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Volatile anesthetics modulate the binding of guanine nucleotides to the α subunits of heterotrimeric GTP binding proteins

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Abstract

The effects of volatile anesthetics on guanine nucleotide binding to the purified α subunits of heterotrimeric GTP binding (G) proteins were studied. At sub-anesthetic doses, halothane, isoflurane, enflurane and sevoflurane inhibit exchange of GTPγS for GDP bound to Gα subunits and markedly enhance the dissociation of GTPγS, but fail to suppress GDPβS release. Nucleotide exchange from non-myristoylated $G\alpha_{i1}$ is similarly inhibited in the absence of any membrane lipid or detergent. The degrees of inhibition of GDP/GTPγS exchange and enhancement of GTPγS dissociation are in the same order: $\alpha_{i2} > \alpha_{i1} > \alpha_{i3} > \alpha_s$. By contrast, $G\alpha_o$, which is closely related to $G\alpha_i$, is completely insensitive to anesthetics. We conclude that volatile agents, at clinically relevant doses, have a direct effect on the conformation and stability of the GTP/Mg^{2+} bound state of some, but not all Gα subunits. By destabilizing this state, volatile agents may uncouple metabotropic and other heptahelical receptors from pathways modulating neuronal excitation.

Keywords: Anesthetic volatile; G-protein; Guanine nucleotide; Gα subunit

1. Introduction

General anesthetic agents include compounds as varied as the noble gases, alkanes, alcohols and ethers (Dilger, 1994; Franks and Lieb, 1994). Those in use today, include the alkane, 2-bromo-2-chloro-1,1,1-trifluoroethane (halothane), and the ether derivatives, such as 1-chloro-2,2,2-trifluoroethyl difluoromethyl ether (isoflurane). Although they are varied in structure, all volatile general anesthetics are moderately hydrophobic and fit the positive Meyer–Overton correlation between anesthetic potency and oil or lipid phase solubility. Because of their high solubility in lipid, volatile anesthetics can reach concentrations in biological membranes on the order of 50–100 mM (Dilger, 1994). These characteristics have led previous investigators to infer that volatile anesthetics alter the membrane's physical properties and thereby perturb its excitability, a hypothesis that still has its proponents (Cantor, 1997). Recently, however, the emphasis has shifted from theories of lipid perturbation to the idea that general anesthetics bind directly to specific proteins (Franks and Lieb, 1994; Eckenhoff and Johansson, 1997; Johansson et al., 1998). New results support this view and point to a group of transmembrane proteins, the γ-amino-*n*-butyric acid (GABA) gated chloride channels, as important anesthetic targets (Mihic et al., 1997; Harris et al., 1998; Kira et al., 1998; Liachenko et al., 1998). By binding directly to GABA and other ligand-gated ion channels, volatile agents could disturb the normal mechanisms controlling these allosteric proteins, thereby altering neuronal excitability.

Additionally, volatile anesthetics could disrupt central pathways that modulate consciousness, memory and nociception by blocking heptahelical receptor signals, which control the responsiveness of both ligand and voltage-gated ion channels (Lambert, 1993; Durieux, 1996a,b). These receptors are coupled to their downstream effectors by heterotrimeric GTP binding (G) proteins, and operate through a circuitous route of second messenger-activated protein kinases and phosphatases, to indirectly regulate ion

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channel activity (Wickman and Clapham, 1995). A less circuitous route involves membrane-delimited association of the channel and active G-protein subunits. Although the volatile agents could act at many different points in this complex network of signaling molecules, as we discuss, most evidence points to the receptor/G-protein coupling step as the most sensitive.

Studies of the central nervous system (CNS) and the myocardium support the view that volatile anesthetics disrupt receptor/G-protein coupling, perhaps at the level of the G-protein, itself. For example, halothane prevents the shift of the muscarinic receptor to a low-affinity state that is observed in the presence of guanylyl-5′-imidodiphosphate (GMPPNP), a nonhydrolyzable GTP derivative (Aronstam et al., 1986). Similarly, halothane affects oxotremorine binding to muscarinic receptors in rat brainstem membranes that is overcome by increasing GMPPNP concentration (Dennison et al., 1987). Persistence of the so-called high affinity receptor state is also found with other volatile agents like chloroform, enflurane, isoflurane and diethylether (Anthony et al., 1988). Volatile anesthetics also cause a rightward shift in the dose of clonidine, a partial α_2-adrenoceptor agonist, to inhibit adenylyl cyclase (Kagawa et al., 1997). This same dose of halothane antagonizes the decrease in clonidine binding affinity observed in the presence of GMPPNP. The results suggest that the volatile agents prevent agonist-induced dissociation of receptor from the heterotrimeric G-protein or decrease the binding of GTP analog or both, thereby preserving the high affinity state and uncoupling heptahelical metabotropic receptors from their downstream effector proteins.

Halothane and most other halogenated anesthetics have a negative inotropic and chronotropic effect on the heart in isolation and in patients (Pavlin and Su, 1994). Paradoxically, these agents, especially halothane, sensitize the myocardium to the effects of exogenous catecholamines through a direct action on the heart. For example, halothane enhances contraction induced by the β-adrenoceptor agonist, isoprenaline, in electrically driven human ventricular preparations (Bohm et al., 1994). In myocardial membranes, halothane increases isoprenaline, sodium fluoride, cholera toxin or GMPPNP stimulation of adenylyl cyclase, but not the stimulation caused by forskolin, which acts directly on this enzyme (Narayanan et al., 1988; Bohm et al., 1994; Schmidt et al., 1994). Treating with pertussis toxin increases isoprenaline, sodium fluoride, or GMPPNP-stimulated adenylyl cyclase activity, but halothane is unable to further increase the response. These observations imply that $G\alpha_i$ is uncoupled from down stream effectors by either pertussis toxin or halothane operating through analogous mechanisms.

In frog oocytes expressing human muscarinic M_1 and 5-HT_{1c} receptors, enflurane (1.8 mM) inhibits agonist-stimulated Ca^{2+} mobilization resulting from activation of phospholipase-C (Lin et al., 1993). The effect is most marked at low levels of receptor occupancy. Likewise, enflurane (albeit at high concentrations) inhibits Ca^{2+} mobilized by GTPγS suggesting that anesthetics block at the level of $G\alpha_q$, or phospholipase-C. An action on $G\alpha_i$ or $G\alpha_o$ is also possible, since stimulation of phospholipase-C is partly pertussis toxin sensitive in oocytes (Quick et al, 1994). Durieux (1996a) also showed that halothane and enflurane (0.2–0.5 mM) inhibit the coupling of muscarinic M_1 receptor to phospholipase-C at low levels of receptor occupancy. Likewise, Minami et al. (1997) have reported that coupling of 5-HT_α and $mGlu_5$ receptors to phospholipase-C are disrupted by volatile anesthetics., but here the stimulation of a negative feedback loop involving protein kinase-C is strongly implicated: Only those receptors possessing a protein kinase-C phosphorylation site are uncoupled from $G\alpha_q$ and phospholipase-C (Minami et al., 1997). This is consistent with direct activation of protein kinase-C, in agreement with stimulation of this kinase by volatile anesthetics in vitro (Hemmings and Adamo, 1997). These results suggest a mechanism for uncoupling of some metabotropic receptors from phospholipase-C, but this is unlikely to account for the uncoupling of all $G\alpha_i$-linked receptors.

Although many observations support involvement of G-proteins in some aspects of anesthetic action, direct evidence concerning the molecular mechanism is lacking. In the present study, we investigate the effects of volatile anesthetics on guanine nucleotide binding to G-proteins, in vitro. The results demonstrate these agents profoundly affect the empty and GTP/Mg^{2+}-charged states of some, but not all Gα subunits, suggesting a plausible mechanism that can explain the disruption in heptahelical receptor signaling. The relevance of these findings to the molecular actions of anesthetics is discussed.

2. Materials and methods

2.1. Materials

Halothane (Halocarbon Laboratories), sevoflurane (Abbot Laboratories), isoflurane and enflurane (Ohmeda PPD) were used in these experiments. [^{35}S]GTPγS, [^{35}S]GDPβS and Scintiverse were purchased from New England Nuclear, MA. Nitrocellulose filters were obtained from Millipore, MA. All other chemicals used in the study were from Sigma, MO.

Gα subunits ($G\alpha_s$ and $G\alpha_{i2}$) were either purified from bovine brain (Sternweis and Robishaw, 1984; Runnels et al., 1996) or expressed in Sf9 cells and purified as previously described (Kozasa and Gilman, 1995) or purchased from Calbiochem ($G\alpha_o$, $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$ and ras). Non-myristoylated $G\alpha_{i1}$ subunit was prepared from *Escherichia coli* bacteria, cotransfected with the vectors, plasmid Qiagen expression-6 (pQE-6) harboring $G\alpha_{i1}$ and plasmid *N*-myristoyltransferase (pNMT) harboring *N*-myristoyltransferase. The degree of myristoylation is con-

trolled by the plasmid copy number of pNMT by regulating kanamycin during induction and separating the myristoylated and non-myristoylated forms by phenylsepharose chromatography as described previously (Lee et al., 1994).

2.2. Measurement of anesthetic concentrations

Volatile drugs in pure liquid form were mixed with assay buffers (1:1 ratio) and equilibrated overnight in 50 ml gas tight vials. Different volumes of the saturated buffer were transferred to 1 ml silanized gas tight vials (Fisher Scientific). Under our conditions, the drug concentrations in the headspace above the aqueous sample reached equilibrium levels within 5 min. After equilibrium was achieved, a 1-μl portion of the headspace was removed with a gas tight Hamilton syringe through the teflon/rubber septum and injected into a Perkin Elmer-990 gas chromatograph (GC) equipped with a 6 ft × 1/8 in. column of carbograph (mesh 60/80) coated with 1% AT-1000 (Alltech). The run temperature was 100°C and detection was by flame ionization. The integrated response (A_s) was compared to standards of vapor saturated with drug (A_{std}) to obtain the percent atmosphere (% Atm) of each drug in the headspace. The aqueous concentration of each drug (C_{aq}) was calculated using the % Atm of each drug and the Ostwald water/gas partition coefficient (λ) appropriate to the temperature and drug as described (Dilger, 1994; Franks and Lieb, 1994).

(I) % Atm of drug at temperature T is calculated from the values obtained by GC analysis.

$$\%\,\mathrm{Atm} = A_s/A_{std} \times \%\,\mathrm{Atm}_{D(20°C)}$$

where A_s is the average peak area of sample from GC; A_{std} is the average peak area of the standard; % $\mathrm{Atm}_{D(20°C)}$ is % Atm drug at 20°C.

(II) λ at the experimental temperature ($\lambda_{expt\text{-}T}$) was calculated by converting $\lambda_{37°C}$ to $\lambda_{30°C}$.

$$\lambda_{expt\text{-}T} = (0.054\,\Delta T + 1)\,\lambda_{37°C}$$

where 0.054 is the fractional change in λ per degree decrease in temperature (Lockwood et al., 1997); ΔT is the difference between 37°C and experimental temperature (30°C).

(III) C_{aq} was calculated using the deduced $\lambda_{expt\text{-}T}$ and % Atm measured as described above.

$$C_{aq} = \left[\left(121.86 \times \lambda_{expt\text{-}T}\right) \times \%\,\mathrm{Atm}\right] / \left(273.15 + T_{expt}\right)$$

C_{aq} is expressed in mmol l^{-1}.

2.3. Guanine nucleotide binding experiments

2.3.1. Exchange reactions

Prior to treatment with anesthetics, Gα subunits were incubated with unlabeled GDP (50 nM) and 5 mM $MgCl_2$ for 2 h at 4°C. Nucleotide exchange was initiated by diluting the GDP-charged Gα subunits (50 ng) into 50 μl of assay buffer (20 mM HEPES, pH 8.0, 1 mM EDTA, 1

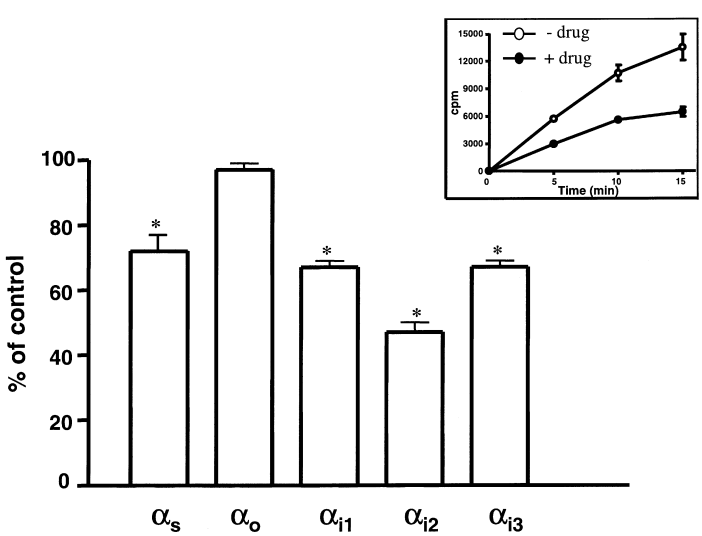

Fig. 1. Effects of sub-anesthetic concentrations (0.1 mM) of halothane on GDP/GTPγS exchange from Gα subunits: Different Gα subunits were preloaded with unlabeled GDP as described in Section 2. Samples were diluted into buffer containing [^{35}S]GTPγS and incubated for 15 min at 30°C, a time at which the exchange of nucleotides in the absence of drug was approximately 50% complete. The exchange of GTPγS for GDP was determined by trapping the protein on nitrocellulose filters and counting the radioactivity bound to protein. Values are percentage of control (Gα subunit incubated without drug). Each value is mean ± SE of at least two experiments assayed in duplicate. * Significant at $p < 0.05$. Insert: Time course effect of halothane (0.1 mM) on nucleotide exchange from $G\alpha_{i2}$. Data are presented as bound GTPγS (cpm) to $G\alpha_{i2}$ in the presence and absence of drug.

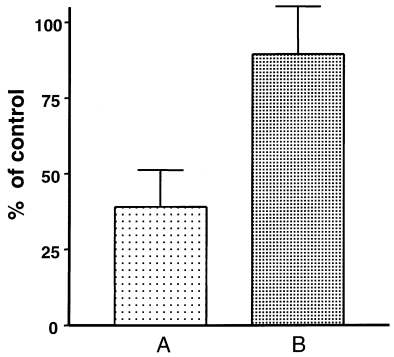

Fig. 2. Reversible effect of halothane on GDP/GTPγS exchange by $G\alpha_{i2}$: The G-α_{i2} subunit is preloaded with GDP as described in Fig. 1. (A) Protein is exposed to halothane (0.2 mM) throughout the course of the experiment (15 min) in a gas-tight vial. (B) Protein is exposed to halothane (0.2 mM) for 5 min. The cap of the vial is then opened for 5 min to let halothane evaporate. Exchange is then initiated by addition of labeled GTPγS. The exchange of GTPγS for GDP was determined by trapping the protein on nitrocellulose filters and counting the bound radioactivity as in Fig. 1. Data are presented as percent of control values (without drug treatment). Each value is the mean ± SE of two experiments performed in duplicate. * Significant at $p < 0.05$.

mM DTT, 100 mM NaCl, 0.2% cholate) containing 5 mM $MgCl_2$, 2.5 μM GTPγS, 2 μCi ^{35}S-GTPγS (10 Ci/μmol) and various concentrations of anesthetic, in a 1-ml silianized glass vial sealed with a teflon/rubber septum. The exchange reaction proceeded at 30°C for stipulated time intervals. Exchange reactions were terminated with ice-cold stop buffer (10 mM HEPES, 100 mM NaCl, 20 mM $MgCl_2$), followed by rapid filtration of the samples through HA nitrocellulose filters (0.45 μm). The filters were then washed with 10 ml of the same buffer, dried, suspended in 5 ml of scintiverse, and counted in a liquid scintillation spectrometer for measurement of the amounts of radiolabeled nucleotide bound to protein.

2.3.2. GDP and GTP dissociation

Prior to treatment with anesthetic, Gα subunits were incubated with either 2 μCi of ^{35}S-GTPγS (10 Ci/μmol) or ^{35}S-GDPβS (10 Ci/μmol) for 10 min at 30°C. Dissociation was initiated by diluting the Gα subunits into assay buffer containing 10 mM EDTA, 100 μM unlabeled GDP or GTP and various concentrations of anesthetic. The dissociation reactions proceeded at 30°C for stipulated time intervals and were terminated with ice cold stop buffer without $MgCl_2$. The amounts of radio-labeled bound guanine nucleotide remaining were determined as described above.

2.4. Statistical analysis

Data are expressed as the mean ± S.E. of at least two independent experiments assayed in duplicate. The significance of the difference between means was determined by Student's *t*-test. Analysis of variance was used to determine significance when multiple comparisons were per-

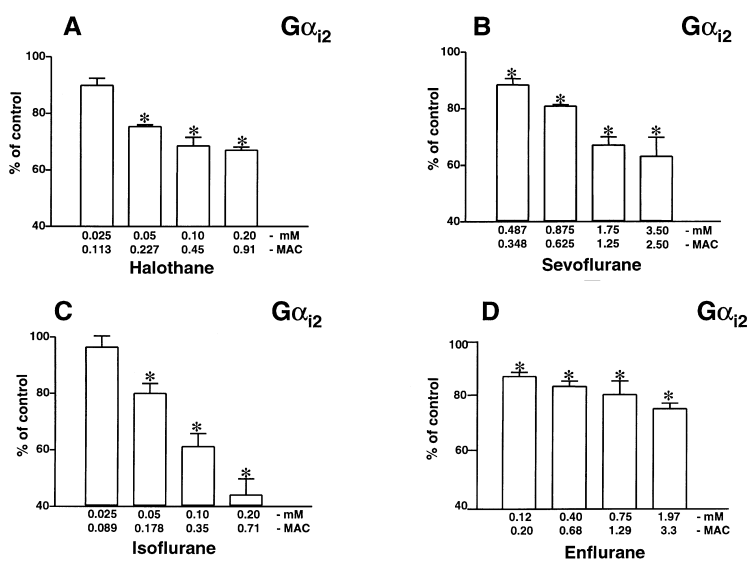

Fig. 3. Effects of halothane (A), sevoflurane (B), isoflurane (C) and enflurane (D) on GDP/GTPγS exchange from $G\alpha_{i2}$: The $G\alpha_{i2}$ subunits were treated as described in Fig. 1. The exchange of GTPγS for GDP was determined by trapping the protein on nitrocellulose filters and counting the bound radioactivity. Values are presented as percent of control (without drug treatment) and each value is mean ± SE of at least two experiments performed in duplicate. Drug concentrations are presented in both millimolar and minimum aveolar concentration (MAC) values. MAC values, the concentration required to immobilize 50% of human patients, are adjusted for the appropriate temperature (30°C) in these experiments (Aronstam et al., 1986; Lin et al., 1993). * Significant at $p < 0.05$.

formed. A value of $p < 0.05$ was accepted as statistically significant.

3. Results

3.1. Volatile anesthetics inhibit GDP / GTP exchange by Gα subunits

To test the idea that volatile anesthetics directly affect Gα subunits, we examined exchange of bound GDP for GTPγS, a reaction normally stimulated by activated receptor (Bourne, 1997). Each purified α subunit was first incubated with an excess of GDP, sufficient to saturate the guanine nucleotide-binding site (Self and Hall, 1995). The protein sample was then diluted into buffer containing [^{35}S]GTPγS and anesthetic. The time course of exchange, measured as the amount of radiolabeled GTPγS bound to the protein, was then monitored over a period of time, up to 30 min. Under these conditions halothane at sub-clinical doses substantially reduced the extent of exchange for α_{i1}, α_{i2}, α_{i3} and α_s, but not α_o (Fig. 1). Of all the subunits tested, $G\alpha_{i2}$ was most sensitive and inhibition of this subunit was reversible (Fig. 2). Other volatile anesthetics, isoflurane, enflurane and sevoflurane, also suppress exchange on α_{i2}, at subclinical doses; isoflurane being the most effective (Fig. 3; Table 1). Compared to isoflurane, its chemical isomer, enflurane, which is nearly as clinically potent and lipid soluble, was not as effective in suppressing exchange (Fig. 3; Table 1). Like halothane, these drugs failed to affect exchange from α_o (not shown).

By contrast to the sensitive α subunits, and similar to $G\alpha_o$, the low molecular weight G-protein, ras, was resistant to high concentrations of each drug (not shown). Thus, the anesthetic agents display measurable degrees of G-protein specificity in vitro.

In the exchange experiments, detergents were used to solubilize and stabilize the lipid modified Gα subunits. To rule out the possibility that volatile drugs disrupt detergent micelles and thereby affect binding of guanine nucleotide to G-proteins, non-myristoylated $G\alpha_{i1}$ subunits were tested. These subunits bind and release guanine nucleotides normally, in the absence of lipid modification (Pomerantz et al., 1997). Because the non-myristoylated protein is soluble, detergents could be omitted during the purification and subsequent assay. We found that under these conditions, halothane inhibits GDP/GTPγS exchange to the same or greater extent (Fig. 4).

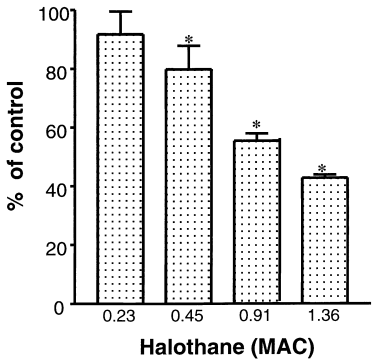

Fig. 4. Concentration dependent effects of halothane on GDP/GTPγS exchange from non-myristoylated $G\alpha_{i1}$: The recombinant non-myristoylated $G\alpha_{i1}$ subunits (soluble protein in the absence of detergents) were treated as described in Fig. 1. The exchange of GTPγS for GDP was determined by trapping the protein on nitrocellulose filters and counting the bound radioactivity. Values are presented as percent of control (without drug treatment) and each value is mean ± SE of two experiments performed in duplicate. * Significant at $p < 0.05$.

3.2. Volatile anesthetics modulate the rate of nucleotide dissociation from Gα subunits

Possible explanations for the diminished guanine nucleotide exchange on α subunits include: (I) decreased rates of GTPγS and Mg^{2+} association, limiting the rate of GTPγS/Mg^{2+} binding to the empty state of the G-protein; (II) a suppressed rate of GDP dissociation, limiting the rate at which GTP can bind; and (III) an enhanced rate of GTPγS dissociation, diminishing the steady-state level of α/GTP complex. As had previously been reported

Table 1
Effects of volatile anesthetics on GDP/GTPγS exchange by Gα subunits
Different Gα subunits were preloaded with unlabeled GDP as described in Section 2. Samples were diluted into buffer containing [^{35}S]GTPγS and incubated for 15 min at 30°C. The exchange of GTPγS for GDP was determined by trapping the protein on nitrocellulose filters and counting the remaining radioactivity. Values are percentage of exchange from Gα subunit incubated without drug (control).
Each value is the mean ± SE of two to four independent experiments assayed in duplicate.
Mean values are significantly different from controls at $p < 0.05$.
ND = not determined.

Drug	% Inhibition of GDP/GTPγS exchange on Gα-subunits at 15 min				
	α_s	α_o	α_{i1}	α_{i2}	α_{i3}
Halothane 0.20 mM (0.91 MAC)	28 ± 6	4 ± 2[a]	29 ± 2	34 ± 2	33 ± 4
Isoflurane 0.20 mM (0.71 MAC)	38 ± 3	6 ± 5[a]	46 ± 5	58 ± 6	49 ± 3
Sevoflurane 1.75 mM (1.25 MAC)	28 ± 4	3 ± 2[a]	29 ± 2	37 ± 4	34 ± 3
Enflurane 0.75 mM (1.25 MAC)	20 ± 3	4 ± 1[a]	ND	22 ± 6	ND

[a] Not significant.

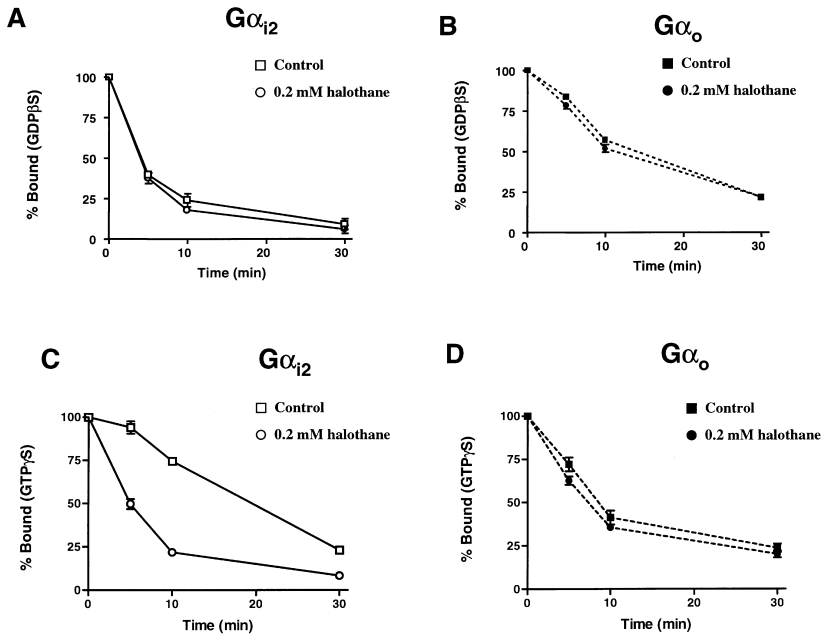

Fig. 5. Effects of halothane (0.2 mM or 0.9 MAC) on guanine nucleotide dissociation from $G\alpha_{i2}$ and $G\alpha_{o}$: Proteins were preloaded with [^{35}S]GTPγS or [^{35}S]GDPβS for 10 min at 30°C in the presence of 5 mM $MgCl_2$. Samples were diluted into buffer containing 100 μM unlabeled GTPγS or GDP with 10 mM EDTA in the presence or absence of the drug. The reactions were terminated at 0, 5, 10 and 30 min and the protein was trapped on nitrocellulose filters and counted for bound radiolabel. Panel A: GDPβS dissociation from $G\alpha_{i2}$. Panel B: GDPβS dissociation from $G\alpha_{o}$. Panel C: GTPγS dissociation from $G\alpha_{i2}$. Panel D: GTPγS dissociation from $G\alpha_{o}$. Circles are values obtained in the presence of 0.2 mM halothane. Squares represent values obtained in the absence of drug (control). Results were normalized to the amount of radioactivity at $t = 0$ (100%). Each value is the mean ± SE of at least two experiments assayed in duplicate.

(Higashijima et al., 1987), we found an extremely slow rate of GTPγS dissociation; only 5% of the labeled nucleotide dissociated in 15 min and this was only slightly affected by anesthetic (not shown). Obviously, an enhanced GTPγS dissociation (possibility III), would not account for the suppressed exchange rate. To test possibility II, the time course of [^{35}S]GDPβS dissociation from the α subunits were measured in the presence or absence of anesthetic. Dissociation was measured in buffer containing 5 mM Mg^{2+}, the same concentration used in the exchange measurements.

Since a decreased GDP dissociation rate would readily account for our results, we were surprised to find the rates of [^{35}S]GDPβS dissociation from $G\alpha_{i2}$ (and $G\alpha_{o}$) were unaffected by anesthetic, whether or not Mg^{2+} was present (Fig. 5A and B), leaving us to conclude that the association rate of GTPγS is suppressed. If true, the anesthetic would likely affect the empty state of the Gα subunit and the conformations it could access. We reasoned that the perturbation by anesthetic could be reflected in an enhanced dissociation of GTPγS (possibility III) and Mg^{2+}, were the free Mg^{2+} concentration to be rapidly decreased. Lowering the Mg^{2+} concentration is well known to enhance the rate of GTP dissociation from Gα subunits (Higashijima et al., 1987).

After loading the Gα subunit with GTPγS and Mg^{2+}, dissociation was induced by dilution into buffer containing an excess of EDTA (5 mM) to complex free Mg^{2+}. Under these conditions, halothane enhanced the dissociation of GTPγS from α_{i2} (Fig. 5C), but had no effect on dissociation from α_{o} (Fig. 5D). Enhanced dissociation of GTPγS from α_{i2} was observed at sub-anesthetic doses of halothane (Fig. 6), as well as isoflurane, enflurane and sevoflurane (Table 2). The results are the mirror image of our exchange measurements, $G\alpha_{i2}$ being the most sensitive and

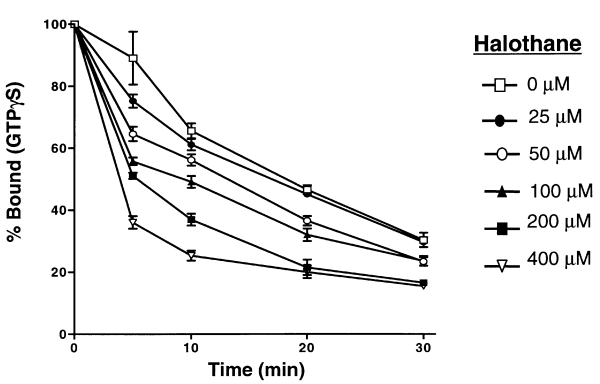

Fig. 6. Effect of halothane concentration on GTPγS dissociation from $G\alpha_{i2}$: $G\alpha_{i2}$ was preloaded with [^{35}S]GTPγS for 10 min at 30°C in the presence of 5 mM $MgCl_2$. Samples were diluted into buffer containing 100 μM unlabeled GTPγS with 10 mM EDTA in the presence or absence of the drug. The reactions were terminated at 0, 5, 10, 20 and 30 min and the protein was trapped on nitrocellulose filters and counted for radioactivity. Results were normalized to the amount of radioactivity at $t = 0$ (100%). Each value is the mean ± SE of at least two experiments assayed in duplicate.

Table 2
Effect of volatile anesthetics on the dissociation of GTPγS from Gα subunits
Proteins were preloaded with [^{35}S]GTPγS for 10 min at 30°C. Samples were diluted into buffer containing 100 μM unlabeled GTPγS in the presence or absence of the drug. The reaction was terminated at 10 min and the remaining radioactivity bound to protein was trapped on nitrocellulose filters and counted for radioactivity. Results were normalized to the amount of radioactivity bound to protein at $t = 0$ (100%) and are expressed as the percent increase in extent of GTPγS dissociation at a single time point (10 min).
Each value is the mean ± SE of two to four independent experiments assayed in duplicate.
Mean values are significantly different from controls at $p < 0.05$.
ND = not determined.

Drug	% Increase in the extent of GTPγS dissociation at 10 min				
	α_s	α_o	α_{i1}	α_{i2}	α_{i3}
Halothane 0.20 mM (0.91 MAC)	32 ± 2	5 ± 2[a]	37 ± 2	50 ± 4	46 ± 5
Isoflurane 0.20 mM (0.71 MAC)	31 ± 2	8 ± 4[a]	38 ± 4	46 ± 3	41 ± 3
Sevoflurane 1.75 mM (1.25 MAC)	ND	6 ± 2[a]	28 ± 5	38 ± 6	32 ± 2
Enflurane 0.75 mM (1.25 MAC)	ND	9 ± 7[a]	30 ± 5	34 ± 4	31 ± 4

[a]Not significant.

$G\alpha_o$ completely insensitive. As found in the exchange experiments, the volatile agents had no effect on either GTPγS or GDPβS dissociation from ras (not shown).

In the absence of drug, an initially slow phase was often observed in the dissociation of GTPγS from α_{i2} (Fig. 5C), and α_{i1}, α_{i3} and α_s (not shown) generally. In our experimental design, the protein sample bound with GTPγS was diluted into buffer containing an excess of EDTA to complex free Mg^{2+}. It is possible that Mg^{2+} dissociation limits the initial off rate of GTP and may therefore account for the initially slow phase of the dissociation in the absence of drug. On the other hand, in the presence of drug, dissociation was too rapid for us to observe a delay.

From our results and previous studies (Higashijima et al., 1987), it is clear that Mg^{2+} ''locks'' the Gα subunit into a stable complex with GTPγS. By preventing the rebinding of Mg^{2+} to the Gα subunit, the rate of GTPγS release is enhanced by volatile anesthetics, reflecting a destabilization of the GTPγS binding site (see Section 4). We speculate that the GTPγS association rate is suppressed in the presence of Mg^{2+} by the same mechanism that enhances dissociation, contributing to slow exchange of GDP for GTPγS.

4. Discussion

Our results demonstrate that volatile anesthetics, at clinically relevant concentrations, inhibit the exchange of GDP for GTPγS on α_i and α_s subunits. Under these conditions, neither the release of GDP nor GTPγS is affected. Hence, the on rate of GTPγS/Mg^{2+} must be suppressed. We also find that, in the absence of free Mg^{2+}, volatile anesthetics increase the release of GTPγS, reflecting a perturbation of the guanine nucleotide-binding site.

The four agents tested, halothane, isoflurane, sevoflurane and enflurane exhibited qualitatively similar, but quantitatively different effects. Especially noteworthy is the effectiveness of isoflurane to inhibit exchange compared to the relatively small suppression by its chemical isomer, enflurane, at nearly equivalent clinical doses. Does this imply that the effects on G-proteins are dissociated from the actions of these drugs as anesthetic agents? No, not necessarily. Although all four drugs produce the same spectrum of broadly defined neurophysiologic responses associated with anesthesia: analgesia, amnesia, sedation and immobility, each agent may act at multiple different sites within the CNS. Differential effects of enflurane and isoflurane on GABA receptors in cerebral cortex and cerebellum have been reported previously (Zhu and Barker, 1996). Similarly, differential effects of isoflurane and enflurane on neurotransmitter release have been noted (Harris et al., 1993).

α_{i2} appears to be most sensitive to volatile anesthetics, yet α_s is also inhibited. In some systems these Gα subunits have diametrically opposed actions on adenylyl cyclase. Nonetheless, one pathway may predominate under certain conditions revealing an action of the anesthetic on G-protein regulated pathways. In ventricular myocardium from failing human ventricles, β-adrenoceptor activation of adenylyl cyclase is desensitized, giving rise to a predominance of α_i mediated suppression (Rockman et al., 1997). Volatile agents appear to intensify β-adrenergic stimulation of adenylyl cyclase and contractile responses by blocking the inhibitory effects of α_i (Bohm et al., 1994), resulting in a net stimulation, even though $G\alpha_s$ may be partly suppressed. Thus, the physiologic response to the volatile agents will depend on the relative tones of the two pathways. Additionally, the actions of $G\alpha_s$ and $G\alpha_i$ on adenylyl cyclase depend on the particular cyclase isotypes present. $G\alpha_i$ directly inhibits adenylyl cyclase isotypes I, V, VI, and VIII, whereas Gβγ subunits, presumably released from activated from $G\alpha_i$ or $G\alpha_o$, directly stimulate types II and IV (Gilman, 1995). In the hippocampus, where type II adenylyl cyclase predominates, cAMP levels are synergistically increased by agonists known to act through both $G\alpha_s$ and $G\alpha_i$ linked

receptors (Mons et al., 1993). Moreover, adenylyl cyclase regulation has additional complexities including Ca^{2+}–calmodulin activation of brain-specific types I and VIII (Katsushika et al., 1992). Although we have used adenylyl cyclase as an example, the actions of volatile agents on $G\alpha_i$ are not limited to this enzyme, since $G\beta\gamma$ subunits released from $G\alpha_i$ also regulate other effectors, including phospholipase-C and ion channels (Wickman and Clapham, 1995). Consistent with this idea, volatile anesthetics perturb the activation of inwardly rectifying K^+ channels by G-proteins. In ventricular myocytes, muscarinic receptor activation of I_k, a $G\beta\gamma$ regulated ion channel is slowed by halothane or isoflurane (Magyar and Szabo, 1996). The slowing of I_k activation is consistent with an uncoupling of receptor/G-protein from the channel. A plausible mechanism for slowed activation could entail either slowed guanine nucleotide exchange or the sequestering of $G\beta\gamma$ by GTP-charged $G\alpha_i$ bound with anesthetic (see discussion below).

The in vitro binding assay, used here, incorporated a detergent, cholic acid, to solubilize the $G\alpha$ subunits containing acyl and prenyl groups. Because the volatile agents partition at high concentrations into lipid, it was plausible that these drugs affected the structures or properties of cholate micelles and thereby perturbed the embedded protein. We eliminated this possibility by showing that non-myristoylated soluble $G\alpha_{i1}$ subunits are similarly affected by volatile anesthetics. Thus, the volatile anesthetics bind directly to the $G\alpha_i$ subunits. By comparing the three-dimensional structures of GDP and GTP/Mg^{2+} states of the $G\alpha$ subunits, a molecular mechanism for the actions of anesthetics on these proteins can be suggested.

The structures of heterotrimeric G-proteins and their function as molecular switches have been deduced from crystallographic studies of the isolated $G\alpha$ subunits in the GDP (Mixon et al., 1995) and GTP/Mg^{2+} (Coleman et al., 1994) states and from their complexes with $G\beta\gamma$ subunits (Wall et al., 1995). The core of all G-protein subunits contains a GTPase fold (Sprang, 1997), consisting of a six-stranded β sheet enclosed by α helices. Loops connecting the helices and strands, designated G1–G5, form the conserved portions of the guanine nucleotide binding pocket. The regions involved, especially G2 (switch I) and G3 (switch II), are highly flexible and form a stable scaffold for binding the γP of GTP and a single Mg^{2+} ion. Switch II interacts with switch III and this interaction is lost upon GTP hydrolysis (Sprang, 1997). Operation of the switches is also coupled to folding of the N- and C-termini, which are crucial to the molecular recognition by $G\beta\gamma$ subunits. In the GDP state of the isolated $G\alpha$ subunit, disorder in switches II and III permits the folding of the N-terminal residues 8–32, and C-terminal residues 344–354, into a stable micro-domain. Conversely, these regions become highly disordered when GTP and Mg^{2+} are bound. Thus, $G\alpha$ subunits use the favorable energy of binding GTP and Mg^{2+} to select a stable protein conformer, flicking open a switch that triggers the release of $G\beta\gamma$ subunits, and modulates downstream effector proteins (Kleuss et al., 1994; Sprang, 1997). The ability of volatile anesthetics to destabilize the GTPγS/Mg^{2+} binding site implies that the conformations of the switch regions are perturbed, potentially interfering with the normal mechanisms governing the binding of $G\alpha$ subunit to $G\beta\gamma$ heterodimer and/or receptor, as well as guanine nucleotides.

By contrast to α_s, α_{i1}, α_{i2} and α_{i3}, both ras (p21) and $G\alpha_o$ subunit are resistant to volatile anesthetics. Thus, these drugs exert a specific action on particular subunits. Resistance of the low molecular weight G-proteins to anesthetics may be explained by the structural differences. In addition, to the absence of the entire α helical domain, ras lacks switch II, and its switch I region, which would otherwise link to the α helical domain, is significantly shorter than those found in the $G\alpha$ subunits (Kjeldgaard et al., 1996; Sprang, 1997). Currently, there is no three-dimensional structure of $G\alpha_o$ that would allow a comparison to the other $G\alpha$ subunits or provide a molecular explanation of our results. Nonetheless, resistance of $G\alpha_o$ to the anesthetics is consistent with the much more rapid GTP dissociation rate which was also described previously (Denker et al, 1995; Busconi and Denker, 1997).

Our results demonstrate a direct action of volatile anesthetics on soluble G-proteins. General anesthetic agents have previously been shown to bind to and directly modify the structures of other soluble proteins, such as firefly luciferase (Franks and Lieb, 1994; Ueda and Suzuki, 1998), albumin and myoglobin (Johansson et al., 1998). Compared to these inherently stable model proteins, ligand-gated ion channels and G-proteins should be more sensitive to anesthetic agents since they have relatively low free energy barriers between alternative native conformations (Eckenhoff and Johansson, 1997). In the case of the $GABA_A$ and NMDA receptors, a single site located at a predicted subunit/subunit interface confers sensitivity to volatile and intravenous general anesthetics, slowing channel closure, and suggesting that a localized perturbation by the anesthetic produces a global change in protein conformation (Harris et al., 1998). Analogous to the changes in ligand-gated channels, volatile anesthetics may affect conformations of the switch regions of some $G\alpha$ subunits. In our model, the normal receptor catalyzed exchange of GDP for GTP is disrupted by binding of anesthetic to a site or sites on the $G\alpha$ subunit that alter switch region conformations without affecting the binding of GDP. Because binding of GTP and Mg^{2+} are linked to the conformations available to the otherwise flexible switch regions, anesthetics may decrease the rates at which GTP and Mg^{2+} associate with the empty state of the $G\alpha$ subunit (normally stabilized by the ligand-activated receptor; Bourne, 1997), thereby disrupting nucleotide exchange, and knocking out critical connections between heptahelical receptors and their effectors. Whether this model and the

results reported here pertain to anesthesia will require experiments to test the effectiveness of these agents to disrupt relevant neurophysiologic responses controlled by G-protein coupled pathways.

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