Anesthesiology 2003; 99:105–11

Effect of Halothane on the Guanosine 5′ Triphosphate Binding Activity of G-Protein α_i Subunits

John Streiff, Ph.D., Kristofer Jones, B.S.,† William J. Perkins, M.D.,‡ David O. Warner, M.D.,§ Keith A. Jones, M.D.‡*

Background: **Receptor-mediated increases in the force produced by airway smooth muscle are attenuated by anesthetics such as halothane. Guanosine 5′-triphosphate (GTP) binding protein α subunits ($G\alpha_i$) are known to participate in the regulation of force in airway smooth muscle. The authors hypothesized that halothane would inhibit the ability of $G\alpha_i$ subunits to bind a nonhydrolyzable analog of GTP ($GTP\gamma S$).**

Methods: **The effect of halothane on both GTPase-specific activity and $[^{35}S]GTP\gamma S$ binding were assayed using purified, recombinant $G\alpha_{i1}$. In separate experiments, $[^{35}S]GTP\gamma S$ binding to $G\alpha_i$ in crude airway smooth muscle membrane preparations was assayed using an immunoprecipitation technique in the presence and absence of halothane.**

Results: **The steady state GTPase-specific activity of the recombinant $G\alpha_{i1}$ was 0.033 ± 0.018 (mean ± SD) mole P_i mole $G\alpha_{i1}^{-1}$ min^{-1} under control conditions and 0.035 ± 0.015 mole P_i mole $G\alpha_{i1}^{-1}$ min^{-1} in the presence of 1.1 ± 0.2 mM halothane, a difference that is not significant. The mole fractions of recombinant $G\alpha_{i1}$ bound to $[^{35}S]GTP\gamma S$ were 0.49 ± 0.02 and 0.60 ± 0.02 at 10 and 20 min, respectively. The addition of halothane (1.26 ± 0.07 mM) did not significantly change these values. Halothane did not affect the binding of $[^{35}S]GTP\gamma S$ to $G\alpha_i$ subunits in membrane fractions of airway smooth muscle as measured using immunoprecipitation. Validity of the assays was confirmed using suramin, an inhibitor of GTP binding.**

Conclusion: **These results suggest that halothane, which inhibits receptor-activated $G\alpha_i$-coupled pathways in intact airway smooth muscle, must functionally target a component of the G protein–coupled receptor complex other than $G\alpha_i$.**

MANY current theories of anesthetic mechanisms posit effects on receptor-mediated processes in cells. One potential target is the function of systems regulated by guanosine 5′-triphosphate (GTP) binding proteins (G proteins). However, the site(s) at which anesthetics act to modify receptor-heterotrimeric G-protein signaling is not yet understood. Pentyala *et al.*[1] found that halothane and other volatile anesthetics at clinically relevant concentrations modulated the binding of guanine nucleotides to purified α subunits in aqueous solution, inhibiting the exchange of guanosine 5′-diphosphate (GDP) for a nonhydrolyzable analog of GTP ($GTP\gamma S$). This finding suggests that anesthetics can interact directly with the guanine-nucleotide binding (catalytic) site. Somewhat to the contrary, Ishizawa *et al.*[2] concluded from photoaffinity labeling studies that halothane incorporates into the receptor rhodopsin but not the coupled G proteins. Our experiments in airway smooth muscle (ASM) suggested but did not prove that halothane acts directly on heterotrimeric G proteins coupled to muscarinic receptors.[3,4]

The overall goal of this study was to determine whether halothane affects the function of the α subunit of heterotrimeric G proteins. We hypothesized that halothane would inhibit the ability of $G\alpha_i$ subunits, which are known to participate in the membrane receptor regulation of calcium sensitivity in ASM, to bind $GTP\gamma S$. We examined the GTP binding properties of $G\alpha_i$ subunits in crude membrane preparations from ASM tissue using an immunoprecipitation technique, and the GTP binding and hydrolysis properties of recombinant, purified $G\alpha_i$ subunits in aqueous solution.

Materials and Methods

$[S^{35}]GTP\gamma S$ Binding to Recombinant $G\alpha_{i1}$

The rate of $[^{35}S]GTP\gamma S$ binding to recombinant $G\alpha_{i1}$ was measured, following a modified version of the method of Sternweis and Robishaw.[5] For each time point in the $[^{35}S]GTP\gamma S$ binding assay, the reaction was carried out at 30°C in a 250-μl tube, which was sealed with a Teflon stopper. Final assay concentrations in 150 μl assay were 50 mM HEPES (pH 8), 1 mM EDTA, 1 mM dithiothreitol, 10 mM $MgCl_2$, 0.1% polyoxyethylene 10 lauryl ether ($C_{12}E_{10}$), 0.7 μM $GTP\gamma S$, 0.4 μCi $[^{35}S]GTP\gamma S$, 30 nM GDP, 8 nM $G\alpha_{i1}$, and 0.25 μM bovine serum albumin. Anesthetic was incorporated when appropriate by bubbling halothane for 15 min with a vaporizer into buffer containing 25 mM HEPES (pH 8), 1 mM EDTA, 1 mM dithiothreitol, and 10 mM $MgCl_2$, which was then added to the assay tubes without changing the final volume. The reactions were quenched with 1 ml ice-cold stop buffer (25 mM Tris [pH 8], 20 mM $MgCl_2$, and 100 mM NaCl). This was immediately filtered under vacuum through 0.45-μm high-affinity nitrocellulose filters from Millipore (Bedford, MA), washed five times with 3 ml stop buffer, dried, and counted in 4 ml liquid scintillation cocktail. Protein-free blanks were run with each assay and subtracted from the total counts before further processing. The $G\alpha_{i1}$ concentration was determined from the binding at 3 h (assuming that binding was complete at this time), and all data were normalized to this value.

* Research Fellow, Department of Anesthesiology, † Research Technician, ‡ Associate Professor of Anesthesiology, § Professor of Anesthesiology.

Received from the Departments of Anesthesiology and Physiology and Biophysics, Mayo Clinic and Mayo Foundation, Rochester, Minnesota. Submitted for publication November 20, 2002. Accepted for publication February 27, 2003. 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. Presented at the annual meeting of the American Society of Anesthesiology, Orlando, Florida, October 15, 2002.

Address reprint requests to Dr. Warner: Mayo Clinic and Foundation, 200 First Street Southwest, Rochester, Minnesota 55905. Address electronic mail to: warner.david@mayo.edu.

Tryptophan Fluorescence

The increase in intrinsic tryptophan fluorescence accompanying GTPγS binding, described by Higashijima *et al.*,[6] was used as a complementary technique to monitor the binding of GTPγS to recombinant subunits. In this technique, 1 μM $G\alpha_{i1}$ in 1.15-ml solution of 25 mM HEPES (pH 8), 1 mM EDTA, 1 mM dithiothreitol, 10 mM $MgCl_2$, 0.1% $C_{12}E_{10}$, 10 μM GTPγS, and 300 nM GDP was sealed in a 1-mm quartz cell with a Teflon stopper. Note that the GTPγS concentration was in excess of the G protein but by an order of magnitude less than that used in the [^{35}S]GTPγS binding assay to minimize the inner filter effect at the excitation wavelength. In both the fluorescence and [^{35}S]GTPγS assays, there was an approximately 25-fold excess of GTPγS to GDP. The intrinsic protein tryptophan fluorescence (λ_{ex} = 290 nm, λ_{em} = 340 nm) was recorded at 20-s intervals for 60 min with a Spex Fluorolog fluorimeter (Jobin Yvon Inc., Edison, NJ). During the assay, the cell holder was maintained at 30°C, and the contents were stirred continuously with a magnetic stir bar. The baseline fluorescence of the GDP-bound subunit, measured just prior to nucleotide addition, was subtracted, and the data were analyzed as net increase in fluorescence. As with the [^{35}S]GTPγS assay, halothane was added from a stock solution prepared by bubbling. Halothane quenches the fluorescence of tryptophan (isolated amino acid) by approximately 1.8%/mM,[7] which was accounted for when analyzing the fluorescence intensity curves.

$G\alpha_{i1}$GTP Hydrolysis

GTPase activity was measured by the release of [^{32}P]-labeled inorganic phosphate, based on the procedure described by Higashijima.[8] $G\alpha_{i1}$, 0.2 μM, was incubated at 30°C in 25 mM HEPES (pH 7.8), 100 mM NaCl, 10 mM $MgSO_4$, 1 mM EDTA, 1 mM dithiothreitol, 0.1 μM GDP, 5 μM GTP, 0.025 mCi [γ^{32}P]GTP, and 0.5 mg/ml bovine serum albumin with and without halothane (control), which was diluted from an aqueous stock solution. At 10 and 20 min after initiation, aliquots from each assay tube were quenched with a solution of 7% (w/v) activated charcoal in 2 N HCL and 0.35 M NaH_2PO_4 and then vortexed for 10 s. The tubes were centrifuged at maximum speed in a microfuge E centrifuge for 10 min to pellet the charcoal. Aliquots of the supernatant from each assay sample time point were transferred to a scintillation vial with 3 ml water, and the amount of [^{32}P]-labeled inorganic phosphate was determined by Cerenkov counting.

Measurement of GTPγS Binding to $G\alpha_i\beta\gamma$ Heterotrimer Immunoprecipitated from ASM Membrane Preparations

GTPγS binding to $G\alpha_i\beta\gamma$ heterotrimeric complex associated in crude membrane prepared from ASM tissue was measured using a modification of previously described methods.[9,10] Porcine tracheal smooth muscle strips (260–280 mg) were frozen and pulverized in liquid N_2 to a fine powder with a mortar and pestle, which was kept cold with dry ice. The powdered tissue was then suspended for 15 min by vigorous mixing in 1.5 ml ice-cold extraction buffer and then centrifuged at 4°C for 10 min at 2,500*g*. The extraction buffer contained 20 mM HEPES (pH 8.0), 1 mM EDTA, and the protease inhibitors 0.1 mM phenylmethanesulfonyl fluoride, 2 μg/ml aprotinin, and 10 μg/ml leupeptin. The crude membrane pellet was washed three times with 1 ml extraction buffer, resuspended in the assay buffer, and homogenized with 20 strokes in a Dounce tissue grinder. The assay buffer contained 50 mM Tris-HCl (pH 7.4), 4.8 mM $MgCl_2$, 2 mM EDTA, 100 mM NaCl, and 1 μM GDP. The crude homogenate was incubated on ice for 1 h and then centrifuged at 4°C for 10 min at 2,500*g*.

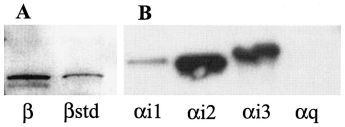

Fig. 1. Western blots of the supernatant from crude airway smooth muscle membrane suspensions. (*A*) Immunoassay for Gβ subunit using Gβ common antibody (Calbiochem). *Left to right:* Supernatant (β) and Gβ standard (βstd; Calbiochem). (*B*) Gα subunit standards Western blotted with anti-$G\alpha_{i3}$ (Calbiochem), showing the cross-reactivity. *Left to right:* $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, and $G\alpha_q$ (*empty lane*) protein standards (Calbiochem).

The resulting supernatant contained intact $G\alpha_i\beta\gamma$ heterotrimeric complex (fig. 1, *A*). To immunoprecipitate, an immunoaffinity purified rabbit antibody directed against the C-terminal nine amino acids of $G\alpha_{i\text{-}3}$ (Calbiochem, San Diego, CA) was used at a 1:200 dilution. This antibody has cross-reactivity with $G\alpha_{i\text{-}1}$, $G\alpha_{i\text{-}2}$, and $G\alpha_{i\text{-}3}$ but not with $G\alpha_{q/11}$ (fig. 1, *B*). Further centrifuging the supernatant at 100,000*g* for 1 h pelleted the membrane completely, which removed any trace of $G\alpha_i\beta\gamma$ heterotrimeric complex from the supernatant, as determined by Western blot (data not shown). Thus, the supernatant can be characterized as a colloidal suspension of small membrane particles containing the $G\alpha_i\beta\gamma$ heterotrimeric complex.

To measure the protein concentration in the supernatant, 50 μl was first solubilized by the addition of 12.5 μl NaOH, 0.1 N, and boiled for 30 min. The protein concentration was measured by the Lowry method, using the detergent compatible assay from Bio-Rad (Hercules, CA). The supernatant was diluted with cold assay buffer to 2.5 mg/ml.

The [^{35}S]-GTPγS binding to $G\alpha_i$ subunits in the supernatant was performed by modification of previously described methods.[9] To minimize variability between tissue preparations, assays were run in duplicate on supernatant from the same tissue preparations. Supernatant (55 μl) was incubated at 30°C without (control) or with 1 mM halothane for 10 min. The reactions were

then initiated by the addition of 6 nM (final concentration) of [^{35}S]GTPγS (≈ 2.7 mCi/fmol) to each assay tube. The reactions were quenched after 1, 5, 10, and 30 min with 0.6 ml ice-cold immunoprecipitation buffer containing 50 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 150 mM NaCl, 0.5% of the nonionic detergent IGEPAL CA630, 100 μM GDP, 100 μM GTP, and 20 μg/ml aprotinin. Then, the suspensions were incubated with 70 μl protein A-Agarose beads that had been precoated with rabbit anti-$G\alpha_{i3}$ antirabbit immunoglobulin directed against an epitope at the C terminus of $G\alpha_{i\text{-}3}$. These mixtures were incubated for 2 h at 4°C on a rocker to ensure continuous mixing. After this incubation, the mixture was centrifuged at 2,500*g*, and the supernatant was removed by aspiration. The pelleted beads were then washed five times with 1 ml immunoprecipitation buffer. The beads were then transferred onto very low protein binding filters (Millipore, Billerica, MA) and washed a final time with 20 ml immunoprecipitation buffer. The dried filter paper retaining the beads was placed in a scintillation vial containing 4 ml liquid scintillant (Ultima Gold; Packard Bioscience, Boston, MA) and counted. The nonspecific binding of [^{35}S]-GTPγS to the beads in the absence of protein was used as the blank value and was determined simultaneously for each study.

Anesthetic Concentrations

Halothane concentrations in assay solutions were estimated from assay tubes handled under identical conditions and run simultaneously with the assay. Halothane concentrations in solution were measured by gas chromatography after hexane extraction by the method of Van Dyke and Wood.[11]

Statistical Analysis

Results are presented as mean ± SD; n refers to the number of pigs for the crude membrane preparations, or the number of assay replicates for recombinant protein. The GTPase-specific activities of recombinant $G\alpha_{i1}$, with and without halothane, were compared by paired *t* test.

The GTPγS binding curves of the recombinant protein, generated with the intrinsic fluorescence and [^{35}S]GTPγS binding assays, and the endogenous protein, generated with [^{35}S]GTPγS immunoprecipitation assay, were fit with the equation $y = a(1 - e^{-kt})$ from reference 12 using nonlinear least squares fitting. In the equation, the independent variable is t, time, the dependent variable is a, the amount of $G\alpha_{i1}$ bound to GTPγS, the parameter k is the on rate of the ligand for the bimolecular reaction, and the parameter a vertically scales the curve. For recombinant $G\alpha_{i1}$, the k parameters were compared for significance by *t* test (n = 3). For the effect of suramin on the endogenous subunit, the k parameter for each curve fit was used to calculate the amount of $G\alpha_i$ bound to GTPγS at 15 min. The amounts of $G\alpha_{i1}$ bound to GTPγS, calculated from the fits to the control and suramin curves, were compared for significance on an experiment by experiment basis by Student paired *t* test.

The effect of halothane or suramin on the amount of recombinant $G\alpha_{i1}$ bound to [^{35}S]GTPγS at specified times was examined using a paired *t* test. The effect of halothane, atropine, or acetylcholine on the amount of endogenous $G\alpha_i$ bound to [^{35}S]GTPγS binding at 10 min was assessed by one-way repeated-measures analysis of variance.

Significance was assigned at $P < 0.05$. Sigma Plot and Sigma Stat (Jandel Scientific, San Rafael, CA), respectively, were used to perform the curve fitting and statistics.

Materials

Hexahistidine-tagged recombinant $G\alpha_{i1}$ subunit, subcloned from rat and expressed in *Escherichia coli*, was generously provided by Alfred G. Gilman,[13] M.D. Ph.D. (Professor of Pharmacology, University of Texas Southwestern Medical Center, Dallas, Texas). [^{35}S]GTPγS was purchased from Amersham Biosciences (San Francisco, CA). Unless specifically mentioned in the text, other materials were purchased from Sigma (St. Louis, MO).

Results

GTPase-Specific Activity of Recombinant $G\alpha_{i1}$ Subunits

The steady state GTPase-specific activity of the recombinant $G\alpha_{i1}$ was 0.033 ± 0.018 mole P_i mole $G\alpha_{i1}{}^{-1}$ min^{-1} under control conditions and 0.035 ± 0.015 mole P_i mole $G\alpha_{i1}{}^{-1}$ min^{-1} in the presence of 1.1 ± 0.2 mM halothane, a difference that is not significant ($P = 0.628$, n = 15).

Binding of [^{35}S]GTPγS to Recombinant $G\alpha_{i1}$ Subunits

$G\alpha_{i1}$ bound [^{35}S]GTPγS in a time-dependent fashion (fig. 2, *A*), with a k of 0.095 ± 0.002 min^{-1} (n = 3).

Suramin, which is known to inhibit GTPγS binding to Gα subunits by inhibiting GDP dissociation,[14] significantly ($P < 0.02$, n = 3) decreased the mole fraction of GTPγS-bound $G\alpha_{i1}$ at 15 min from 0.62 ± 0.10 to 0.49 ± 0.03 (fig. 2, *A*). However, because GTPγS is a nonhydrolyzable analog of GTP, which is more tightly bound, near-stoichiometric binding was achieved within 3 h even in the presence of suramin (n = 3).

The mole fractions of $G\alpha_{i1}$ bound to [^{35}S]GTPγS were 0.49 ± 0.02 and 0.60 ± 0.02 at 10 and 20 min, respectively. The addition of halothane (1.26 ± 0.07 mM) did not significantly change these values (0.48 ± 0.005 and 0.63 ± 0.016, respectively, $P = 0.14$, n = 3; fig. 2, *A*).

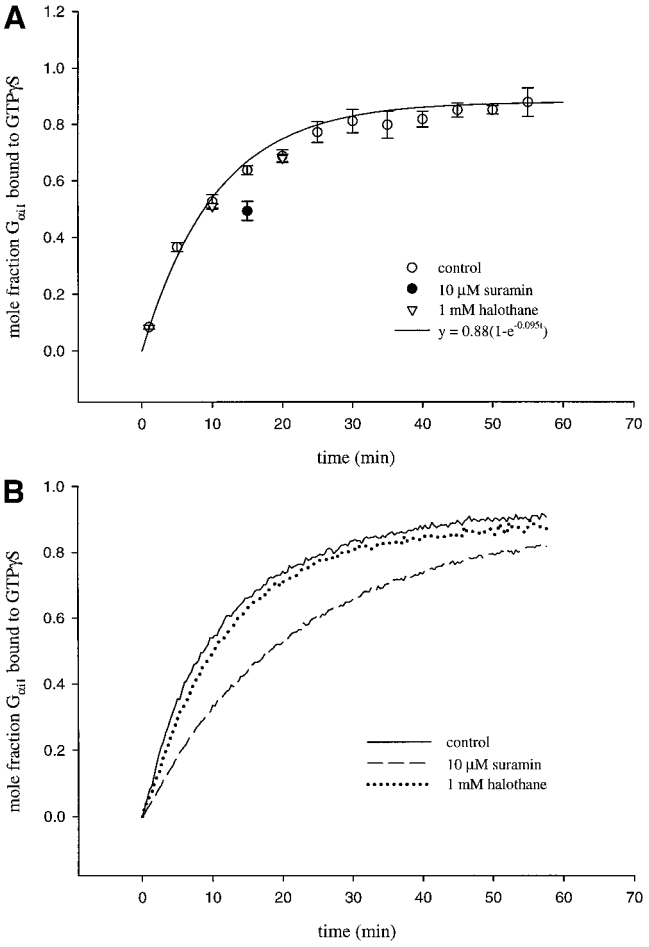

Fig. 2. (*A*) [^{35}S]GTPγS binding of recombinant $G\alpha_{i1}$. Data are the mean of three measurements. *Open circles* = control data; *solid line* = control data fit with $y = a(1 - e^{-kt})$; *closed circle* = amount of [^{35}S]GTPγS bound by $G\alpha_{i1}$ in the presence of 10 μM suramin; *filled triangles* = amount of [^{35}S]GTPγS bound by $G\alpha_{i1}$ in the presence of 1 mM halothane. The ordinate is the amount of $G\alpha_{i1}$ bound to [^{35}S]GTPγS relative to the amount of [^{35}S]GTPγS bound after 3 h. (*B*) GTPγS binding of recombinant $G\alpha_{i1}$ assayed by the intrinsic fluorescence increase. Each curve is the average of three measurements. *Solid line* = intrinsic fluorescence increase of the control; *dotted line* = curve in the presence of 0.6 mM halothane; *dashed line* = curve in the presence of 10 μM suramin. The fluorescence curves were fit with the equation $y = a(1 - e^{-kt})$ and scaled by the a parameter of the fit to the control [^{35}S]GTPγS binding time course to place them on the same ordinate scale. GTP = guanosine 5'-triphosphate.

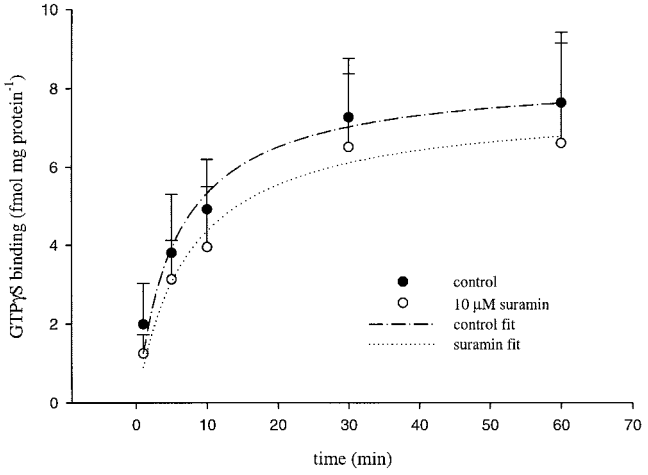

Fig. 3. [^{35}S]GTPγS binding, expressed as femtomoles [^{35}S]GTPγS bound per milligrams total protein, of endogenous $G\alpha_i$ immunoprecipitated from membrane preparation. Data are the mean of seven experiments (done in duplicate). *Filled circles* = control data collected at 1, 5, 10, 30, and 60 min; *open circles* = data collected in tandem with the control and containing 10 μM suramin. GTP = guanosine 5'-triphosphate.

Binding of GTPγS to Recombinant $G\alpha_{i1}$ Subunits Assayed by Intrinsic Fluorescence

The a parameter from the curve fit to the [^{35}S]GTPγS-binding time course was substituted into the control fluorescence curve fit equation, which scales the fluorescence curve fit to the same ordinate units for direct comparison. An assumption that the observed fluorescence intensity change corresponds exclusively to GTPγS binding at the $G\alpha_{i1}$ active site was made and is justified on the basis of previous observations.[6,8] Furthermore, on the basis of our [^{35}S]GTPγS binding experiments, which demonstrate that inhibition of binding with suramin changes the rate of GTPγS binding (k) but not the final stoichiometry (a), it was possible to scale all subsequent fluorescence curve fits with the a parameter from the control [^{35}S]GTPγS-binding time course. This allowed the comparison of the radioactive and fluorescence data on the same ordinate scale. Under control conditions, the intrinsic fluorescence intensity curve fit had a k of 0.091 ± 0.015 min^{-1}(n = 3, fig. 2, *B*), which is nearly identical to that obtained in the control [^{35}S]GTPγS-binding time course. Thus, the two methods provide comparable results, which are consistent with previously reported values for this subunit.[15,16]

Suramin (10 μM) significantly decreased the value of k (to 0.046 ± 0.004 min^{-1}) compared with control conditions (n = 3, $P < 0.01$; fig. 2, *B*). The increase in fluorescence observed at 15 min in the presence of suramin was on average 33% smaller than the increase in the control fluorescence at that time. This is similar to the 22% decrease in the mean mole fraction of [^{35}S]GTPγS bound to $G\alpha_i$ in the presence of 10 μM suramin at 15 min. In contrast to the effect of suramin, halothane (0.59 ± 0.03 mM) did not significantly (n = 3, $P = 0.1$) affect the rate of fluorescence increase (k = 0.083 ± 0.002 min^{-1}; fig. 2, *B*).

[^{35}S]GTPγS Binding to Endogenous $G\alpha_i\beta\gamma$ Heterotrimer in Membrane Preparations from ASM Tissue Assayed via *Immunoprecipitation*

[^{35}S]GTPγS binding increased in a time-dependent manner with a mean rate that was approximately twice that measured in the recombinant $G\alpha_{i1}$ (k = 0.17 ± 0.08 min^{-1}, n = 7).

Suramin (10 μM) did not significantly affect the mean of the fitted k values (0.18 ± 0.13 min^{-1}, $P = 0.92$, n = 7; fig. 3). However, when compared on a per-experi-

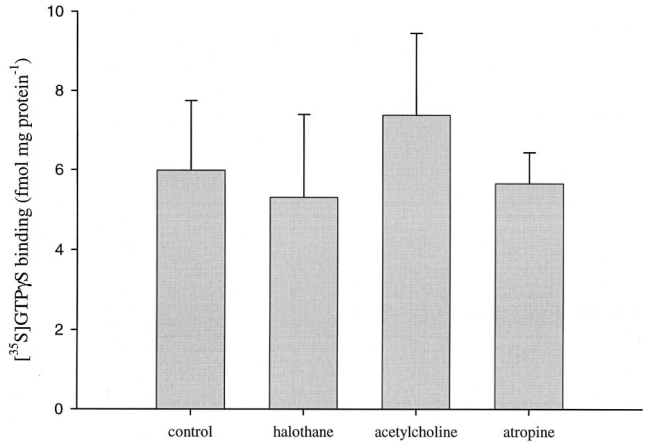

Fig. 4. The effects of 10 μM atropine (n = 4), 100 μM acetylcholine (n = 4), and 1 mM halothane (n = 6) on the amount of [35S]GTPγS bound by endogenous Gαi immunoprecipitated from membrane preparation, assayed at 10 min. The amount of bound [35S]GTPγS, expressed as femtomoles [35S]GTPγS bound per milligrams total protein, are the mean of the specified number of measurements, done in duplicate, and with a paired control. None of the three significantly affected the [35S]GTPγS binding as judged by one-way repeated-measures analysis of variance. GTP = guanosine 5'-triphosphate.

ment basis with Student *t* test, suramin significantly reduced the amount of [35S]GTPγS bound at 10 min, as calculated from the fit parameters (5.7 ± 1.7 and 4.2 ± 1.4 fmol/mg for control *vs.* suramin, $P < 0.02$, n = 7).

To further characterize this preparation, muscarinic receptor function was examined in a separate set of studies. Neither 100 μM acetylcholine nor 10 μM atropine affected [35S]GTPγS binding at 10 min of assay (n = 4; fig. 4), suggesting that muscarinic receptors are not functionally coupled to heterotrimeric G proteins in this preparation.

Halothane (0.99 ± 0.17 mM) did not significantly affect [35S]GTPγS binding measured at 10 min (n = 6; fig. 4).

Discussion

The major finding of this study is that halothane, at concentrations sufficient to produce both anesthesia *in vivo* and relaxation of intact ASM *in vitro*, does not inhibit the binding of GTPγS to recombinant $G\alpha_{i1}$ subunits in aqueous solution or its subsequent hydrolysis. Likewise, similar concentrations of halothane do not inhibit the binding of GTPγS by endogenous $G\alpha_i\beta\gamma$ heterotrimer in crude membrane preparations from ASM tissue.

In the current study, we examined the effects of halothane on the $G\alpha_i$ subunit to explore the mechanism responsible for the observed inhibition of muscarinic receptor–induced increases in calcium sensitivity by halothane in ASM.[3] Our prior work localized the site of halothane's action to the muscarinic G protein–coupled receptor complex.[4] We and others have also shown that the $G\alpha_i$ subunit is responsible at least in part for mediating these effects.[4,17–19]

The anesthetic target is not currently known, but is assumed to be a component of the G protein–coupled receptor complex. Studies of halothane interaction with rhodopsin-transducin have failed to demonstrate direct binding of the anesthetic to the G protein.[2,20] However, Pentyala *et al.*[1] found that volatile anesthetics (including halothane, isoflurane, and sevoflurane) at low concentrations (approximately 0.25 mM, equivalent to approximately 1 minimum alveolar concentration) modulated the binding of guanine nucleotides to purified Gα subunits in aqueous solution, inhibiting the exchange of GTPγS for bound GDP. This effect was observed for several subunits studied (including G_i and G_s) but not all (including G_o and monomeric G proteins). This finding suggested potential anesthetic effects directly on the guanine-nucleotide binding (catalytic) site that may be specific to Gα subunit isotype. However, these isoform-specific results are not totally consistent with the behavior of the corresponding isoforms in cardiac muscle membranes, where $G\alpha_i$ and $G\alpha_S$ couple M_2 muscarinic and β-adrenergic receptors, respectively, to adenylate cyclase. In preparations of these membranes, halothane interferes with muscarinic receptor inhibition of adenylate cyclase through $G\alpha_i$ but does not affect β-adrenergic receptor stimulation *via* $G\alpha_S$.[17,21,22]

Recombinant $G\alpha_{i1}$ Subunits

In the current study, the GTPase-specific activity and the [35S]GTPγS binding kinetic profile of recombinant $G\alpha_{i1}$ under control conditions (fig. 2) were similar to those previously reported for $G\alpha_i$ isoforms.[15,16] The results of the [35S]GTPγS binding assays were verified by comparison to the results of the increase in intrinsic fluorescence, run under similar conditions. At times less than 60 min, $G\alpha_{i1}$ in the presence of suramin bound less GTPγS than the control, presumably because suramin inhibited the off rate of GDP,[14] which is the rate-limiting step to GTP binding and hydrolysis.[8] These findings suggest the validity of our GTPγS binding assay.

On the other hand, halothane did not significantly affect the amount of GTPγS bound to $G\alpha_{i1}$ compared with control conditions. Our results are not consistent with the conclusion of Pentyala *et al.*[1] that clinically relevant concentrations of halothane reduce the binding of GTP. We observed neither an inhibition of GTPγS binding nor an inhibition of GTP hydrolysis.

What could explain the apparent contradiction between our results and those of Pentyala *et al.*[1]? The [35S]GTPγS binding results were obtained with similar assay methods. One possible point of departure is different sources of Gα subunits. However, the binding kinetics and hydrolysis rate that we measured for the $G\alpha_{i1}$ are in accordance with published literature and are invariate in the presence of up to 1 mM halothane. Thus, we

cannot explain the reason for the differences in our results.

GTPγS Binding to Endogenous $G\alpha_i$ in Crude Membrane Preparation from ASM

To confirm our results, we also studied the ability of endogenous membrane–associated G-protein heterotrimer from crude membrane preparations of ASM tissue to bind GTPγS. This preparation also provided a milieu in which to test whether halothane might affect unstimulated $G\alpha_i\beta\gamma$ GTPγS binding through direct interaction on the $G\alpha_i$ subunit or indirect effects through the heterotrimer. The lack of effect of acetylcholine, a receptor agonist, or atropine, a receptor antagonist, on $G\alpha_i\beta\gamma$ GTPγS binding demonstrated that the heterotrimer was functionally uncoupled from the muscarinic receptor. Thus, the measured $G\alpha_i\beta\gamma$ GTPγS binding was not regulated by membrane receptors. However, this membrane preparation probably contains other regulators of G-protein activity that may affect the GTP binding activity of $G\alpha_i$. For example, the basal GTPγS binding activity of recombinant $G\alpha_q$ in aqueous solution is undetectable but is measurable in membrane preparations.[23,24] This factor may explain the more rapid kinetics of binding observed in the current immunoprecipitation study compared with the kinetics measured for recombinant $G\alpha_i$ subunits in aqueous solution.

Despite these differences, the pattern of results was similar between the two preparations. Suramin had an inhibitory effect on GTPγS binding by $G\alpha_i\beta\gamma$ in both the membrane preparation and the recombinant $G\alpha_i$, confirming the ability of both assays to detect a decrease in GTPγS binding. However, unlike the recombinant protein, suramin did not significantly alter the mean rate (k) of binding to endogenous protein in the membrane preparation compared to the control. This may arise partly because of the presence of intracellular effectors, which enhance the apparent rate of GTPγS binding by $G\alpha_i$ in the membrane preparation compared to the isolated recombinant protein. If these effectors act to enhance the off rate of GDP from $G\alpha_i$, they might partially counteract the effect of suramin, which is to inhibit the GDP off rate. Halothane did not inhibit the basal GTPγS binding activity of endogenous $G\alpha_i$ in the membrane preparation (fig. 4). Taken together with a similar lack of effect on recombinant $G\alpha_i$, this finding suggests that factors such as the presence of $G\beta\gamma$ subunits do not modulate any effects of halothane on G-protein function *in situ*. This finding does not appear to lend support to the proposal that anesthetics stabilize the binding of the $G\alpha_i$ subunit to the $G\beta\gamma$ dimer, although a more detailed study of such an effect is required to definitively address this.[25] It is possible that features necessary for an effect of halothane on basal GTPγS binding activity (such as functional coupling to receptors) are lost in the membrane preparation assayed, so this conclusion cannot be definitive. It is also possible that halothane could affect the activity of other $G\alpha$ subunits that regulate contractility in ASM, such as $G\alpha_q$.[26,27] However, the current results suggest that the target of anesthetic effects to inhibit G protein–coupled receptor responses is the receptor itself, or the interface between receptor and the heterotrimeric G protein, rather than a site on the $G\alpha$ subunit that regulates guanine nucleotide exchange.

Conclusion

Halothane did not inhibit the basal GTPγS binding or hydrolysis activity of isolated, recombinant $G\alpha_{i1}$ subunits or the unregulated GTPγS binding of endogenous $G\alpha_i$ subunits in a crude membrane preparation of ASM. These results suggest that halothane, which inhibits receptor-activated $G\alpha_i$-coupled pathways in ASM, must functionally target a component of the G protein–coupled receptor complex other than $G\alpha_i$.

The authors thank Alfred G. Gilman, M.D. Ph.D. (Professor of Pharmacology, University of Texas Southwestern Medical Center, Dallas, Texas), for generously supplying the $G\alpha_{i1}$; Dr. Franklyn Prendergast, M.D., Ph.D. (Professor of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, Minnesota) for the use of spectroscopic instrumentation; and Darrell Loeffler (Technician, Department of Anesthesiology, Mayo Clinic, Rochester, Minnesota) for expert technical assistance.

References

1. Pentyala SN, Sung K, Chowdhury A, Rebecchi MJ: Volatile anesthetics modulate the binding of guanine nucleotides to the alpha subunits of heterotrimeric GTP binding proteins. Eur J Pharmacol 1999; 384:213–22
2. Ishizawa Y, Sharp R, Liebman PA, Eckenhoff RG: Halothane binding to a G protein coupled receptor in retinal membranes by photoaffinity labeling. Biochemistry (Mosc) 2000; 39:8497–502
3. Bremerich DH, Hirasaki A, Jones KA, Warner DO: Halothane attenuation of calcium sensitivity in airway smooth muscle: Mechanisms of action during muscarinic receptor stimulation. ANESTHESIOLOGY 1997; 87:94–101
4. Kai T, Jones KA, Warner DO: Halothane attenuates calcium sensitization in airway smooth muscle by inhibiting G-proteins. ANESTHESIOLOGY 1998; 89:1543–52
5. Sternweis PC, Robishaw JD: Isolation of two proteins with high affinity for guanine nucleotides from membranes of bovine brain. J Biol Chem 1984; 259: 13806–13
6. Higashijima T, Ferguson KM, Sternweis PC, Ross EM, Smigel MD, Gilman AG: The effect of activating ligands on the intrinsic fluorescence of guanine nucleotide-binding regulatory proteins. J Biol Chem 1987; 262:752–6
7. Lopez MM, Kosk-Kosicka D: Spectroscopic analysis of halothane binding to the plasma membrane Ca2+-ATPase. Biophys J 1998; 74:974–80
8. Higashijima T, Ferguson KM, Smigel MD, Gilman AG: The effect of GTP and Mg2+ on the GTPase activity and the fluorescent properties of Go. J Biol Chem 1987; 262:757–61
9. Barr AJ, Manning DR: Agonist-promoted [^{35}S]GTPγS-binding as a Probe of Receptor-G Protein Communication in Reconstituted Sf9 Cells, G Proteins: Techniques of Analysis. Edited by Manning DR. New York, CRC Press, 1999, pp 227–46
10. Cao WB, Harnett KM, Chen Q, Jain MK, Behar J, Biancani P: Group I secreted PLA2 and arachidonic acid metabolites in the maintenance of cat LES tone. Am J Physiol 1999; 277:G585–98
11. Van Dyke RA, Wood CL: Binding of radioactivity from 14 C-labeled halothane in isolated perfused rat livers. ANESTHESIOLOGY 1973; 38:328–32
12. Ferguson KM, Higashijima T, Smigel MD, Gilman AG: The influence of bound GDP on the kinetics of guanine nucleotide binding to G proteins. J Biol Chem 1986; 261:7393–9
13. Linder ME, Gilman AG: Purification of recombinant Gi alpha and Go alpha proteins from Escherichia coli. Methods Enzymol 1991; 195:202–15
14. Freissmuth M, Boehm S, Beindl W, Nickel P, Ijzerman AP, Hohenegger M, Nanoff C: Suramin analogues as subtype-selective G protein inhibitors. Mol Pharmacol 1996; 49:602–11

15. Carty DJ, Padrell E, Codina J, Birnbaumer L, Hildebrandt JD, Iyengar R: Distinct guanine nucleotide binding and release properties of the three Gi proteins. J Biol Chem 1990; 265:6268–73

16. Linder ME, Ewald DA, Miller RJ, Gilman AG: Purification and characterization of Go alpha and three types of Gi alpha after expression in Escherichia coli. J Biol Chem 1990; 265:8243–51

17. Schmidt U, Schwinger RH, Bohm M: Interaction of halothane with inhibitory G-proteins in the human myocardium. ANESTHESIOLOGY 1995; 83:353–60

18. Nietgen GW, Honemann CW, Durieux ME: Influence of anesthetics on endogenous and recombinantly expressed G protein-coupled receptors in the Xenopus oocyte. Toxicol Lett 1998; 100–101:319–27

19. Yoshimura H, Jones KA, Warner DO, Perkins WJ: Dual effects of hexanol and halothane on the regulation of calcium sensitivity in airway smooth muscle (abstract). ANESTHESIOLOGY 2001; 93:A1346

20. Ishizawa Y, Pidikiti R, Liebman PA, Eckenhoff RG: G protein-coupled receptors as direct targets of inhaled anesthetics. Mol Pharmacol 2002; 61: 945–52

21. Narayanan TK, Confer RA, Dennison RL Jr, Anthony BL, Aronstam RS: Halothane attenuation of muscarinic inhibition of adenylate cyclase in rat heart. Biochem Pharmacol 1988; 37:1219–23

22. Bohm M, Schmidt U, Gierschik P, Schwinger RH, Bohm S, Erdmann E: Sensitization of adenylate cyclase by halothane in human myocardium and S49 lymphoma wild-type and cyc- cells: Evidence for inactivation of the inhibitory G protein Gi alpha. Mol Pharmacol 1994; 45:380–9

23. Barr AJ, Brass LF, Manning DR: Reconstitution of receptors and GTP-binding regulatory proteins (G proteins) in Sf9 cells: A direct evaluation of selectivity in receptor: G protein coupling. J Biol Chem 1997; 272:2223–9

24. Selkirk JV, Price GW, Nahorski SR, Challiss RA: Cell type-specific differences in the coupling of recombinant mGlu1alpha receptors to endogenous G protein sub-populations. Neuropharmacology 2001; 40:645–56

25. Rebecchi MJ, Pentyala SN: Anaesthetic actions on other targets: protein kinase C and guanine nucleotide-binding proteins. Br J Anaesth 2002; 89:62–78

26. Croxton TL, Lande B, Hirshman CA: Role of G proteins in agonist-induced Ca2+ sensitization of tracheal smooth muscle. Am J Physiol 1998; 275:L748–55

27. Hirshman CA, Lande B, Croxton TL: Role of M2 muscarinic receptors in airway smooth muscle contraction. Life Sci 1999; 64:443–8