imidazole-HCl (pH 8.0), and 0.5% polyoxyethylene 10-laurylether followed by three column volumes of 0.2% cholate wash composed of 20 mM HEPES-NaOH (pH 8.0), 50 mM NaCl, 3 mM MgCl<sub>2</sub>, 9.8 mM 2-mercaptoethanol, 10 μM GDP, 10 mM imidazole-HCl (pH 8.0), and 0.2% sodium cholate. The column was warmed to room temperature and washed again with three column volumes of cholate wash.

G $\alpha_{i-3}$  was eluted from the  $\beta_1\gamma_{2HF}$  dimer with Ni-NTA elution buffer composed of 20 mM HEPES-NaOH (pH 8.0), 50 mM NaCl, 50 mM MgCl<sub>2</sub>, 9.8 mM 2-mercaptoethanol, 10 μM GDP, and 1% sodium cholate. Alternatively, the intact G $\alpha_{i-3}\beta_1\gamma_{2HF}$  heterotrimer was eluted from the column with Ni-NTA cholate wash containing 150 mM imidazole-HCl (pH 8.0) and 1% sodium cholate. One-milliliter elution fractions were collected, and samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (10% acrylamide) to screen for protein (fig. 1). The fractions containing G $\alpha_{i-3}$  or G $\alpha_{i-3}\beta_1\gamma_{2HF}$  were pooled and concentrated with Microcon-10 centrifugal concentrators (Millipore, Bedford, MA). Finally the buffer was exchanged into storage buffer composed of 20 mM HEPES-NaOH (pH 8.0), 100 mM NaCl, 2 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 1 mM dithiothreitol, 11 mM CHAPS, and 1 μM GDP. The procedure typically yielded 150–200 μg purified G-protein subunit from a 10-g Sf9 cell pellet.

#### *Nuclear Magnetic Resonance Saturation Transfer Difference*

One-dimensional nuclear magnetic resonance (<sup>1</sup>H NMR) and saturation transfer difference (STD)<sup>31</sup> spectra were recorded with a 500-MHz Bruker BioSpin NMR (Billerica, MA) as previously described and validated by our laboratory for the purpose of detecting anesthetic-protein binding.<sup>28</sup> This technique is based on the nu-

clear Overhauser effect between bound anesthetic protons and all protein protons. G $\alpha$  subunit samples were prepared in deuterated water containing 20 mM phosphate buffer (pH 7.0) with 100 mM NaCl, 50 μM dithiothreitol, 2 mM MgSO<sub>4</sub>, and 0.5 μM GDP. Samples were placed in 5-mm NMR tubes (Wilmad Labglass, Buena, NJ) and sealed with a capillary stem insert tube containing 10 mM sodium acetate in deuterated water as an external standard. The external standard was used to quantify the aqueous concentration of ligands and also functioned as a negative control because it does not bind to protein. As a control, the <sup>1</sup>H NMR and STD spectra of 1 mM halothane, 1 mM suramin, and 10 mM hexanol were recorded to confirm that the pulse sequence did not directly saturate the ligands. Suramin binds and inhibits G $\alpha_i$  activity<sup>32</sup> and was used as a positive control for ligand-protein binding. Then the spectra of these compounds were recorded in the presence of 40 μM G $\alpha_{i-3}$ .

#### *G $\alpha_{i-3}$ and G $\alpha_{i-3}\beta_1\gamma_{2HF}$ GTP Hydrolysis*

Guanosine-5'-triphosphatase (GTPase) activity was measured using a standard phosphohydrolase assay with radioactive inorganic phosphate ([<sup>32</sup>Pi])-labeled GTP<sup>33</sup> as previously described by our laboratory.<sup>27,34</sup> G $\alpha_{i-3}$  or G $\alpha_{i-3}\beta_1\gamma_{2HF}$  (0.2 μM final concentration in the assay) was incubated (30°C, 5 min) with or without halothane (control) in an assay buffer composed of 25 mM HEPES (pH 7.8), 100 mM NaCl, 10 mM MgSO<sub>4</sub>, 1 mM EDTA, 1 mM dithiothreitol, 0.1 μM GDP, 5 μM GTP, and 0.5 mg/ml bovine serum albumin. After initiating the reactions 0.025 mCi [<sup>32</sup>Pi]GTP for 3, 6, 9, and 12 min, aliquots from each assay tube were quenched on ice with 7% (wt/vol) activated charcoal in 2N HCl and 0.35 M NaH<sub>2</sub>PO<sub>4</sub> and vigorously vortexed. The tubes were centrifuged (12,000g, 10 min, room temperature) to pellet the charcoal and the amount of radioactivity (from <sup>32</sup>Pi) in supernatant was determined by liquid scintillation counting.

#### *Culture and Transfection of COS-7 Cells*

COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (10% DMEM) and penicillin and streptomycin (50 U/ml each). The day before transfection, confluent cells were trypsinized and seeded in 10-cm tissue culture plates to reach 90% confluence in 24 h (approximately 4.5 × 10<sup>6</sup> cells per 10-cm plate). The cells were then 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 mixed (5 min, room temperature) with 1.5 ml Opt-MEM I in a 50-ml Falcon tube. For cotransfection, 10 μg M<sub>2</sub> cDNA plus 5 μg G $\alpha_{i-3}$  cDNA were used, and for transfection of G $\alpha_{i-3}$  alone, 5 μg G $\alpha_{i-3}$  cDNA plus 10 μg vector pcDNA3.1 were used. Lipofectamine 2000 was mixed in



another Falcon tube with 1.5 ml Opt-MEM (2.5  $\mu\text{l}/\mu\text{g}$  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 DMEM, 10%, without penicillin or streptomycin. The transfection mixture was replaced with 7 ml fresh DMEM, 10%, plus penicillin and streptomycin after 12 h. Twenty-four hours after transfection, the cells were washed twice with phosphate-buffered saline (room temperature), scraped in ice-cold phosphate-buffered saline, transferred to 1.5-ml microfuge tubes, and pelleted by centrifugation (500g, 2 min, 4°C). The cells were flash-frozen in liquid nitrogen and stored at -70°C until they were used to prepare crude membranes.

#### Crude Membrane Preparation

Frozen cells from three 10-cm plates were suspended for 15 min in ice-cold lysis buffer (500  $\mu\text{l}/\text{plate}$ ) composed of 20 mM HEPES (pH 8.0), 1 mM EDTA, 0.1 mM phenylmethanesulfonyl fluoride, 10  $\mu\text{g}/\text{ml}$  leupeptin, and 2  $\mu\text{g}/\text{ml}$  aprotinin. Then the cells were 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 (400g, 10 min, 4°C) to remove intact cells and nuclei, and then to ultracentrifugation (87,000g, 30 min, 4°C) to pellet the crude membrane. The membrane pellet was washed with lysis buffer and then resuspended by repeated passage through a 27-gauge needle in assay buffer (100  $\mu\text{l}/\text{plate}$ ) composed of 10 mM HEPES (pH 8.0), 100 mM NaCl, 10 mM  $\text{MgCl}_2$ , and 10  $\mu\text{M}$  GDP. A portion of the crude membrane (50  $\mu\text{l}$ ) was solubilized in 12.5  $\mu\text{l}$  NaOH, 0.1N, and boiled (3 min) to determine protein concentration.<sup>35</sup> The crude membrane suspension was then diluted with assay buffer to a protein concentration of 2–3 mg/ml, frozen in liquid nitrogen, and stored at -70°C until used for the assay.

#### Quantification of $M_2$ Muscarinic Receptor Expression

$M_2$  muscarinic receptor was quantified in the crude membranes by saturation binding with the muscarinic receptor antagonist *l*-[benzyl-4,4'-<sup>3</sup>H]-quinuclidinyl benzilate (<sup>3</sup>H-QNB).<sup>36,37</sup> Specific binding was determined in triplicate assays with a single saturating concentration of <sup>3</sup>H-QNB of 5 nM. Nonspecific binding was determined by performing triplicate assays in the presence of 5  $\mu\text{M}$  atropine. Aliquots of crude membranes containing 20  $\mu\text{g}$  protein from  $M_2$ - $G\alpha_{i-3}$  cotransfected COS-7 cells, or untransfected COS-7 cells were incubated (90 min, room temperature) in 0.5 ml buffer containing 5 nM <sup>3</sup>H-QNB, 50 mM Tris (pH 7.4), and 10 mM  $\text{MgCl}_2$ , with and without 5  $\mu\text{M}$  atropine. After the 90-min incubation period, reactions were applied to prewetted GF/B

glass fiber filters sheets using a Brandel cell harvester (Brandel, Gaithersburg, MD) and washed three times with buffer containing 50 mM Tris (pH 7.4) and 10 mM  $\text{MgCl}_2$ . Bound radioactivity on the filters was quantified by liquid scintillation counting.

#### $G\alpha_{i-3}$ Guanosine Nucleotide Exchange Assay in COS-7 Cell Membranes

$G\alpha_{i-3}$  guanosine nucleotide exchange was assayed in crude membrane prepared from  $M_2$ - $G\alpha_{i-3}$  cotransfected COS-7 cells using previously described methods.<sup>38</sup> Reaction mixtures containing 18–20  $\mu\text{g}$  membrane protein, 10 mM HEPES, 100 mM NaCl, 10 mM  $\text{MgCl}_2$ , and 10  $\mu\text{M}$  GDP were incubated with and without halothane and with and without acetylcholine in a total volume of 62  $\mu\text{l}$  for 5 min at 30°C. To minimize the loss of volatile anesthetics, reactions were performed in narrow 0.25-ml polypropylene tubes. The reactions were initiated by the addition of 5  $\mu\text{l}$  of the radioactive, nonhydrolyzable form of GTP, [<sup>35</sup>S]GTP $\gamma$ S, to the assay mixture (1,250 Ci/mmol; 20 nM final concentration in assay). Reactions were terminated at times according to experimental design with 900  $\mu\text{l}$  ice-cold assay buffer. The reaction tubes were then centrifuged (16,000g, 10 min, 4°C), and the pellets were resuspended by vigorous vortexed mixing (2 min, room temperature) in 50  $\mu\text{l}$  solubilization buffer composed of 100 mM Tris-HCl (pH 7.4), 1 mM EDTA, 200 mM NaCl, 1.25% (vol/vol) IGEPAL CA-630, and 0.2% sodium dodecyl sulfate. Then the samples were gently rocked (30 min, 4°C), diluted in 50  $\mu\text{l}$  solubilization buffer without sodium dodecyl sulfate, vortex mixed, and centrifuged at (16,000g, 10 min, 4°C). The supernatant (100  $\mu\text{l}$ ) containing detergent-solubilized [<sup>35</sup>S]GTP $\gamma$ S-bound  $G\alpha_{i-3}$  was transferred to 1.5-ml microfuge tubes and incubated (1 h, 4°C) with rabbit polyclonal antibody raised against a peptide mapping at the carboxy terminus of rat  $G\alpha_{i-3}$  (1:100 vol/vol dilution). Then 40  $\mu\text{l}$  protein A-agarose beads was added to each sample for an additional hour (4°C). The beads were then washed three times by repeated pelleting (2,000g, 1 min, 4°C) followed by resuspension in 0.5 ml solubilization buffer without sodium dodecyl sulfate and rotated on an orbital rocker (30 min, 4°C). Finally, the washed beads were placed in 4 ml Ultimate Gold scintillation cocktail (Packard Bioscience, Meriden, CT), and radioactivity was quantified by liquid scintillation counting.

Background radioactivity measurements were determined by performing tandem experiments with the same amount of protein, except that the assay was terminated immediately. The amount of background radioactivity was less than 40% of the radioactivity of the basal  $G\alpha_{i-3}$  nucleotide exchange measurements. In preliminary work, halothane had no effect on this nonspecific background radioactivity. Data were normalized to the amount of membrane protein and the specific activity of

the [ $^{35}$ S]GTP $\gamma$ S in the assay, and each experimental condition was assayed in triplicate.

#### *Preparation of Anesthetic Solutions*

Saturated aqueous stocks of halothane were prepared by stirring 5 ml liquid anesthetic with 10 ml assay buffer for 30 min in an airtight, ground-glass flask as previously described.<sup>14,26-28</sup> After the stirring had been stopped for 5 min, 5 ml of the saturated assay buffer was transferred to a 5-ml glass vial. Aliquots of the anesthetic-saturated stocks were added directly to the reaction tubes in volumes that produced the desired final anesthetic concentration. To account for any loss of volatile anesthetic in the assay tubes, tandem experiments were conducted under the same assay conditions, and anesthetic concentrations were measured after hexane extraction by gas chromatography using an electron capture detector (Hewlett-Packard, Waltham, MA; model 5880A).<sup>39</sup> In preliminary studies, we found that after an initial loss of approximately 20% after transfer and mixing, the amount of volatile anesthetic in the reaction tubes was relatively stable, with less than 10% additional loss occurring during the longest assay time in this report (10 min).

#### *Experimental Protocols*

**Characterization of the  $G\alpha_{i-3}$  Guanosine Nucleotide Exchange Assay in COS-7 Cell Membranes.** To determine the dependence of the  $G\alpha_{i-3}$  [ $^{35}$ S]GTP $\gamma$ S-GDP exchange measurements on  $M_2$  muscarinic receptor expression and activation by acetylcholine, assays were performed using crude membranes prepared from COS-7 cells transfected with the cDNA constructs encoding for  $G\alpha_{i-3}$  only, or both the  $M_2$  muscarinic receptor and  $G\alpha_{i-3}$ . The crude membranes were incubated without (for basal  $G\alpha_{i-3}$  [ $^{35}$ S]GTP $\gamma$ S-GDP exchange measurements) or with (for acetylcholine-promoted  $G\alpha_{i-3}$  [ $^{35}$ S]GTP $\gamma$ S-GDP exchange measurements) 10  $\mu$ M acetylcholine for 5 min, and then the reactions were initiated with [ $^{35}$ S]GTP $\gamma$ S. The reactions were terminated after 10 min, and the samples were subjected to the immunoprecipitation step of the assay using either non-immune rabbit serum (for nonspecific background measurements) or rabbit anti- $G\alpha_{i-3}$  immunoglobulin G. These data were not subjected to nonspecific background subtraction.

**Effect of Acetylcholine on  $G\alpha_{i-3}$  Guanosine Nucleotide Exchange in COS-7 Cell Membranes.** These protocols were conducted to help guide the experimental approach of subsequent protocols that examined possible anesthetic effects on  $G\alpha_{i-3}$  [ $^{35}$ S]GTP $\gamma$ S-GDP exchange. To determine the time course for  $G\alpha_{i-3}$  [ $^{35}$ S]GTP $\gamma$ S-GDP exchange, crude membrane prepared from  $M_2$ - $G\alpha_{i-3}$  cotransfected COS-7 cells was incubated for 5 min with or without 10  $\mu$ M acetylcholine, and then the reactions were initiated with [ $^{35}$ S]GTP $\gamma$ S. The reac-

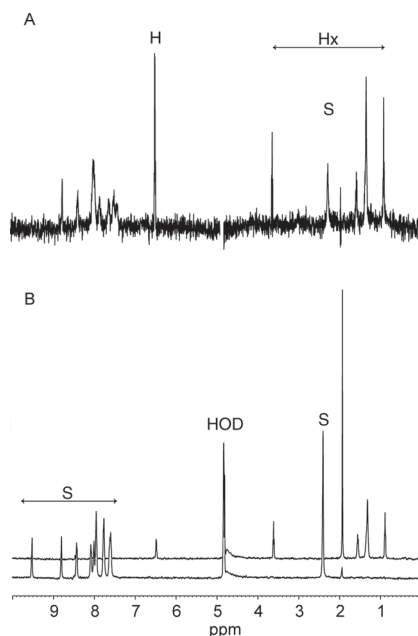
tions were terminated after 1, 2, 5, or 10 min, and then the samples were subjected to the immunoprecipitation step of the assay.

To determine the acetylcholine concentrations that produced half-maximal and maximal promotion of  $G\alpha_{i-3}$  [ $^{35}$ S]GTP $\gamma$ S-GDP exchange, crude membrane was incubated with or without various concentrations of acetylcholine (0.001-100  $\mu$ M) for 5 min. The reactions were then terminated 5 min after initiation with [ $^{35}$ S]GTP $\gamma$ S, and the samples were subjected to the immunoprecipitation step of the assay. The acetylcholine-promoted  $G\alpha_{i-3}$  [ $^{35}$ S]GTP $\gamma$ S-GDP exchange was expressed as the percentage of the difference between the values measured in the absence of acetylcholine and that measured in the presence of the acetylcholine concentration that produced the maximal effect (*i.e.*, percentage acetylcholine-promoted).

**Effect of Halothane on  $G\alpha_{i-3}$  Guanosine Nucleotide Exchange in COS-7 Cell Membranes.** Crude membranes prepared from  $M_2$ - $G\alpha_{i-3}$  cotransfected COS-7 cells were incubated for 5 min with or without 0.5, 1, or 3 mM halothane and with or without the acetylcholine concentration determined to promote an increase in  $G\alpha_{i-3}$  [ $^{35}$ S]GTP $\gamma$ S-GDP exchange of approximately 80-90% of the maximum value. These concentrations of anesthetics include those studied in our previous work with crude membranes prepared from porcine tracheal smooth muscle,<sup>14,26</sup> thereby allowing comparisons with these previous data. The reactions were terminated at a time determined to produce an approximately 70-80% increase in maximal  $G\alpha_{i-3}$  [ $^{35}$ S]GTP $\gamma$ S-GDP exchange, and then the samples were subjected to the immunoprecipitation step of the assay. Basal  $G\alpha_{i-3}$  [ $^{35}$ S]GTP $\gamma$ S-GDP exchange values are expressed as a percentage of the values measured in the absence of halothane (*i.e.*, basal control). The acetylcholine-promoted  $G\alpha_{i-3}$  [ $^{35}$ S]GTP $\gamma$ S-GDP exchange values are expressed as a percentage of the difference between the basal control values (*i.e.*, absence of acetylcholine or halothane in the assay) and the values measured in the presence of the chosen acetylcholine concentration but the absence of halothane in the assay (*i.e.*, acetylcholine-promoted control).

#### *Materials*

The pET28a plasmid, BL21 (DE3) *E. coli* bacteria, and the FastPlax Titer kit were purchased from Novagen (Madison, WI). The sepharose chromatography columns were purchased from Amersham Biosciences (Piscataway, NJ), and the hydroxyapatite CHT10-I column was purchased from Biocompare (San Francisco, CA). The Sapphire insect transfection kit and Sf9 cells were obtained from Orbigen (San Diego, CA). COS-7 cells were purchased from American Type Culture Collection (Manassas, VA). Deuterated water was purchased from CDN

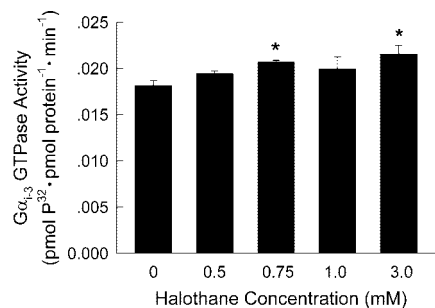


**Fig. 2.** Representative saturation transfer difference (A) and one-dimensional nuclear magnetic resonance (B) spectra recorded for a solution containing 1 mM suramin (S), 1 mM halothane (H), 10 mM hexanol (Hx), and the  $\alpha$  isoform-3 G-protein subunit ( $G\alpha_{i,3}$ ).  $G\alpha_{i,3}$  was expressed in and purified from *Spo-doptera frugiperda* insect cells. The region of the saturation transfer difference spectra where deuterated water (HOD) absorbs (approximately 4.8 ppm) was deleted from the figure for clarity of presentation.

Isotopes (Pointe-Claire, Quebec, Canada). All chemicals and supplies required for cell culture and cDNA transfection of COS-7 cells, pCDNA 3.1 plasmid, pCR-BluntII TOPO vector, and baculovirus transfer vector pVL1392 were purchased from Invitrogen (Carlsbad, CA). EndoFree maxi-prep kits used for cDNA preparation and Ni-NTA resin were purchased from Qiagen Science (Valencia, CA). Halothane was purchased from Ayerst Laboratories, Inc. (New York, NY). 1-[Benzilic-4,4'-3H]-quinuclidinyl benzilate (42 Ci/mmol) and [ $^{35}$ S]GTP $\gamma$ S (1,250 Ci/mmol) were purchased from Perkin-Elmer (Boston, MA). Rabbit nonimmune serum was purchased from Calbiochem (EMD Biosciences, Inc. Affiliate, San Diego, CA). Protein A-agarose beads and anti-rabbit immunoglobulin G were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Lowry protein assay kits were purchased from Bio-Rad Life Science Research Products (Hercules, CA). All other chemicals were purchased from Sigma Chemical Company (St. Louis, MO). All drugs and chemicals were prepared in distilled, filtered water.

#### Data Analysis and Statistics

Data are reported as mean  $\pm$  SE; n represents the number of independent times an assay was performed. All time- and concentration-dependent effects on acetylcholine on  $G\alpha_{i,3}$  [ $^{35}$ S]GTP $\gamma$ S-GDP exchange were determined by nonlinear regression analysis as previously



**Fig. 3.** Concentration-dependent effect of halothane on guanosine-5'-triphosphate (GTP) hydrolysis rate (GTPase activity) of the nonmyristoylated  $\alpha$  isoform-3 G-protein subunit ( $G\alpha_{i,3}$ ) expressed in and purified from *E. coli*. GTPase activity was determined using a standard phosphohydrolyase assay with radiolabeled [ $\gamma$ - $^{32}$ P]GTP as described in detail in the Materials and Methods. Assays were performed in the absence or presence of 0.5, 0.75, 1.0, and 3.0 mM halothane. Data are presented as mean  $\pm$  SE of 3–14 assays. \* Significant difference from values measured in the absence of halothane.

described (SigmaPlot 8; Systat Software, Point Richmond, CA).<sup>14,26,40</sup> Data were statistically compared by either repeated-measures analysis of variance with *post hoc* testing performed using the Student-Newman-Keuls test or paired Student *t* test as appropriate for experimental design. For all statistical comparisons, a value of  $P < 0.05$  was considered significant.

## Results

### Anesthetic Binding to $G\alpha_{i,3}$ by STD- $^1$ H NMR

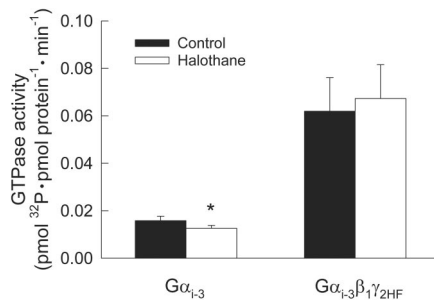
Saturation transfer from  $G\alpha_{i,3}$  to halothane, hexanol, and suramin was observed with STD (fig. 2A) and confirmed with  $^1$ H NMR (fig. 2B). The spectra showed intensity from resonances from all three molecules indicating that each binds  $G\alpha_{i,3}$ . Halothane had a single peak at approximately 6.3 ppm, whereas the remaining resonances are from suramin and hexanol. Because the STD spectrum is dependent on the interaction geometry,<sup>31</sup> the STD spectrum contains some but not all of the ligand- $^1$ H NMR peaks, and the relative intensities of these peaks are not identical to those in the  $^1$ H NMR spectra. The peak at approximately 4.8 ppm in the  $^1$ H NMR spectrum is from residual water, and its peak shape is due to suppression.

### Effect of Halothane on $G\alpha_{i,3}$ and $G\alpha_{i,3}\beta_1\gamma_2$ GTPase Activity

$G\alpha_{i,3}$  expressed in and purified from *E. coli* exhibited an intrinsic GTPase activity of  $0.0182 \pm 0.0005$  pmol  $^{32}$ Pi  $\cdot$  pmol protein $^{-1} \cdot$  min $^{-1}$ , which was similar to that reported by other investigators.<sup>41,42</sup> The presence of halothane in the assay tended to cause a slight increase in  $G\alpha_{i,3}$  GTPase activity, reaching statistical significance only at 0.75 and 3 mM halothane (fig. 3).

The GTPase activities of the  $G\alpha_{i,3}$  and  $G\alpha_{i,3}\beta_1\gamma_{2HF}$  heterotrimer expressed in and purified from membranes



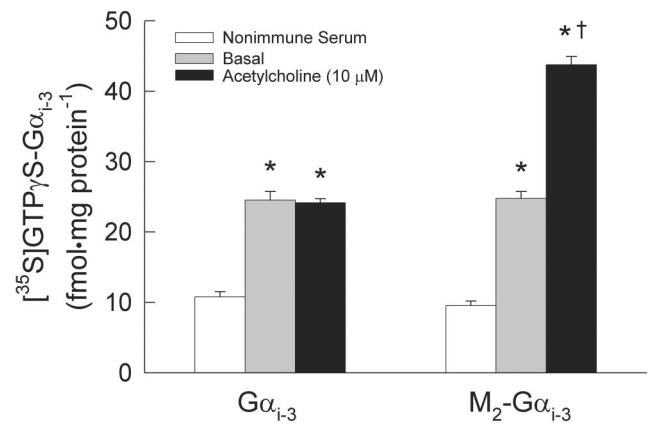


**Fig. 4.** Effect of halothane on guanosine-5'-triphosphate (GTP) hydrolysis rate (GTPase activity) of the  $\alpha$  isoform-3 G-protein subunit ( $G\alpha_{i-3}$ ) and the heterotrimer composed of  $G\alpha_{i-3}$ , and the untagged  $\beta$  isoform-1 ( $\beta_1$ ) and amino-terminus hexahistidine-tagged, Flag-tagged  $\gamma$  isoform-2 G-protein subunits ( $G\alpha_{i-3}\beta_1\gamma_{2HF}$ ). Proteins were expressed in and purified from *Spo. doptera frugiperda* insect cells. GTPase activity was determined using a standard phosphohydrolase assay with radiolabeled [ $^{32}$ P]GTP as described in detail in the Materials and Methods. Assays were performed in the absence (control) or presence of 3 mM halothane. Data are presented as mean  $\pm$  SE of 9–12 assays. \* Significant difference from values measured in the absence of halothane (control).

of Sf9 insect cells were also comparable to those previously reported.<sup>43</sup> As expected, the GTPase activity of the heterotrimer was approximately fourfold greater than that of the  $G\alpha_{i-3}$  subunit ( $0.0158 \pm 0.009$  and  $0.0619 \pm 0.0141$  pmol  $^{32}$ Pi · pmol protein<sup>-1</sup> · min<sup>-1</sup> for  $G\alpha_{i-3}$  and  $G\alpha_{i-3}\beta_1\gamma_{2HF}$ , respectively).<sup>43</sup> The presence of 3 mM halothane in the assay had no significant effect on the GTPase activity of the  $G\alpha_{i-3}\beta_1\gamma_{2HF}$ , whereas it caused a small but significant inhibition of  $G\alpha_{i-3}$  GTPase activity (fig. 4).

#### Characterization of Cotransfected COS-7 Cells

No muscarinic receptors could be detected above the nonspecific background measurements in crude membranes prepared from untransfected COS-7 cells as expected, because these cells do not express endogenous muscarinic receptors.<sup>44,45</sup> However, the membranes prepared from COS-7 cells cotransfected with the cDNA constructs encoding for the  $M_2$  muscarinic receptor and  $G\alpha_{i-3}$  expressed the  $M_2$  muscarinic receptor at 2–3.4 pmol/mg protein. In crude membranes prepared from  $G\alpha_{i-3}$ -only transfected cells or  $M_2$ - $G\alpha_{i-3}$  cotransfected cells, there was a significant increase in  $G\alpha_{i-3}$  [ $^{35}$ S]GTP $\gamma$ S-GDP exchange above that of the nonspecific background measurements even when acetylcholine was omitted from the assay (fig. 5). The coexpression of the  $M_2$  muscarinic receptor with  $G\alpha_{i-3}$  did not promote an additional increase in this basal  $G\alpha_{i-3}$  [ $^{35}$ S]GTP $\gamma$ S-GDP exchange (fig. 5). These data indicate that the expressed  $M_2$  muscarinic receptor does not possess constitutive activity, as described by others,<sup>38</sup> but rather reflects the constitutive activity of endogenously expressed receptors that are functionally coupled to the expressed  $G\alpha_{i-3}$ . This assertion is supported by the finding that adenosine-5'-diphosphate ribosylation of  $G\alpha_{i-3}$  by pertussis toxin in crude membranes prepared from  $G\alpha_{i-3}$ -alone transfected

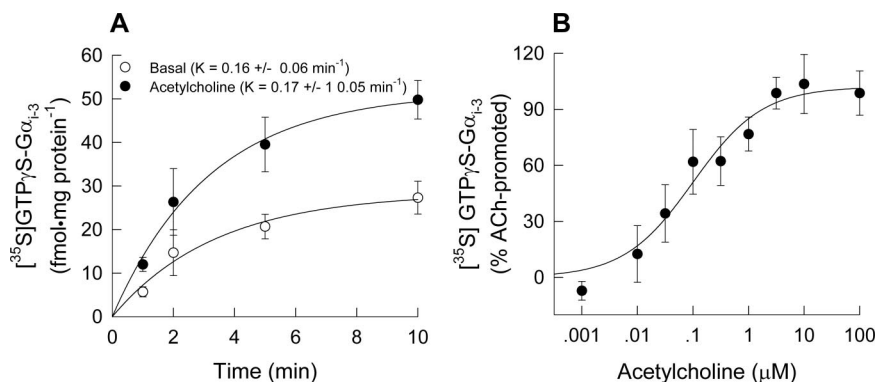


**Fig. 5.** Effect of acetylcholine on the exchange of the radioactive, nonhydrolyzable form of guanosine-5'-triphosphate, [ $^{35}$ S]GTP $\gamma$ S, for guanosine-5'-diphosphate ([ $^{35}$ S]GTP $\gamma$ S-GDP exchange) at the  $\alpha$  isoform-3 G-protein subunit ( $G\alpha_{i-3}$ ). Assays were performed using crude membranes prepared from COS-7 cells transfected with the complementary DNA that encodes for human  $G\alpha_{i-3}$  only, or human  $M_2$  muscarinic receptor and human  $G\alpha_{i-3}$ . Measurements were made in the absence (basal exchange) and presence of 10  $\mu$ M acetylcholine and were terminated 10 min after initiation of the assays with [ $^{35}$ S]GTP $\gamma$ S. The immunoprecipitation step of the assays was performed using nonimmune serum (for nonspecific background measurements) or antiserum specific for  $G\alpha_{i-3}$ . Data are presented as mean  $\pm$  SE of six assays. \* Significant difference from background radioactivity. † Significant difference from basal  $G\alpha_{i-3}$  [ $^{35}$ S]GTP $\gamma$ S-GDP exchange.

cells abolished the  $G\alpha_{i-3}$ -specific basal [ $^{35}$ S]GTP $\gamma$ S-GDP exchange from  $24.7 \pm 1.1$  (fig. 5) to  $6.0 \pm 0.8$  fmol/mg protein. The inclusion of 10  $\mu$ M acetylcholine in the assay buffer of membranes prepared from  $M_2$ - $G\alpha_{i-3}$  cotransfected cells caused a further approximately twofold increase in the magnitude of the  $G\alpha_{i-3}$ -specific [ $^{35}$ S]GTP $\gamma$ S-GDP exchange (fig. 5). However, when the receptor was omitted from the transfection (*i.e.*, crude membranes prepared from  $G\alpha_{i-3}$ -alone transfected cells), acetylcholine did not promote  $G\alpha_{i-3}$  [ $^{35}$ S]GTP $\gamma$ S-GDP exchange above the basal exchange levels.

#### Effect of Acetylcholine on $G\alpha_{i-3}$ Guanosine Nucleotide Exchange

In the absence of acetylcholine in the assay, membranes prepared from  $M_2$ - $G\alpha_{i-3}$  cotransfected cells incorporated [ $^{35}$ S]GTP $\gamma$ S into  $G\alpha_{i-3}$  in a time-dependent manner and could be adequately fit with to a monoexponential association equation with a K of  $0.16 \pm 0.06$  min<sup>-1</sup> (fig. 6A). The presence of acetylcholine in the assay promoted a significant increase in the magnitude of  $G\alpha_{i-3}$  [ $^{35}$ S]GTP $\gamma$ S-GDP exchange at each time point but had no significant effect on K, which was  $0.17 \pm 0.05$  min<sup>-1</sup> ( $P = 0.8$ ). The acetylcholine-promoted increase in  $G\alpha_{i-3}$  [ $^{35}$ S]GTP $\gamma$ S-GDP exchange above basal exchange levels was concentration dependent, with an EC<sub>50</sub> value of  $0.72 \pm 0.10$   $\mu$ M acetylcholine (fig. 6B). Based on these initial investigations, subsequent experiments examining the effect of halothane on  $G\alpha_{i-3}$



**Fig. 6.** (A) Time-dependent change in the exchange of the radioactive, nonhydrolyzable form of guanosine-5'-triphosphate, [<sup>35</sup>S]GTPγS, for guanosine-5'-diphosphate [<sup>35</sup>S]GTPγS-GDP exchange) at the  $\alpha$  isoform-3 G-protein subunit ( $G\alpha_{i-3}$ ). Assays were performed using crude membranes prepared from COS-7 cells cotransfected with the complementary DNA constructs encoding for the human  $M_2$  muscarinic receptor and human  $G\alpha_{i-3}$ .  $G\alpha_{i-3}$  [<sup>35</sup>S]GTPγS-GDP exchange was measured in the absence (basal exchange) or presence of 10  $\mu$ M acetylcholine in the assay. The reactions were terminated 1, 2, 5, and 10 min after initiation of the assays with [<sup>35</sup>S]GTPγS. Data are presented as mean  $\pm$  SE of six assays. (B) Concentration-dependent effect of acetylcholine on  $G\alpha_{i-3}$  [<sup>35</sup>S]GTPγS-GDP exchange measured in crude membranes prepared from  $M_2$ - $G\alpha_{i-3}$  cotransfected COS-7 cells. Assays were performed in the absence (basal exchange) and presence of acetylcholine (ACh, 0.001–100  $\mu$ M) in the assays and the reactions were terminated 10 min after initiation with [<sup>35</sup>S]GTPγS. Data are the percentage of the difference between the basal exchange values and those measured in the presence of the acetylcholine concentration that produced the maximal effect. Data are presented as mean  $\pm$  SE of four assays.

[<sup>35</sup>S]GTPγS-GDP exchange were performed using 10  $\mu$ M acetylcholine to optimize the ability to detect modest shifts in acetylcholine-promoted  $G\alpha_{i-3}$  [<sup>35</sup>S]GTPγS-GDP exchange and were measured after 10 min of stimulation.

#### Effect of Halothane on $G\alpha_{i-3}$ Nucleotide Exchange

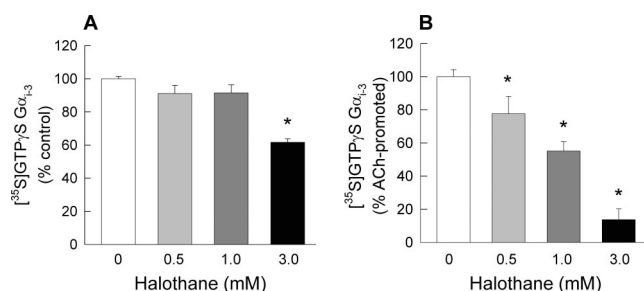
The presence of 0.5 or 1 mM halothane in the assay had no significant effect on basal  $G\alpha_{i-3}$  [<sup>35</sup>S]GTPγS-GDP exchange measured in the absence of acetylcholine; however, 3 mM halothane inhibited basal  $G\alpha_{i-3}$  [<sup>35</sup>S]GTPγS-GDP exchange by approximately 40% (fig. 7A). Halothane caused a concentration-dependent inhibition of  $G\alpha_{i-3}$  [<sup>35</sup>S]GTPγS-GDP exchange when 10  $\mu$ M acetylcholine was included in the reactions (fig. 7B). The amounts of inhibition were  $22.7 \pm 8.2$ ,  $44.9 \pm 6.5$ , and  $86.3 \pm 4.8\%$  for 0.5, 1, and 3 mM halothane, respectively.

## Discussion

The major new finding of this study is that halothane, at concentrations typically achieved when administered to patients to treat acute, severe bronchospasm (0.5–1 mM), inhibits the biochemical coupling between the  $M_2$  muscarinic receptor and  $G\alpha_{i-3}$  without direct inhibition of the intrinsic GTPase activity of the  $G\alpha_{i-3}\beta_1\gamma_{2HF}$  heterotrimer. Although halothane binds to  $G\alpha_{i-3}$ , this binding either has biochemically significant consequences on the heterotrimer only when it is regulated by the  $M_2$  muscarinic receptor or has no consequences at all for heterotrimer function.

In the simplest two-state model for heterotrimeric G protein-coupled receptors, the  $M_2$  muscarinic receptor exists as two conformations, active and inactive.<sup>46</sup> In the absence of ligand binding, these two forms are in dy-

namic equilibrium, with the inactive form being predominant. Receptor agonists induce or select for the active conformation, which promotes (or stabilizes) the nucleotide-free  $G\alpha_i$  subunit. The high intracellular ratio of GTP to GDP ensures that activated receptor promotes GTP binding to  $G\alpha_i$ , which ultimately reduces its affinity for the receptor and the  $G\beta\gamma$  dimer. This results in its dissociation from the receptor-ligand complex. The GTP-bound  $G\alpha_i$  is thought to couple to the downstream signaling proteins, such as RhoA,<sup>47–49</sup> that increase  $Ca^{2+}$



**Fig. 7.** Concentration-dependent effect of halothane on the exchange of the radioactive, nonhydrolyzable form of guanosine-5'-triphosphate, [<sup>35</sup>S]GTPγS, for guanosine-5'-diphosphate [<sup>35</sup>S]GTPγS-GDP exchange) at the  $\alpha$  isoform-3 G-protein subunit ( $G\alpha_{i-3}$ ). Assays were performed using crude membranes prepared from COS-7 cells cotransfected with the complementary DNA constructs encoding for the human  $M_2$  muscarinic receptor and human  $G\alpha_{i-3}$ . Measurements were made in the absence (basal exchange, A) or presence (B) of 10  $\mu$ M acetylcholine (ACh) in the assay, and the reactions were terminated 10 min after initiation with [<sup>35</sup>S]GTPγS. Basal  $G\alpha_{i-3}$  [<sup>35</sup>S]GTPγS-GDP exchange values are expressed as the percentage of the values measured in the absence of halothane (basal control exchange), whereas the acetylcholine-promoted exchange values are expressed as a percentage of the difference between the basal control values and those measured in the presence of the 10  $\mu$ M acetylcholine but in the absence of halothane in the assay (*i.e.*, percentage acetylcholine-promoted). Data are presented as mean  $\pm$  SE of 9–42 assays. \* Indicates significant difference from the values obtained in the absence of halothane.



sensitivity in ASM.<sup>19,25</sup> Hydrolysis of the bound GTP by the intrinsic GTPase activity of  $G\alpha_i$  permits reassociation of the subunits into a heterotrimer and terminates the activation of the signaling cascade that promote  $Ca^{2+}$  sensitivity and ASM contraction. Our findings are consistent with this two-state receptor model. Acetylcholine increased the fraction of receptors in the active conformation, as demonstrated by a concentration-dependent increase in the magnitude, but not the rate, of  $G\alpha_{i-3}$  [<sup>35</sup>S]GTP $\gamma$ S-GDP exchange.

The rationale for conducting the current study was derived from a series of physiologic studies of ASM indicating that the mechanism by which halothane inhibits the acetylcholine-induced increase in  $Ca^{2+}$  sensitivity is due, at least in part, to a direct inhibitory effect on the  $M_2$  muscarinic receptor- $G\alpha_i$  heterotrimeric G-protein complex.<sup>16,17,19</sup> For example, in one study, the magnitude of relaxation caused by halothane was partially attenuated by adenosine-5'-diphosphate ribosylation of  $G\alpha_i$  with pertussis toxin.<sup>19</sup> Accordingly, the original goal of the current study was to determine, through direct biochemical assessment, whether halothane inhibits guanosine nucleotide exchange at  $G\alpha_i$  in porcine ASM and, if so, whether this inhibition depended on whether the  $G\alpha_i$  heterotrimer was regulated by the  $M_2$  muscarinic receptor. However, in a previous study of crude membrane prepared from this tissue, we could not reliably measure acetylcholine-promoted  $G\alpha_i$  guanosine nucleotide exchange using the techniques presented in the current study.<sup>14</sup> Consequently, we used an experimental approach that investigated anesthetic effects on increasingly more complex protein models beginning with the recombinant  $G\alpha_{i-3}$  monomer and the  $G\alpha_{i-3}\beta_1\gamma_{2HF}$  heterotrimer (isoforms expressed in ASM) and ending with a well-established, mammalian protein expression model in which the  $M_2$  muscarinic receptor and  $G\alpha_{i-3}$  heterotrimeric G protein are transiently expressed and are functionally coupled.<sup>38</sup>

Halothane (0.5–1.0 mM) had no effect on basal  $G\alpha_{i-3}$  [<sup>35</sup>S]GTP $\gamma$ S-GDP exchange in membranes prepared from the  $M_2$ - $G\alpha_{i-3}$  cotransfected COS-7 cells. This result is consistent with our previous findings that halothane, at similar concentrations, had no effect on the  $G\alpha_i$  heterotrimer extracted from porcine ASM membrane in a preparation without functional receptor coupling.<sup>27</sup> These data are also consistent with the findings of the current study that halothane, while binding to and causing a small but statistically significant inhibition of the intrinsic GTPase activity of the isolated  $G\alpha_{i-3}$  monomer expressed in Sf9 insect cells (studied at 3 mM), had no effect on the  $G\beta_1\gamma_{2HF}$ -regulated GTPase activity of  $G\alpha_{i-3}$  (*i.e.*, the heterotrimer), the biologically relevant protein complex. When interpreted in aggregate, these data suggest that halothane does not directly inhibit guanosine nucleotide exchange at  $G\alpha_{i-3}$  of the  $G\alpha_{i-3}\beta_1\gamma_{2HF}$  heterotrimeric protein.

Interestingly, we also observed that in contrast to the more clinically relevant halothane concentrations, very high concentrations (3 mM) inhibited basal  $G\alpha_{i-3}$  [<sup>35</sup>S]GTP $\gamma$ S-GDP exchange in the  $M_2$ - $G\alpha_{i-3}$  coexpressing membranes. It is likely that this effect was due to inhibition of the constitutive coupling between endogenous receptors in COS-7 cells and the transiently expressed  $G\alpha_{i-3}$ . This assertion is based on the observations that the basal  $G\alpha_{i-3}$  guanosine exchange was not due to constitutive  $M_2$  muscarinic receptor activity, because there was no difference in the magnitude of [<sup>35</sup>S]GTP $\gamma$ S incorporation into  $G\alpha_{i-3}$  between membranes prepared from cells expressing  $G\alpha_{i-3}$  alone or  $G\alpha_{i-3}$  and the  $M_2$  muscarinic receptor. In addition, treatment of membranes prepared from cells expressing  $G\alpha_{i-3}$  alone with pertussis toxin abolished basal  $G\alpha_{i-3}$  [<sup>35</sup>S]GTP $\gamma$ S-GDP exchange, implying that the observed basal activity was promoted by constitutively active endogenous receptors.

In contrast to the lack of a direct effect on the intrinsic, receptor-unregulated GTPase activity of the  $G\alpha_{i-3}$  heterotrimer, we found that incubating the crude membrane prepared from the  $M_2$ - $G\alpha_{i-3}$  cotransfected cells with 0.5–1 mM halothane caused a concentration-dependent inhibition of  $G\alpha_{i-3}$  [<sup>35</sup>S]GTP $\gamma$ S-GDP when promoted by acetylcholine binding to the  $M_2$  muscarinic receptor. These data are similar to our previous studies of crude membrane prepared from differentiated porcine ASM, where halothane and the anesthetic hexanol inhibited acetylcholine-promoted guanosine nucleotide exchange at  $G\alpha_{q/11}$ , but also had no effect on basal exchange.<sup>14,26</sup> A possible direct anesthetic effect on native, recombinant  $G\alpha_q$  subfamily protein activity could not be examined in that study because of the essentially unmeasurable intrinsic GTPase activity of these proteins.<sup>50</sup>

Protein-anesthetic binding can be determined with STD,<sup>28</sup> which is an NMR technique used to probe low-affinity interactions ( $K_d \approx 10^{-8}$  to  $10^{-3}$  M) between small molecules and soluble proteins.<sup>51</sup> With this technique, samples are alternately irradiated at a frequency that only certain protons within the protein absorb (on-resonance) and a frequency far from resonance with any component of the protein (off-resonance). The absorbed on-resonance radiation (*i.e.*, saturation) rapidly distributes throughout the network of protein protons by spin diffusion.<sup>52</sup> The saturation is also transferred from the protein protons to the protons of bound molecules (ligands) with a rate that depends on the protein mobility, ligand-protein complex lifetime, and geometry.<sup>28</sup> The transfer of saturation to the ligand is detected from the difference spectrum, which is calculated by subtracting the spectrum recorded with on-resonance irradiation from the spectrum collected with off-resonance irradiation. Because the chemical shifts of small molecules are distinctive in high-resolution spectroscopy, it is possible

to screen several molecules for binding to a single protein simultaneously.<sup>51</sup>

Using STD spectroscopy, we observed saturation transfer from  $G\alpha_{i-3}$  to suramin, a positive control for the technique, and both halothane and hexanol, evidence of anesthetic binding to  $G\alpha_{i-3}$ . However, this binding was not associated with inhibition of the  $G\alpha_{i-3}$  heterotrimer activity. Halothane did not inhibit the GTPase activity of nonmyristoylated  $G\alpha_{i-3}$  purified from *E. coli*; in fact, halothane tended to cause a slight activation at the higher concentrations. In addition, although 3 mM halothane (approximately 15 minimum alveolar concentration) caused a small but statistically significant inhibition of the intrinsic GTPase activity of myristoylated  $G\alpha_{i-3}$  purified from Sf9 cells, there was no halothane effect on the GTPase activity of the  $G\alpha_{i-3}\beta_1\gamma_{2HF}$ , the more physiologically and biochemically relevant form. Therefore, halothane binding either has biochemically significant consequences on the heterotrimer only when it is regulated by the  $M_2$  muscarinic receptor or has no consequences at all for heterotrimer function. When interpreted in aggregate, these data suggest that in this receptor-heterotrimer complex, the salient, biochemically relevant protein target might be  $M_2$  muscarinic receptor in contrast to the  $G\alpha_{i-3}$  heterotrimer. This assertion is consistent with previous studies showing photoaffinity labeling of [<sup>14</sup>C]halothane to a specific tryptophan of the prototypical G protein-coupled receptor, rhodopsin.<sup>53,54</sup>

In summary, this study demonstrated for the first time through direct biochemical assessment that halothane, in concentrations used clinically to treat severe bronchospasm, interacts with the  $M_2$  muscarinic receptor- $G\alpha_{i-3}$  heterotrimeric complex in a manner that prevents acetylcholine-promoted guanosine nucleotide exchange the  $G\alpha_{i-3}$ . This novel effect of halothane was observed at near maximal activation of the  $M_2$  muscarinic receptor (10  $\mu$ M acetylcholine), a finding consistent with the ability of halothane to inhibit isolated ASM contraction even when maximally stimulated<sup>9</sup> and to cause direct bronchodilation of airways.<sup>5,6</sup> Halothane had no direct inhibitory effect on the intrinsic biochemical activity of the  $G\alpha_{i-3}\beta_1\gamma_{2HF}$  heterotrimer, despite evidence of anesthetic binding to the  $G\alpha_{i-3}$  subunit.

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